BW GOLD LTD
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Blackwater Gold Project

Follow-up Programs for Condition 3.14 of the Blackwater Gold Project Decision Statement Issued under Section 54 of the Canadian Environmental Assessment Act, 2012

May 2023

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Follow-up Programs for Condition 3.14 of the Blackwater Gold Project Decision Statement Issued under Section 54 of the Canadian Environmental Assessment Act, 2012

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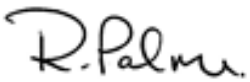

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ACRONYMS AND ABBREVIATIONS

Terminology used in this document is defined where it is first used. The following list will assist readers who may choose to review only portions of the document.

AEMP	Aquatic Effects Monitoring Plan
ATU	accumulated thermal units
BC	British Columbia
Blackwater or Project	Blackwater Project or Blackwater Gold Project
BW Gold	BW Gold LTD.
CAHS	Centre for Aquatic Health Sciences
CEA Agency	Canadian Environmental Assessment Agency (now Impact Assessment Agency of Canada)
CPUE	catch-per-unit-effort
DFO	Fisheries and Oceans Canada
DS	Decision Statement
EA	Environmental Assessment
EAO	British Columbia Environmental Assessment Office
FWSS	freshwater supply system
FWR	freshwater reservoir
GAUC	Gaussian Area-Under-The-Curve model
IAAC	Impact Assessment Agency of Canada
IFN	instream flow needs
Lake 15	Lake 01538UEUT
Lake 16	Lake 01682LNRS
MaxN	maximum fish count in a video frame
NAAHP	National Aquatic Animal Health Program
OWM	Overwintering Mortality
Project	Blackwater Gold Project
QA/QC	quality assurance and control
RT-qPCR	Real time Polymerase Chain Reaction
SOPs	Standard operating procedures
UAV	Unmanned Aerial Vehicle

1. INTRODUCTION

The Blackwater Gold Project (Project) received a Decision Statement (DS) on April 15, 2019, under the Canadian Environmental Assessment Act, 2012 (CEA Agency 2019) and an Environmental Assessment Certificate #M19-01 on June 21, 2019, under the 2002 Environmental Assessment Act (EAO 2019).

Condition 3.14 of the DS requires the BW Gold Ltd.'s (BW Gold) to develop a Fish and Fish Habitat Follow-up Program as follows:

3.14 The Proponent shall develop, prior to construction and in consultation with Indigenous groups, Fisheries and Oceans Canada (DFO), and other relevant authorities, a follow-up program to verify the accuracy of the environmental assessment and determine the effectiveness of the mitigation measures as it pertains to adverse environmental effects of the Designated Project on fish and fish habitat. The Proponent shall implement the follow-up program during all phases of the Designated Project and shall apply conditions **2.9** and **2.10** when implementing the follow-up program. As part of the follow-up program, the Proponent shall:

3.14.1 conduct parasite and pathogen inventories in Lake 01538UEUT and Lake 01682LNRS prior to enlarging Lake 01682LNRS and connecting it to Lake 01538UEUT pursuant to Condition 3.13 and compare the results of the parasite and pathogen inventories for the two lakes;

3.14.2 monitor, starting when the Proponent starts to pump water into Davidson Creek and continuing through until the freshwater supply system has been decommissioned, Rainbow Trout (*Oncorhynchus mykiss*) and kokanee (*Oncorhynchus nerka*) populations in Davidson Creek, including:

3.14.2.1 community composition of Rainbow Trout (*Oncorhynchus mykiss*) and kokanee (*Oncorhynchus nerka*), their absolute abundance, genetic structure and diversity;

3.14.2.2 absolute abundance of overwintering Rainbow Trout juveniles; and

3.14.2.3 characteristics of spawner populations through surrogate monitoring metrics including size at 50% maturity, redd counts and spawner distribution.

Condition 2.9 of the DS requires follow-up programs to verify the accuracy of the environmental assessment, determine whether modified or additional mitigation measures are required, and timely implementation if required.

Condition 2.10 of the DS requires consultation with Indigenous groups on the follow-up programs regarding opportunities for participation in their implementation.

As with avoidance measures, effluent treatment as a mitigation measure is not a monitoring requirement of Condition 3.14. Effluent monitoring is part of the Mine Site Water and Discharge Monitoring and Management Plan (MSDP). The MSDP details the monitoring procedures for each phase of mine life for the effective interception, conveyance, diversion, storage, and discharge of water (e.g., contact and non-contact) on the mine site. The MSDP also provides the operational and monitoring plans for all discharges of mine contact water to the receiving environment.

1.1 Purpose and Objectives

The purpose of Fish and Fish Habitat Follow-up Program described herein is to fulfill Condition 3.14 of the federal DS.

The Fish and Fish Habitat Follow-up Programs are designed to first characterize baseline conditions for each of the indicators listed in the condition (e.g., characteristics of spawner populations). These indicators will then be monitored during all phases of the Project to determine, to the extent possible, if (a) variation from baseline conditions is occurring, (b) mitigation measures are effective, (c) if the environmental assessment was accurate in terms of anticipated effects on the indicators, and (d) determine if additional mitigations should be taken pursuant to Condition 2.9.

2. FOLLOW UP PROGRAM 3.14.1: LAKES 15 AND 16 PARASITES AND PATHOGENS STUDY

2.1 Background and Approach

The Application/EIS determined fish habitat under the mine footprint and upstream of it will be permanently altered or lost during construction, operations, closure, and post-closure phases (Section 5.3.8 Fish of the Application/EIS). Mitigation for this lost fish habitat in upper Davidson Creek includes diversion of Lake 16 through a constructed stream channel to Lake 15, one of two headwater lakes in the Creek 705 Watershed. Lakes 15 and 16 are located at high elevation (~1345 m), in separate sub-watersheds that are divided by a narrow (~500 m) strip of land (**Figure 1**), with a 0.4 m difference between lake elevations. Lake 16 is the headwater lake of Davidson Creek, which is the main creek draining the mine site. Construction of the connector channel between the Lake 15 and Lake 16 is proposed (and required under Condition 3.13 of the DS) to preserve the Rainbow Trout population in Lake 16 that would otherwise be entirely isolated by the mine development in the upper and middle reaches of Davidson Creek (**Figure 1**). The purpose of Condition 3.14.1 is to evaluate the risk of introducing new parasites or pathogens to either of the lakes when they are connected, which could harm the populations of Rainbow Trout in either lake. There are no direct mitigation measures for this potential effect, but there are several lines of evidence that indicate possible historical exchange of fish between the two watersheds (Section 5.3.8.3.2.5.2 of the Application/EIS). This potential effect was not carried forward into a residual effects assessment in the Application/EIS.

A parasite-pathogen study was designed by the BC Center for Aquatic Health Sciences (CAHS). Due to potential low fish numbers in Lake 15 and Lake 16, a non-lethal sampling approach was used (i.e., no sampling of Rainbow Trout tissue, which would have required sacrificing of fish). The study was initiated in September 2021 with a field program to collect gill swabs and mucus samples from Rainbow Trout in Lakes 15 and 16, as well as water samples. The purpose of the study is to test the samples for a suite of viruses, bacteria, and pathogens and thereby establish the degree of overlap between the parasite and pathogens present in each lake prior to construction of the offsets.

2.2 Field Methods

Standard fish sampling protocols (RISC 2001) were adhered to during the September 2021 field program. Rainbow Trout in Lakes 15 and 16 were captured primarily by angling due to high mortality with gillnetting. A combination of angling techniques (i.e., predominantly trolling and to a lesser extent shore casting) and gear (i.e., fly rods equipped with sinking line and flies; spinning rods equipped with lures) were used to capture fish. Additional details on the field methods and results for the September 2021 field program are

available in the Blackwater Gold Project Fish and Aquatic Resources 2021 Field Survey Report (Palmer 2022a).

Due to potential low fish numbers in Lake 15 and Lake 16, a non-lethal sampling approach was used (i.e., no sampling of Rainbow Trout tissue, which would have required sacrificing of fish). Gill swabs and mucus samples were collected from captured Rainbow Trout, and water samples were collected from each lake. The gill swabs and mucus samples were be taken by a trained technician from CAHS. Prelabelled microtubes and sample bags were used for each fish and swabs specific to viral or bacterial sampling were used for skin mucous and gills. More information on the methods used to obtain gill swabs and mucus samples are available in the laboratory analysis report in Appendix A.

2.3 Laboratory Methods

Molecular assays for a list of NAAHP (National Aquatic Animal Health Program) parasites and pathogens of Rainbow Trout have been performed by CAHS. At CAHS, molecular assays are routinely used for fish screening using kidney tissues. However, these assays needed to be optimised for the non-lethal samples being used in this study which would contain low levels of parasites/pathogens genetic material relative to fish tissue samples.

A Real time Polymerase Chain Reaction (RT-qPCR) molecular assay was used that targets genetic material of the pathogen of concern. Specific primers (forward and reverse) and a probe had to be designed for each of the NAAHP diseases of concern for Rainbow Trout and kokanee found in the lakes. Primers and probes already exist for testing samples for several well-known fish diseases, including Ceratomyxosis (*Ceratomyxa shasta*) and Whirling disease (*Myxobolus cerebralis*). Additionally, primers and probes have been developed for this study by CAHS for three major fish bacteria: *Renibacterium salmoninarum*; *Aeromonas salmonicida*; *Yersinia ruckeri*; and for four major fish viruses: Infectious Hematopoietic Necrosis Virus, Infectious Pancreatic Necrosis Virus, Infectious Salmon Anaemia Virus, and Viral Haemorrhagic Septicaemia Virus.

To prepare for analysis, each isolate was filtered and diluted 5 times in 10-fold dilution. Control swabs were dipped in each dilution to mimic the sample swabs. DNA was extracted from each swab using the Qiagen DNeasy blood and tissue kit (Cat# 69506) and as per CAHS SOP #17 v2.1. RT-qPCR was performed on the extracted DNA using the TaqMan qPCR kit (Applied Biosystems).

2.4 Reporting and Follow-up

Testing of the gill swab, mucus, and water samples from the two lakes was completed and results of this study are available in the Blackwater Gold Project Fish and Aquatic Resources 2021 Field Survey Report (Palmer 2022a).

The final parasite and pathogen report has been completed (provided in Appendix A of this document) and presented to Indigenous groups and DFO for consideration prior to construction of the connector channel between Lakes 15 and 16 (CAHS 2022). No additional testing at Lakes 15 and 16 is included as part of Condition 3.14 because the study concluded that parasites and pathogens causing disease in fish are not present in either of the two lakes. Therefore, joining these lakes poses minimal risk of introducing new harmful parasites or pathogens to either lake. As such, Artemis proposes that additional studies or monitoring for parasites or pathogens will not be required.

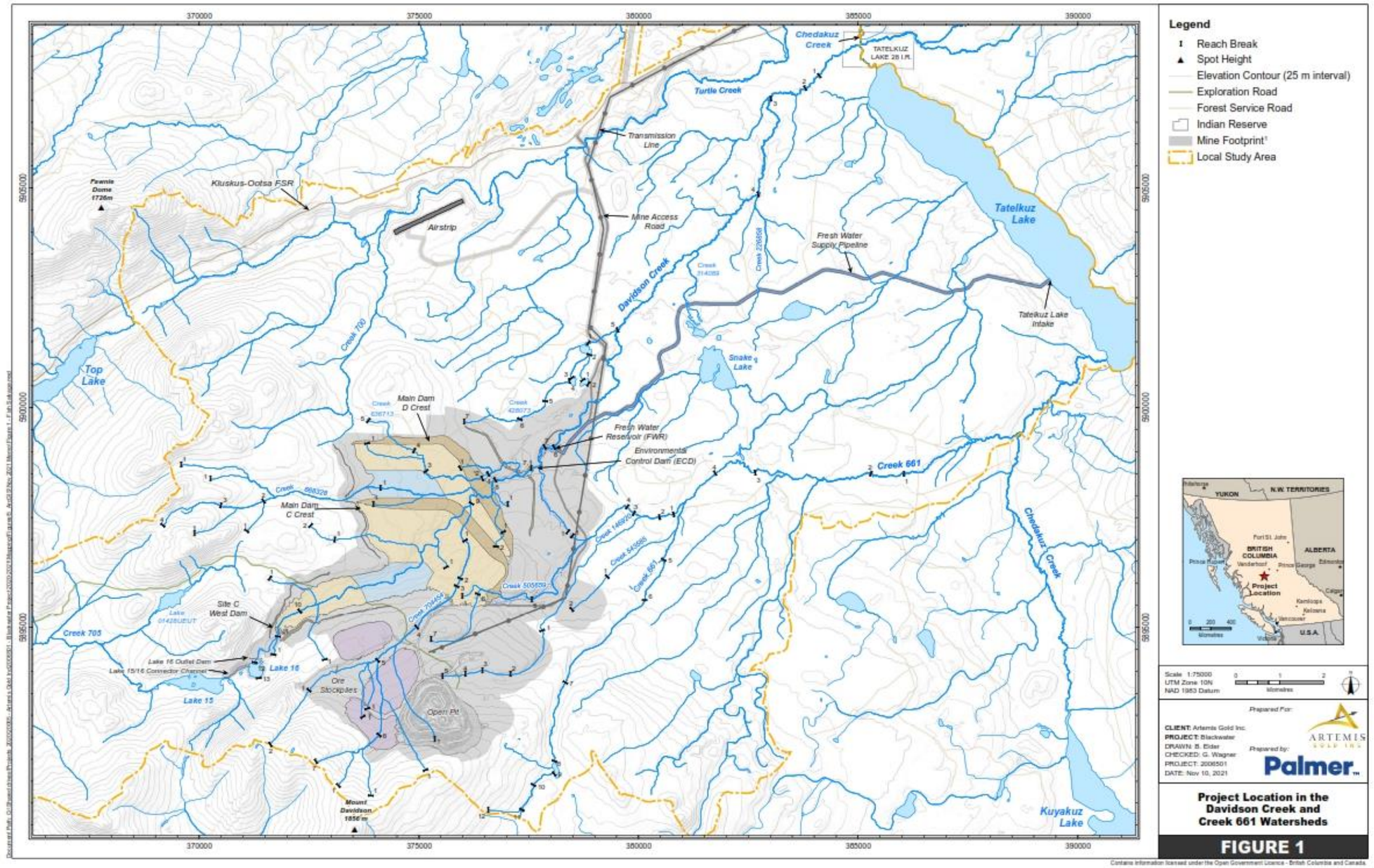

Figure 1. Project Location in the Davidson Creek and Creek 661 Watersheds

3. FOLLOW UP PROGRAM 3.14.2: DAVIDSON CREEK FISH POPULATIONS

3.1 Background and Approach

The Application/EIS determined fish habitat under the mine footprint and upstream of it will be permanently altered or lost during construction, operations, closure, and post-closure phases (Section 5.3.8 Fish of the Application/EIS). Mitigation for this lost fish habitat includes offsetting by constructing overwintering and rearing ponds near the mid-reaches of Davidson Creek and by creating or restoring additional fish habitat elsewhere in the local and regional area, including the lake connector channel described in **Section 2 Follow Up Program 3.14.1: Lakes 15 and 16 Parasites and Pathogens Study**. With mitigation, these fish habitat losses were determined to be not significant largely because the lost habitat will be replaced by new fish habitat.

Development of the Project also will cause a reduction in the catchment area of Davidson Creek, and, if un-mitigated, a corresponding reduction in flows in Davidson Creek downstream of mine (Section 5.3.8 Fish of the Application/EIS). To mitigate this effect on flows, flow augmentation is proposed using a Freshwater Supply System (FWSS) that will pump water from Tatelkuz Lake to a freshwater reservoir (FWR) adjacent to Reach 6 of Davidson Creek (**Figure 1**).

In the Application/EIS, water temperature in Davidson Creek during Operations and Closure was predicted to increase in the winter and decrease in the summer as a result of flow augmentation using water pumped from below the epilimnion of Tatelkuz Lake. These temperature changes were predicted to reduce incubation times of kokanee embryos and result in earlier emergence of kokanee fry, increase growth rates of juvenile Rainbow Trout overwintering in Davidson Creek, lengthen incubation time of Rainbow Trout embryos and delay fry emergence, and reduce growth of juvenile Rainbow Trout. These potential residual effects were predicted to be not significant because they are low in magnitude and local in extent, and are just as likely to produce positive effects on fish growth, survival, and reproduction as negative effects. Updated modelling indicates that water temperatures in lower Davidson Creek under a variety of modelled flow scenarios are expected to be within ±1°C of the baseline temperatures between 93% and 99% of the time (Palmer 2022b). Water temperature monitoring is part of the Aquatic Effects Monitoring Program (AEMP)(ERM 2022). The AEMP details the monitoring procedures for each phase of mine life for the effective interception, conveyance, diversion, storage, and discharge of water (e.g., contact and non-contact) on the mine site. In addition, in-situ water quality measurements, including temperature, will be collected at each monitoring site, as indicated in **Section 3.2.1.1 Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance** and in **Section 3.2.3.2 Kokanee Spawner Abundance**.

Another potential effect of reduction in Davidson Creek water flows, and flow augmentation to mitigate the loss, is potential disruption of salmonid fish homing in Davidson Creek (Section 5.3.8 Fish of the Application/EIS). The likelihood of some disruption of homing in both Rainbow Trout and kokanee is high, although it is difficult to predict the magnitude of that disruption. The residual Project effect of flow augmentation using Tatelkuz Lake water on homing behaviour is assessed as not significant for both indicator species, because it is assessed as unlikely, and capable of being managed should it occur.

For the first 5 years of mine operations, the FWR will store water diverted from the upper portions of Davidson Creek, and mine contact water that is suitable for release into Davidson Creek. Water will be released from the FWR into Davidson Creek to meet a defined Instream Flow Need (IFN) for Rainbow Trout and kokanee life stages.

Starting in Year 6 of Operations, water will be pumped from Tatelkuz Lake, and the FWR will also store and release water that is a mix of water diverted from the upper catchment, treated effluent, and Tatelkuz Lake water. The IFN identifies the timing of the streamflow rate in Davidson Creek that would be required to provide adequate habitat to instream aquatic species throughout the year. The IFN is also to be monitored

as part of the MSDP. Water flow data collected for the IFN program also will be used in this monitoring program for Rainbow Trout overwintering habitat (**Section 3.2.2.1**) and kokanee fry outmigration (**Section 3.2.3.3**). Commitments as they relate to IFN for Davidson Creek will be addressed under a separate cover in response to Condition 3.8 of the Decision Statement. Further information on the Instream Flow Needs study is available through a memo entitled *Blackwater Fisheries Offsetting Plan Instream Flow Needs for Davidson Creek* (Palmer 2021a). This memo contains a table presenting the fish species, life stages, and periodicity for Davidson Creek.

The primary purpose of Condition 3.14.2 follow-up program is to determine if the Project, and specifically flow augmentation from the FWSS, is affecting fish community composition, overwintering abundance and habitat use, and spawner populations during different Project phases. No specific changes to these variables were predicted in the EIS/EA because all were assessed as being not significant after mitigation. Several indictors require monitoring, with the aim of detecting changes that may be attributable to flow augmentation. The follow-up program currently proposes monitoring of the following:

- Community Composition
    - Young-of-Year (YoY) and juvenile Rainbow Trout abundance (Condition 3.14.2.1);
    - Adult kokanee and Rainbow Trout abundance and genetics (Condition 3.14.2.1 and 3.14.2.3);
- Rainbow Trout overwintering abundance and habitat availability (Condition 3.14.2.2);
- Spawner Populations
    - Rainbow Trout adult spawner and redd abundance (Condition 3.14.2.3);
    - Kokanee adult spawner and redd abundance (Condition 3.14.2.3); and
    - Kokanee fry outmigration assessment (surrogate metric for Condition 3.14.2.3).

Periphyton and benthic invertebrate sampling is outside the scope of 3.14 Fisheries Follow-up Program. However, both variables will be monitored as part of the Aquatic Effects Monitoring Program for the Project. The findings of other monitoring programs (e.g., the AEMP, ERM 2022], 3.15 Follow-up Program), including periphyton and benthic invertebrate monitoring, will be considered when interpreting the results of the Fisheries Follow-up Programs.

Field programs were completed in 2021 and 2022 (Palmer 2022a) and are planned for 2023 to build upon and validate baseline information on fish populations in Davidson Creek (AMEC 2013), and to refine and select the field sampling methods that will be carried forward for the follow-up program through the life of the mine. Results of the 2021 field program are available in the Blackwater Gold Project Fish and Aquatic Resources 2021 Field Survey Report (Palmer 2022a). The scope of 2022 field programs and the long-term 3.14.2 Follow-up Program study design will be further developed in consultation with First Nations.

3.2 Study Design

3.2.1 Community Composition (Sub-condition 3.14.2.1)

3.2.1.1 Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance

To estimate absolute abundance of YoY (age 0+) and juvenile (and 1+ to 3+) Rainbow Trout, electrofishing surveys will be conducted at ten sites in Davidson Creek. At least one site will be located in each of the six reaches of Davidson Creek, as shown in **Figure 2.**

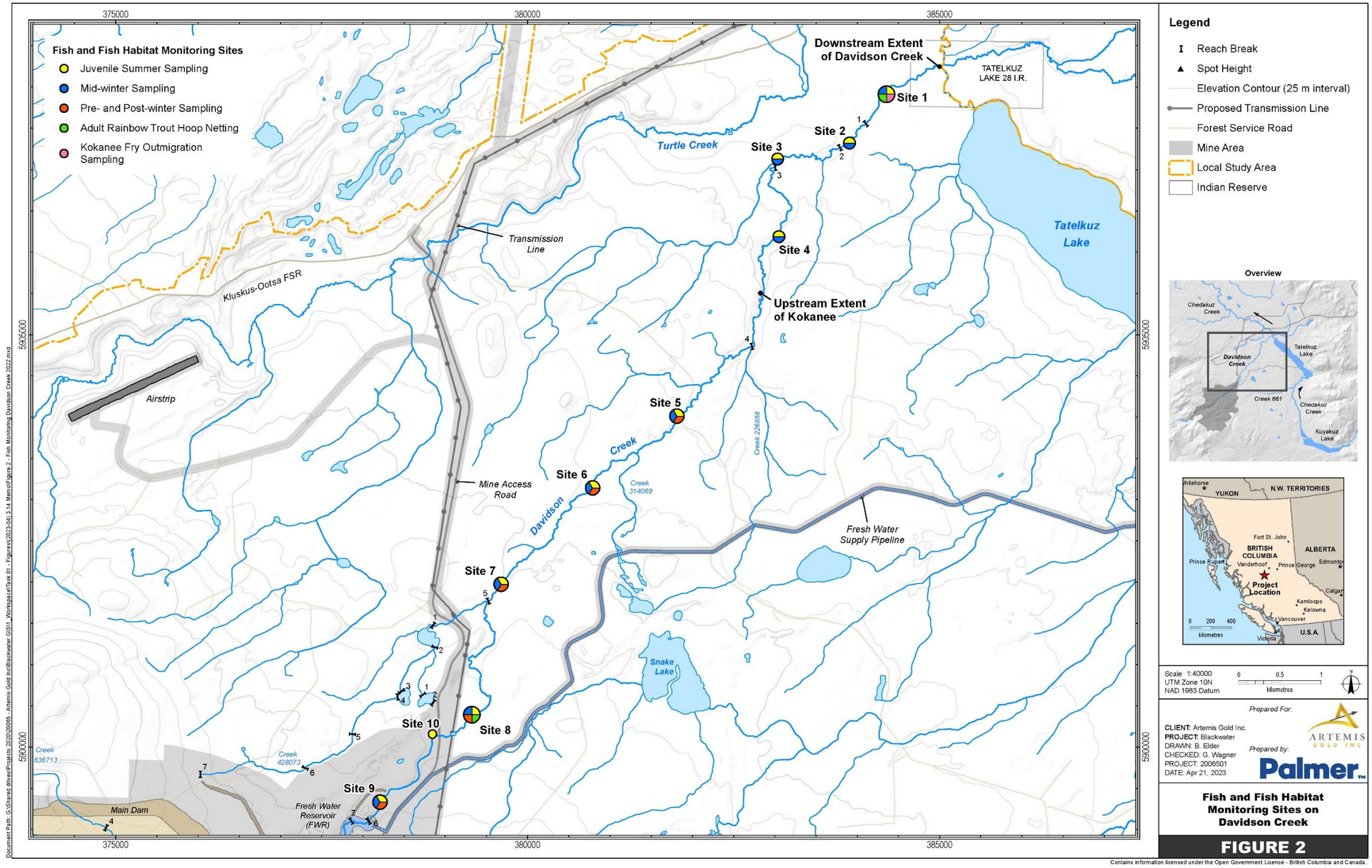

Figure 2. Fish and Fish Habitat Monitoring Sites on Davidson Creek.

At each site, sampling will include:

- Three-pass depletion electrofishing of accessible stream lengths of 100 m, isolated with block nets;
- Identification of species and collection of length, weight, and body condition data for all fish captured;
- Scale samples from a subset of fish for ageing analysis
- Measurement of channel dimensions and calculation of mean values of bankfull width, wetted width, water depth, and gradient; and,
- In-situ water quality measurements (i.e., temperature, dissolved oxygen, pH, and conductivity).

Sampling will occur during in late July, during the period after Rainbow Trout YoY emergence and before the arrival of spawning kokanee adults in the downstream reaches of Davidson Creek. Sampling timing will be confirmed by calculating accumulated thermal units (ATU) using local stream and air temperature data. This calculated value will be compared to available emergence timing and ATU literature to estimate the timing window and variability of Rainbow Trout emergence dates. Results will be used to refine the juvenile sampling window for a given year. Observed emergence timing from field surveys will be compared to modelled emergence dates to validate and refine estimated emergence and ATU calculations.

At each site, sampling effort, electrofisher specifications, and catches will be recorded. Catch per unit effort (CPUE), relative abundance (number of fish per unit effort) and density metrics (number of fish per m^2) will be calculated and analyzed, as described in **Section 3.3.1.1 Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance**. Sampling frequency is described in **Section 3.4 Frequency and Duration**. Additionally, fish habitat will be assessed at each site via Fish Habitat Assessment Procedure (FHAP) habitat assessments (Johnston and Slaney 1996).

Scale samples will be collected from a subset of fish for juvenile Rainbow Trout abundance study (captured using 3-pass depletion electrofishing) to estimate fish age. Scales will be collected to align with the BC RISC Fish Collection Methods and Standards. Scales will be collected above the lateral line and behind the dorsal fin with a sharp knife. At least 10 scales will be collected from each fish. Using the knife, scales will be pulled from posterior to anterior such that scales can be separated. Scales will be placed individually in labelled coin envelopes and air dried for laboratory analysis.

The summer fish inventory measurement endpoints will include an inventory of the fish community and fish health (**Table 3-1**).

Table 3-1: Measurement and Assessment Endpoints for the Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance

Measurement Endpoint	Assessment Endpoint
Fish community and abundance	Catch Per Unit Effort (CPUE), relative abundance, and fish density (fish/100 m^2) for each identified species, population structure
Fish health	Length, weight, condition, age
Fish habitat	Mesohabitat unit boundaries, channel dimensions, stream morphology, cover, vegetation, substrate composition, riparian habitat

3.2.1.2 Davidson Creek Adult Kokanee and Rainbow Trout Abundance

Adult Rainbow Trout and kokanee abundance will be estimated from spawner surveys, described in **Section 3.2.3 Davidson Creek Spawner Populations (Sub-condition 3.14.2.3)**.

3.2.1.3 Rainbow Trout and Kokanee Genetic Structure and Diversity

The requirement (3.14.2.1) to monitor Rainbow Trout and kokanee genetic structure and diversity will be met by employing the genetic study methodology used in the baseline studies to the extent possible and where feasible, as described in Taylor (2012). This sampling methodology is described here, although it is recognized that adaptations may be warranted to account for advances in laboratory methods and genetic analysis techniques, based on subject matter expert guidance.

Samples of genetic material will be taken from Rainbow Trout and kokanee populations that occur in lower Davidson Creek. As described in the Project's baseline studies, two sub-populations of Rainbow Trout are understood to occur in Davidson Creek, separated by a cascade barrier in Reach 11: one in the lower reaches (i.e., downstream of the barrier) and one in the upper reaches and Lake 16 (i.e., above the barrier). This monitoring condition is specific to the effects on fish residing in Davidson Creek downstream of the mine site. Therefore, only the lower Davidson Creek sub-population of Rainbow Trout will be assessed as part of this program. Only a single identified population of kokanee occur in Davidson Creek, so this population will also be assessed.

Rainbow Trout tissue samples will be taken from stream-resident juvenile fish caught in Davidson Creek during summer electrofishing sampling (**Section 3.2.1.1 Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance**). Kokanee tissue samples will be taken from mature adult fish returning to spawn. Adult, pre-spawn kokanee will be sampled, rather than juvenile lake-resident kokanee, to ensure that fish sampled are part of the Davidson Creek spawning population, since Tatelkuz Lake likely contains fish from multiple stream-spawning populations. Migrating pre-spawn kokanee adults will be captured in lower Davidson Creek using seine nets over an area with no observed actively spawning fish. A minimum of 30 samples will be collected for each species (i.e., 30 Rainbow Trout and 30 kokanee). All fish sampled will be identified to the species level, measured for length and weight, sampled for tissue, then released back into stream site from which they were captured.

Tissue samples will be adipose and/or caudal fin clips, depending on tissue analysis volume requirements. Fin clips will be immediately placed into labelled vials containing 95% ethanol to minimize DNA degradation.

Polymerase chain reactions (PCR) of microsatellite DNA will be carried out on ten microsatellite loci of Rainbow Trout and six loci of kokanee, previously identified in the baseline genetic analysis (Taylor 2012).

Measurement and assessment endpoints have been selected with a focus on non-lethal monitoring of the fish community. The genetic measurement endpoint is a deviation from a population equilibrium (i.e., allele frequency is stable between generations; **Table 3-2**).

Table 3-2: Measurement and Assessment Endpoints for the Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance

Measurement Endpoint	Assessment Endpoint
Genetic structure	Deviation from population equilibrium

3.2.2 Juvenile Rainbow Trout Overwintering (Sub-condition 3.14.2.2)

DS Condition 3.14.2 requires "absolute abundance of overwintering Rainbow Trout juveniles". However, sampling of fish populations under ice during winter is logistically challenging and can present risks to human and fish health. Therefore, following discussion with representatives of IAAC and DFO (17 November, 2021), a technically feasible alternate assessment strategy was developed.

Juvenile Rainbow Trout overwintering surveys will include two assessment methods:

1. Mid-winter assessment of relative fish abundance using underwater cameras and evaluation of habitat characteristics at nine sites in Davidson Creek; and
2. Fall pre-overwintering and spring post-overwintering relative abundance surveys using three-pass electrofishing at five sites in Davidson Creek.

The first year of monitoring occurred in March 2022 in accordance with the methodology presented in **Section 3.2.2.1 Mid-winter Assessment**. Results of this study are available in the Blackwater Gold Project Fish and Aquatic Resources 2021 Field Survey Report (Palmer 2022a). Future years of monitoring will consider the methods and outcomes of previous years' of monitoring to optimize data collection.

3.2.2.1 Mid-winter Assessment

Winter surveys will be conducted to assess overwintering abundance and habitat use at ten sites in Davidson Creek (Site 1 to Site 10; **Figure 2**). Potential overwintering sites were identified using winter 2022 field survey information, field reconnaissance and unmanned aerial vehicle (UAV) imagery from summer 2021 surveys (**Section 3.2.3 Davidson Creek Spawner Populations (Sub-condition 3.14.2.3)**), and baseline stream habitat data. The mid-winter overwintering abundance and habitat assessment program will include measurement and assessment of juvenile Rainbow Trout overwintering abundance at each selected site using underwater cameras. Cameras and underwater lights will be placed in an overwintering deep pool habitat within each site. Stationary high-quality video will be recorded for a standardized period (e.g., 60 minutes) during daytime. The video will be reviewed to determine relative abundance using the established metric of maximum count of individuals observed simultaneously in a video frame (MaxN; Hitt et al. 2020).

At each site, recording time, camera and lighting specifications, habitat measurements, and water chemistry parameters will be recorded. MaxN values will be determined and habitat suitability will be assessed, as described in **Section 3.3.2.1 Mid-winter Assessment**. Sampling frequency is described in **Section 3.4 Frequency and Duration**. The mid-winter overwintering survey measurement endpoints will include assessment of fish abundance and habitat suitability (

Table 3-3).

Table 3-3: Measurement and Assessment Endpoints for the Mid-winter Juvenile Rainbow Trout Overwintering Assessment

Measurement Endpoint	Assessment Endpoint
Fish abundance	Maximum count of individuals observed simultaneously in a video frame (MaxN)
Habitat suitability	Flowing water is present and dissolved oxygen levels are greater than 5 mg/L

3.2.2.2 Fall Pre-overwintering and Spring Post-overwintering Assessment

Pre-and post-overwintering surveys will include three-pass electrofishing assessments of five sites in Davidson Creek (Site 5 to Site 9; **Figure 2**). The methodology will follow that previously described in **Section 3.2.1.1 Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance** for summer Rainbow Trout assessment.

Sampling timing will vary, depending on seasonal differences in water temperature, ice cover, and discharge. Fall sampling will generally be conducted in late September before water temperatures drop below 5°C and before ice cover forms. Spring sampling will be conducted shortly after ice-off once temperatures reach 5°C and before spring freshet precludes effective electrofishing.

Notably, the five sites selected for assessment are less than the nine identified for summer YoY and juvenile sampling and overwintering assessment. This is due to the expected presence of incubating kokanee embryos in the gravel substrates of the lower reaches of Davidson Creek. Electrofishing sampling will not be conducted in potential kokanee spawning areas to minimize mortalities. Therefore, the four furthest-downstream sites (DCOH1 to DCOH 4) will not be sampled in fall or spring.

At each site, sampling effort, electrofisher specifications, and catches will be recorded. CPUE, relative abundance (number of fish per unit effort) and density metrics (number of fish per m^2) will be calculated and analyzed, as described in **Section 3.3.2.2 Fall Pre-overwintering and Spring Post-overwintering Assessment**. Sampling frequency is described in **Section 3.4 Frequency and Duration**. The pre- and post-winter overwintering survey measurement endpoints will include assessment of fish abundance fish health (**Table 3-4**).

Table 3-4: Measurement and Assessment Endpoints for Fall Pre-overwintering and Spring Post-overwintering Assessment

Measurement Endpoint	Assessment Endpoint
Fish community and abundance	Catch Per Unit Effort (CPUE), relative abundance, and fish density (fish/100 m^2) for each identified species, population structure
Fish health	Length, weight, condition

3.2.3 Davidson Creek Spawner Populations (Sub-condition 3.14.2.3)

DS Condition 3.14.3 requires assessment of "characteristics of spawner populations through surrogate monitoring metrics including size at 50% maturity". However, evaluating size at 50% maturity can require intensive lethal sampling to assess gonad development. Such an effort would require annual lethal sampling to achieve a sample size that can be used for statistical interpretation. This loss could negatively impact the Rainbow Trout and kokanee population. Further, such sample targets (i.e., sample sizes of spawner populations) require fishing efforts that would encompass multiple passes to achieve the desired metrics during sensitive timing windows for the target species, and fishing efforts will be confounded by the migratory life history and lake-residence period of Rainbow Trout. Therefore, following discussion with representatives of IAAC and DFO (17 November, 2021) and additional consultation with First Nations and their technical consultants, an assessment of size at 100% maturity is proposed as an alternative to size at 50% maturity. To measure size at 100% maturity, body size (i.e., fork length and weight) of migrating pre-spawn fish (in the case of Rainbow Trout) or postorbital-hypural length of post-spawn mortalities (in the case of kokanee), that are assumed to be 100% mature, will be measured and evaluated. This metric will be used to directly evaluate change in body size of mature fish that spawn in Davidson Creek.

Additional annual data collection will occur in support of the Effectiveness Monitoring Plan (EMP) (Palmer 2023a) for dissolved free amino acids (DFAA) that are used by salmonids for olfactory imprinting and homing back to natal streams. These DFAA data also support CEAA Decision Statement Sub-condition 3.14.2.3 to characterize spawner populations. Analysis will involve evaluating the concentrations of up to 22 different DFAAs in Davidson Creek. DFAA results from 2021-2022 (Palmer 2022a, Palmer 2023b) represent pre-construction data and will be used to determine if those levels in Davidson Creek are altered by the addition of Tatelkuz Lake water once the FWSS is operational.

3.2.3.1 Rainbow Trout Spawner Abundance

Adult Rainbow Trout spawner abundance and distribution will be directly assessed with two methods: capture of migrating spawners using hoop nets and visual assessment of spawners and redds.

The first year of sampling occurred in spring 2021 in accordance with the methodology described below. Results of this study are available in the Blackwater Gold Project Fish and Aquatic Resources 2021 Field Survey Report (Palmer 2022a). Future years of monitoring will consider the methods and outcomes of previous years' of monitoring to optimize data collection.

Hoop Net Sampling

Direct sampling of migrating fish will involve intercepting mature fish during their annual pre-spawning migration into Davidson Creek. Migrating adult fish will be captured and counted using bi-directional hoop nets located at two sites: one site the furthest downstream reach of Davidson Creek, near its confluence with Chedakuz Creek (Site 1) and one site (Site 8) immediately downstream of the proposed mine access road in middle Davidson Creek (**Figure 2**).

The hoop netting program will include the following tasks:

- Installation and maintenance of bi-directional (i.e., upstream- and downstream-facing) hoop nets at each site;
- Regular (i.e., at least twice-daily) hoop net checks for fish, including:
    - Marking mature Rainbow Trout (i.e., those producing milt or eggs) with movement direction- and site-specific coloured and numbered floy tags to evaluate residence time and movement;
    - Enumeration of Rainbow Trout and measurement of weight, length, sex, and body condition;
    - Testing for spawning ripeness (i.e., exuding milt or eggs) by lightly pressing on the abdomen; and
    - Scale samples from a subset of 30 fish for ageing.
- In-situ daily water quality measurement (i.e., temperature, dissolved oxygen, pH, and conductivity)
- Daily velocity measurements at the hoop net sites.

Scale samples will be collected from a subset of 30 fish for the Rainbow Trout spawner survey (captured using hoop nets). Scales will be collected to align with the BC RISC Fish Collection Methods and Standards. Scales will be collected above the lateral line and behind the dorsal fin with a sharp knife. At least 10 scales will be collected from each fish. Using the knife, scales will be pulled from posterior to anterior such that scales can be separated. Scales will be placed individually in label coin envelopes and air dried for laboratory analysis.

Fish capture data will be used to evaluate spawner numbers and distribution, as described in **Section 3.3.3.1 Rainbow Trout Spawner Abundance**. Sampling frequency is described in **Section 3.4 Frequency and Duration**. The Rainbow Trout spawner abundance measurement endpoints will include the number of migrating adult fish captured in hoop nets (**Table 3-5**).

Table 3-5: Measurement and Assessment Endpoints for Rainbow Trout Spawning Hoop Net Sampling

Measurement Endpoint	Assessment Endpoint
Fish abundance	Total number of adult spawners captured in hoop nets returning to Davidson Creek, and a subset of ageing samples to characterize the population
Fish distribution	Number of adult spawners captured at each hoop net site in Davidson Creek
Fish size at 100% maturity	Weight, length, and age at 100% maturity of Rainbow Trout captured in hoop nets returning to Davidson Creek

Bank Walk Visual Assessment

Bank walk surveys also will be conducted during the peak spawning period (i.e., May and June) and post-spawn to estimate redd counts, to identify areas of high spawning activity, and to determine the spawning distribution of Rainbow Trout within Davidson Creek. Bank walk surveys will be completed on an annual basis during Construction and Operation (more details on frequency and duration are discussed in **Section 3.4 Frequency and Duration**).

The bank walk surveys will be completed at the two hoop netting sites in Davidson Creek, downstream of the FWR (Site 1 and Site 8; Figure 2). Each survey zone will be located within the same reach as the hoop net site, although some site-specific adjustment will be needed to establish effective monitoring locations. Each survey zone will consist of a 200-m long section that contains likely spawning locations based on baseline FHAP and RISC 1:20,000 habitat assessments from the baseline program (Johnston and Slaney 1996; RISC 2001; AMEC 2013) and field reconnaissance.

An additional eight bank walk survey sites will be performed at each of the remaining survey sites (Site 2 to Site 7, Sites 9 and 10). Thes same survey methodology as detailed above will be performed, assessing 2 km (i.e., 10 x 200 m) of the stream. This information will provide an improved understanding of the distribution of Rainbow Trout spawning locations. Snorkel surveys may be completed in addition to the bank walks if safe access is possible. However, abundant woody debris and high freshet flows are expected to limit the feasibility of snorkel surveying.

Each section will be surveyed at all sites once per week during the annual Rainbow Trout spawning period. Exact survey timing and duration will depend on annual variability in run timing. Each surveyed section will be walked once in an upstream direction by a pair of trained observers. Snorkel surveys will be conducted, if stream conditions allow for safe snorkelling, by the same pair of trained observers, with one crew member floating in a downstream direction while enumerating spawning Rainbow Trout and redds and one crew member providing safety oversight from the bank. Using both methods, observers will record the number of redds and adult fish in each section. Geographic coordinates will be recorded for each redd (or redd complex if multiple redds occur in close proximity).

Fish and redd observation data will be used to evaluate spawner numbers and distribution, as described in **Section 3.3.3.1 Rainbow Trout Spawner Abundance**. Sampling frequency is described in **Section 3.4 Frequency and Duration**. The Rainbow trout bank walk assessment measurement endpoints will include assessment of fish and redd abundance and density (**Table 3-6**).

Table 3-6: Measurement and Assessment Endpoints for Rainbow Trout Spawning Bank Walk

Measurement Endpoint	Assessment Endpoint
Fish abundance	Number of adult spawners observed and their density at each survey sites
Fish distribution	Relative number of adult spawners observed at each survey site
Redd abundance	Relative number of redds observed and their density in each survey reach.

3.2.3.2 Kokanee Spawner Abundance

Spawner Survey

Kokanee spawner surveys in Davidson Creek will include underwater camera surveys and bank walks to determine the total abundance of mature kokanee entering Davidson Creek. The surveys will begin at the

confluence of Chedakuz and Davidson creeks, extending through Reaches 1 to 4 of Davidson Creek (**Figure 2**), representing the full extent of documented kokanee spawning.

Bank walk surveys will be conducted approximately once per week for the duration of the annual pre-spawning migration and spawning periods, typically beginning in late July and ending in early September, depending on environmental factors. Bank walk spawner and redd surveys that encompass the entire migration and spawning period allows the use of an area-under-the-curve (AUC) estimate of kokanee spawner abundance, described further in **Section 3.3.3.2 Kokanee Spawner Abundance**.

The kokanee spawner survey field program will include the following tasks:

- Installation of an underwater camera (that will operate 24/7) paired with a fish fence near the confluence of Davidson Creek and Chedakuz Creek to estimate the total abundance of fish entering Davidson Creek;
- Visual assessment of kokanee spawners and redds via bank walks. Information collected during the bank walk surveys will include:
    - Kokanee counts (characterizing fish as either holding/migrating, spawning, spent, or carcass); and
    - Redd abundance, approximate location, and substrate type and size.
    - Depending on the distribution (grouped versus evenly spread), the downstream and upstream extent of spawning groups and redds can be marked to delineate active spawning areas of the stream.
- Measurement of fork and postorbital-hypural lengths from carcasses;
- Collection of tissue samples (i.e., otoliths and fin clips) from carcasses; and
- In-situ water quality (i.e., temperature, turbidity, dissolved oxygen, pH, and conductivity).
- Measurement of sediment composition.

Surveys will be conducted in the upstream direction to reduce disturbing the sediment to maximize fish observability. As observers advance along the creek, live and dead observations will be recorded; live fish will be classified as holding/migrating (lively, fresh in appearance, absence of wear, unpaired male and female) or spawning (paired male and female) or spent (lethargic, presence of wear). Male and female mortalities will be counted and marked by chopping the fish posteriorly above the adipose fin to avoid recounts in subsequent surveys. Environmental conditions (e.g., percent bankfull, water temperature, water clarity, brightness, cloud cover, and precipitation intensity) will be documented.

A value of observer efficiency (OE) will be applied to each reach on each day to account for negative bias associated with spatial/temporal changes in observability. OE is a value determined each day that is calculated by comparing daily survey counts made by two observers, with multiple consecutive counts being performed. The mean of all paired observer counts at site is then compared to the highest point count to determine observer efficiency. This factor is then applied to the observed values for each reach / stratum on each day. These correction factors are critical to AUC estimates because it allows inter- and intra-annual comparisons of fish counts within and between streams.

Substrate size will be determined by taking at least three grab samples along transects at the upstream and downstream ends of each spawning reach (minimum 6 total samples). Sediments from each sample will be sieved to determine substrate size ratios. Transect locations will be marked with fixed stakes for year-over-year comparison of sediment transport.

The first year of sampling occurred between July and September, 2021 in accordance with the methodology described above to capture the start and end of the kokanee spawner migration into Davidson Creek. Results of this study are available in the Blackwater Gold Project Fish and Aquatic Resources 2021 Field

Survey Report (Palmer 2022a). Future years of monitoring will consider the methods and outcomes of previous years' of monitoring to optimize data collection.

Tissue Sampling

Tissue samples collected opportunistically from carcasses and from live fish will be used for age measurement and for genetic analysis.

Otolith samples will be opportunistically gathered for age analyses to determine the age ranges of kokanee spawners. The proposed target minimum sample size for otoliths is n=100 samples, although more samples could be collected, if carcasses are available, to increase the confidence of statistical analyses. Effort will be made to collect samples throughout different times of the migration period where possible (e.g., 10 per day for 10 days) to reduce temporal bias. These ageing structures will be removed and prepared (e.g., mounted, polished, or otherwise treated) as necessary. Age will be determined by counting the number of annuli through a compound microscope. Age data will be analyzed to calculate a length-at-age relationship for mature fish. This information will be used to determine length and age at 100% maturity for the population.

Fin clip samples will also be taken from kokanee spawners during the spawning survey to perform genetic analysis of the populations, as described in **Section 3.2.1.3 Rainbow Trout and Kokanee Genetic Structure and Diversity**. Similar to otoliths, the proposed target minimum sample size for fin clips is n=100 samples. Fin clips will be taken from live, pre-spawn, adult fish, gathered using seine net pulls from Davidson Creek. Migrating and holding fish will be targeted; no fish will be collected from active spawning areas. Fin clips will be stored in 95% ethanol for genetic analysis. PCR testing will be performed, including identification of microsatellite loci to be compared. Statistical tests will be performed on these data to estimate deviation from population equilibrium and population differentiation.

Kokanee spawner counts, redd counts, length measurements, and age data will be used to evaluate total escapement, distribution of spawners, and size at maturity, as described in **Section 3.3.3.2 Kokanee Spawner Abundance**. Sampling frequency is described in **Section 3.4 Frequency and Duration**. The kokanee bank walk visual assessment measurement endpoints will include assessment of fish abundance and size at 100% maturity (**Table 3-7**).

Table 3-7: Measurement and Assessment Endpoints for Kokanee Spawning Bank Walk

Measurement Endpoint	Assessment Endpoint
Fish abundance	Relative number of adult spawners and their density observed in each survey reach
Redd abundance	Relative number of redds and their density observed in each survey reach; Substrate size at upstream and downstream ends of each survey reach
Fish size at 100% maturity	Length and age at 100% maturity of kokanee returning to Davidson Creek
Substrate composition	Mean percent composition of substrate within each spawning reach of Davidson Creek

3.2.3.3 Kokanee Fry Outmigration Survey

Kokanee fry outmigration abundance is an indicator of the success of the previous year's kokanee spawning activity and the overwinter survival of in-gravel kokanee embryos. As such, a spring kokanee fry outmigration survey in Davidson Creek will be completed to assess abundance as a surrogate metric for adult kokanee spawning success.

Kokanee fry outmigration assessment will be completed using a sub-sampling mark-recapture method at Site 1 (Figure 2). Sampling will involve deploying fine-mesh nets of known dimensions into the channel at predetermined locations, according to the methods of Fraley and Clancey (1984). Each net will be sampled at a set interval and the fry captured will be enumerated and recorded. The duration of the sampling period will be adjusted based on the numbers of fry netted and/or the amount of debris present, although it is expected to last approximately four weeks, based on literature review. Data including date, time, water depth, water temperature and weather conditions will be recorded. Sampling will be conducted once per week, between 19:00 hours and 02:00 hours as most (>90%) fry emigration occurs during this period (Thorp 1987, Manson 2005).

Capture efficiency of the nets will be determined using a mark-recapture approach by marking captured fry with Bismarck Brown Y and releasing them upstream of the capture location. Recaptured marked fish will be counted and the proportion of recaptured fish will indicate the trap's effectiveness.

To inform sampling timing, accumulated thermal units (ATU) will be calculated using continuously measured stream and air temperature data. The results will be compared to available emergence timing and ATU literature to estimate the timing window and variability of kokanee fry emergence dates. Results of this calculation will inform an approximate fry sampling window for a given year. Observed emergence timing data will be compared to modelled emergence to help validate and refine estimated emergence and ATUs.

Total fry emigration for each sampling period will be calculated and estimates for the entire emigration period extrapolated using flow rates, stream channel dimensions, and recapture rates, as described in **Section 3.3.3.3 Kokanee Fry Outmigration Survey**. Sampling frequency is described in **Section 3.4 Frequency and Duration**. The kokanee fry outmigration measurement endpoints will include assessment of relative fish abundance and timing (**Table 3-8**).

Table 3-8: Measurement and Assessment Endpoints for Kokanee Fry Outmigration Survey

Measurement Endpoint	Assessment Endpoint
Fish abundance	Calculated total fry abundance for the outmigration period
Outmigration timing	Estimated start, finish, and peak timing of outmigration movement

An underwater camera was deployed in Davidson Creek in late May, 2021 to estimate timing of kokanee fry outmigration to Tatelkuz Lake (Palmer 2022a). This pilot program was important to determine the optimal window for future outmigration surveys. No kokanee fry were detected on the camera recordings in 2021. Therefore, these results will be considered for future years of monitoring and the timing of the surveys will be adjusted (Palmer 2022a). Future years of monitoring will consider the methods and outcomes of previous years' of monitoring to optimize data collection.

3.3 Data Analysis

Statistical analyses will be used to evaluate changes over time. Power analyses have been performed, as appropriate, to support a study design that will allow for between-year comparisons and longer-term trend analysis. Between-year comparison (i.e., paired comparison between two separate annual datasets) will be completed to identify statistically significant differences in mean values using Analysis of Variance (ANOVA), or if the data are not normally distributed the equivalent non-parametric statistical test (e.g., Wilcoxon Signed-Rank Test). Prior to performing the ANOVA, tests were run to ensure that ANOVA assumptions were satisfied (i.e., normality, homogeneity of variance) for the CPUE data by year. (Shapiro-Wilk test for normality W=0.935, p=0.1565; Levene's test for Homogeneity of Variance F=1.0338 p>F=0.40). All statistical analyses will be performed using the R statistical system (R Development Core Team 2011). The significance level (α) = 0.05

will be used for all statistical tests, except as noted in specific analyses. In addition, year over year change will be assessed qualitatively, by comparing with the baseline data.

For longer time scale (i.e., five years and onwards), non-parametric Mann-Kendall temporal trends testing will be used to determine if there are significant temporal trends in any given monitoring metric, and if so, the direction and statistical significance of temporal trend. The sensitivity of the Mann-Kendall trends test increases with an increasing number of time steps (i.e., consecutive years of data) and it is considered that somewhere between five- and ten-time steps are a minimum requirement. Trends analyses will therefore begin following the fifth year of this monitoring program.

Additional metric-specific tests are described in the following section.

3.3.1 Community Composition

3.3.1.1 Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance

Fish data will be transcribed from field notes and submitted to the ENV Fisheries Data Submission site.

Catch-Per-Unit-Effort (CPUE)

Fish community data will be summarized by calculating CPUE for each individual fishing effort and fish species captured. The CPUE will be calculated as the number of fish captured per sampling device per unit time as follows:

Electrofishing:

$$CPUE = \text{number of fish caught} * [100/(\text{electrofishing effort, hr})]$$

The CPUE is an index of relative abundance that can be used to compare fish populations over time with the assumption that catch is proportional to the amount of effort for each gear-type used. For effects assessment, a Mann-Kendall temporal trends test will be undertaken for each site and compared between control and impact sites of the AEMP (ERM 2022). This trends analysis will require a minimum of five years of sequential monitoring data.

Density

Fish density values will be calculated by dividing the total number of fish caught in a closed site by the area of that site. Site area will be determined by multiplying a mean wetted width of the site by the length of the site. A minimum of three width values will be used to calculated mean wetted width.

Density values will be calculated for the total number of fish captured (i.e., all species), Rainbow Trout life stages (i.e., YoY and juveniles), and for any other species encountered.

Population Structure

Population structures of fish will be assessed using length and weight frequency distributions and length-weight regressions.

Fish Condition

Length-weight data will be plotted to visually assess the entire data set and to identify outliers. Once outliers are visually identified, potential explanations for the outlier values will be investigated and decisions will be made to either repair the outlier, include the outlier in data analysis, or remove the outlier from further analysis.

The length/weight data from reference sites and from historical data (Appendix 2-O, Fish and Aquatic Resources 2011 – 2012 Baseline Report; Appendix 2-P, Fish and Aquatic Resources 2013 Baseline Report) will be combined and a normal reference range will be calculated using specific length increments and the associated average weight data. The following equation will be used for definition of normal range:

$$log_{10}(W) = b * log_{10}(L) + a$$

where *W* = weight (g), *L* = length (mm), *a* = the intercept of the regression defined from the reference and historical data, and *b* = the slope of the regression defined from the reference and historical data.

The regression equation for the normal range will then used to calculate the log_{10} of expected weight as:

$$log_{10}(W_E) = b * log_{10}(L) + a$$

where W_E = expected weight (g), *L* = measured fork length (mm), *a* = the defined intercept of the regression and *b* = the defined slope of the regression. Residual log_{10}(weight) values will then be calculated as the difference between expected and measured weight as:

$$W_R = W - W_E$$

where W_R = residual weight, *W* = measured weight, and W_E = expected weight. The median, 25th percentile, 75th percentile, and the interquartile range (IQR) for both negative and positive residuals will then be calculated. The upper and lower limits of the normal range of the residuals will then be calculated as:

$$NR_{UL} = 75\%ile + 1.5 \times IQR$$

$$NR_{LL} = 25\%ile - 1.5 \times IQR$$

where NR_{UL} = the upper limit of the normal range of the residuals, NR_{LL} = the lower limit of the normal range of the residuals, *IQR* = the interquartile range, 25%ile = 25th percentile value for the negative residuals, and 75%ile = 75th percentile value for the positive residuals.

The upper and lower limits of normal range for the length/weight linear regression will be calculated as:

$$log_{10}(W_{UL}) = (a - NR_{UL}) + b \times log_{10}(L\ \ )$$

$$log_{10}(W_{LL}) = (a + NR_{LL}) + b \times log_{10}(L\ \ )$$

where W_{UL} = normal range upper limit for weight (g), W_{LL} = normal range lower limit weight (g), *L* = fork length (mm), *a* = the intercept of the regression and *b* = the slope of the regression. The lower limit and upper limit of normal range will be used to assess the length/weight fit of fish from assessed sites relative to the normal range, both among years and among sites.

The relative condition (K_n) will be used as the metric for condition and will be calculated by comparing the measured weight to the expected weight from the measured length as:

$$K_n = \frac{W}{W_E}$$

where *W* = measured fish weight (g) and W_E = expected fish weight (g).

Relative condition will be statistically compared between sites. First, the distributions will be tested for normality using an Anderson-Darling test and if normally distributed, a single factor ANOVA followed by a Tukey's multiple comparison test will be computed to compare relative condition. If the data are not normally distributed, a Kruskal-Wallis test by ranks will be used with a Steel-Dwass test for multiple comparisons. Significance will be assumed when $p < 0.05$.

Site Selection Power Analysis

The number of monitoring sites was selected to provide adequate statistical power to detect a year-over-year change in the mean relative abundance value.

To inform this power calculation, the relative abundance of YoY and juvenile Rainbow Trout captured at the three 2021 survey sites are presented in **Table 3-9**. The two age groups were combined due to the low numbers captured in each reach.

Table 3-9. Relative Abundance of Combined Young-of-Year and Juvenile Rainbow Trout in Davidson Creek, 2021

Survey Site	Abundance (number of captured fish)	Relative Abundance (catch per unit effort)[1]
DCJUVRB1 – Reach 1	13	0.87
DCJUVRB2 – Reach 5	32	0.56
DCJUVRB3 – Reach 6	24	0.56

Notes:
[1] CPUE is reflected as the number of fish / 100 seconds of electrofishing.

To standardize the data used in this evaluation, single-pass electrofishing data was used for determining relative abundance, incorporating data from the original baseline work conducted in 2011 to 2013, and recent sampling in 2021. This data comparison provided a relative abundance mean/standard deviation (SD) from a total of 16 sites, for which ETA squared (i.e., measure of effect size that is commonly used in Analysis of Variance [ANOVA] models) was determined, as well as Cohens f statistic (i.e., a measure of standardized average effect in the population across all the levels of the independent variable; Cohen 1988) to use as an effect size derived from the sample population relative abundance data. An ANOVA was completed for CPUE by year using the Sum of Squares value for year and residuals from the ANOVA to evaluate an effect size of 0.576. Based on this value, the sample size required for 80% power (alpha = 0.05) is nine survey sites per year in Davidson Creek.

The abundance data collected using triple-pass depletion approach is expected to have a lower coefficient of variation over time when compared to the single-pass approach (used in the 2011-2013 baseline studies, and in 2021). As a result, the triple-pass approach has more statistical power to detect any trends or changes in abundance (George et al 2021). The proposed triple-pass method will lower the observed variance and allow for more powerful statistical evaluation. The data from the first pass of the triple-pass sampling also will be comparable to the single-pass baseline (2011-2013) and 2021 data to allow for historical comparison.

Baseline and Control Site Comparisons

Davidson Creek YoY and juvenile Rainbow Trout relative abundance and fish condition results from each site will be compared against baseline results obtained for the Application/EIS and in 2021, 2022, and 2023 (Palmer 2022a, 2023b). As well, these results will be compared with AEMP control/reference sites (ERM 2022). Deviation from control/reference levels will indicate any changes are not due to the mine.

3.3.1.2 Davidson Creek Adult Kokanee and Rainbow Trout Abundance

Adult kokanee and Rainbow Trout abundances are addressed in **Section 3.3.3 Davidson Creek Spawner Populations**.

3.3.1.3 Rainbow Trout and Kokanee Genetic Structure and Diversity

Data analysis methods for genetic structure and diversity will broadly follow those described in Taylor (2012), summarized here. However, adjustments may be required to account for laboratory-specific analysis variation.

Tests will be performed using GENEPOP (Raymond and Rousset 2001). Tests for deviations from Hardy-Weinberg equilibrium will be performed for each locus-population combination using an exact test in which probability (P) values will be estimated using a Markov chain method. Tests for genotypic linkage disequilibrium for all combinations of locus pairs within a population will also be made using a Markov chain method with GENEPOP default values. Tests for population differentiation between all pairs of populations will be performed using FST estimated as θ (Weir and Cockerham 1984) as implemented in GENETIX (Belkhir et al. 2004). Significance levels will be determined correcting for multiple simultaneous tests following Narum (2006). Basic descriptive statistics of sample size (N), number of alleles (NA), observed (HO) and expected (HE) will be compiled using FSTAT (Goudet 2001). A factorial correspondence analysis (FCA) will be used to depict genetic similarity amongst all individuals in genetic space as inferred from variation in allele frequencies using GENETIX. A hierarchical partitioning of allele frequency variation to that between drainages, among localities within drainages, and within localities will be conducted using the analysis of molecular variance approach as implemented in ARLEQUIN (Excoffier et al. 2006).

The model-based Bayesian clustering analysis within STRUCTURE (Pritchard et al. 2000) will be used to assess population structure employing the admixture model with correlated allele frequencies and a burnin of 50,000 iterations followed by an additional 100,000 iterations, replicated three times. The simulations will be run with hypothesized numbers of populations (K) ranging from K = 1 to K = s+2 where s = the number of localities sampled (i.e., a total of K = 9). STRUCTURE HARVESTER will be used to process the results from multiple runs of STRUCTURE (Earl 2011). Given the relatively small spatial scale of the study (and an expected low level of genetic differentiation) and that fish were sampled largely within what are likely to be spawning tributaries, the STRUCTURE analysis will also use the locality prior option which employs prior knowledge of where each sample was collected to assist in clustering. The LOCPRIOR model works on the principle that individuals from the same sampling location often come from the same genetic population. Therefore, the LOCPRIOR operate to assume that the sampling locations can be informative about ancestry (Hubisz et al. 2009).

These calculations will be used to generate a description of the genetic condition of the assessed populations of Rainbow Trout and kokanee. This data will be analyzed to evaluate change in allele frequency within the population over time.

3.3.2 Juvenile Rainbow Trout Overwintering

3.3.2.1 Mid-winter Assessment

Fish abundance will be estimated using the metric of the maximum count of individuals observed simultaneously in a video frame (MaxN), as described in Hitt et al. (2021). This value will be obtained by an observer reviewing the video footage while recording the number of fish present. Still frames will be captured to document the MaxN values.

Habitat suitability will be determined based on the presence of ice-free water beneath ice at the deepest point of the assessment site and adequate dissolved oxygen to support aquatic life. This threshold will be

defined as 5 mg/L, based on BC's Approved Water Quality Guidelines for Aquatic Life for the instantaneous minimum water quality guidelines for all life stages other than buried embryo / alevin (BC MOECCS 2021).

3.3.2.2 Fall Pre-overwintering and Spring Post-overwintering Assessment

Methods for analyzing fall pre-overwintering and spring post-overwintering data will follow those described in **Section 3.2.1.1 Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance**.

The fall pre-overwintering and spring post-overwintering relative abundance data will be used to determine overwintering survival. The estimated abundance and survival, will be determined with the relative abundance catch data from pre- and post-winter electrofishing effort adopting the approach of Trudel et al. (2012) to determine overwinter mortality (OWM) outlined in below equation:

$$OWM = \left[1 - \frac{(CPUE_{spring})}{(CPUE_{fall})}\right] * 100$$

where $CPUE_{spring}$ and $CPUE_{fall}$, respectively, define the pre-overwintering and post-overwintering estimates of catch per unit effort.

3.3.3 Davidson Creek Spawner Populations

3.3.3.1 Rainbow Trout Spawner Abundance

Catches for each hoop net site and each movement direction (i.e., upstream and downstream) will be tabulated and used to calculate the number of Rainbow Trout spawners entering Davidson Creek.

Total adult abundance (i.e., the total number of unique adult fish captured migrating into Davidson Creek) will be determined based on the total number of unique floy tags applied to fish captured in hoop nets. Adult spawner distribution will be assessed by tabulating the relative numbers of fish captured at each hoop net site, moving in each direction. Fish size at 100% maturity will be calculated by determining the mean length and weight values of all mature spawners encountered.

Bank walk survey data for each site will be evaluated to calculate relevant metrics. Total spawner abundance will be calculated for pooled and individual sites. Fish distribution will be assessed by comparing the relative number of fish observed at each site. Redd abundance will be determined by the maximum number of redds observed at each site during the survey period.

3.3.3.2 Kokanee Spawner Abundance

Spawner Abundance

An area-under-the-curve (AUC) method will be used to estimate escapement of spawning kokanee based on periodic counts obtained through visual surveys. AUC methods calculate an escapement (E) estimate by taking the integral of the count data (expanded by observer efficiency) and dividing it by a value of survey life (SL; synonymous with residence time or stream life): E = AUC ÷ SL

Specifically, total escapement for Davidson Creek will using a gaussian area-under-the-curve (GAUC) model developed by Millar et al. (2012) using the R Studio Environment (R Core Team, 2021). This method involves using a generalized linear model (GLM) with a quasi-Poisson family to fit to the OE-expanded count data. This method subsequently incorporates empirically derived stream-specific values of SL and OE and their uncertainty into the estimator. There is no historical information available for these values and their calculation would require a weir and a comprehensive study design that is outside the scope of the

present study. Rather, OE was implicit through its application in periodic surveys, while a literature value of SL and its uncertainty was used similarly to Holt and Cox (2008).

This GAUC method will be used to generate estimates of total escapement and standard error, start and end dates of the spawning period, and peak spawning timing.

Redd Abundance

Redd abundance data will be tabulated for each surveyed reach and for each survey day. Redds may overlap in Davidson Creek given the channel morphology, abundance of cover, cattle crossings, and woody debris that exists within the area. As such, the highest count of redd abundance for each reach will be identified as used as the metric value for that reach. The total number of redds within Davidson Creek will be calculated as the total of maximum redd counts per reach. Effort will be made to categorize individual redds as 'definitive' and 'potential' with individual coordinates where possible for data recording purposes.

Size at Maturity

Size at maturity will be calculated using the length, weight, and age data gathered for adult spawning kokanee. This metric will be calculated by determining the mean postorbital-hypural length values of all mature spawners encountered. In addition, age at 100% maturity also will be calculated by determining the mean age of kokanee, based on otolith ageing.

3.3.3.3 Kokanee Fry Outmigration Survey

Total daily catch data will first be expanded, using an estimate of trapping efficiency, based on the mark-recapture-based estimate of trap effectiveness. To calculate total daily catch estimate, the following formula for the adjusted Peterson estimate will be applied (Ricker 1975):

$$N = \frac{(M + 1\,) * (C + 1)}{R + 1}$$

Where: *N* = Daily fry estimate, *C* = Daily Catch, *R* = Number of Marks Recaptured, and *M* = Number of Marks Released

Kokanee fry outmigration abundance will then be estimated, based on the efficiency-adjusted daily fry estimate, using an AUC calculation method, similar to that used for adult kokanee, described in **Section 3.3.3.2 Kokanee Spawner Abundance**.

3.4 Frequency and Duration

The monitoring programs for community composition, overwintering abundance and habitat use, and spawner populations will occur during all phases of the Project.

Annual monitoring will occur in the Construction phase of the project for all sampling components for Condition 3.14.2. Annual monitoring will also continue for Rainbow Trout YoY and juvenile abundance surveys (**Section 3.2.1.1 Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance**) and Rainbow Trout overwintering surveys (**Section 3.2.2 Juvenile Rainbow Trout Overwintering (Sub-condition 3.14.2.2)**) during Operation.

Rainbow Trout spawning surveys and kokanee spawning surveys and escapement surveys (**Section 0At each site, sampling will include:**

- Three-pass depletion electrofishing of accessible stream lengths of 100 m, isolated with block nets;

- Identification of species and collection of length, weight, and body condition data for all fish captured;
- Scale samples from a subset of fish for ageing analysis
- Measurement of channel dimensions and calculation of mean values of bankfull width, wetted width, water depth, and gradient; and,
- In-situ water quality measurements (i.e., temperature, dissolved oxygen, pH, and conductivity).

Sampling will occur during in late July, during the period after Rainbow Trout YoY emergence and before the arrival of spawning kokanee adults in the downstream reaches of Davidson Creek. Sampling timing will be confirmed by calculating accumulated thermal units (ATU) using local stream and air temperature data. This calculated value will be compared to available emergence timing and ATU literature to estimate the timing window and variability of Rainbow Trout emergence dates. Results will be used to refine the juvenile sampling window for a given year. Observed emergence timing from field surveys will be compared to modelled emergence dates to validate and refine estimated emergence and ATU calculations.

At each site, sampling effort, electrofisher specifications, and catches will be recorded. Catch per unit effort (CPUE), relative abundance (number of fish per unit effort) and density metrics (number of fish per m2) will be calculated and analyzed, as described in **Section 3.3.1.1 Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance**. Sampling frequency is described in **Section 3.4 Frequency and Duration**. Additionally, fish habitat will be assessed at each site via Fish Habitat Assessment Procedure (FHAP) habitat assessments (Johnston and Slaney 1996).

Scale samples will be collected from a subset of fish for juvenile Rainbow Trout abundance study (captured using 3-pass depletion electrofishing) to estimate fish age. Scales will be collected to align with the BC RISC Fish Collection Methods and Standards. Scales will be collected above the lateral line and behind the dorsal fin with a sharp knife. At least 10 scales will be collected from each fish. Using the knife, scales will be pulled from posterior to anterior such that scales can be separated. Scales will be placed individually in labelled coin envelopes and air dried for laboratory analysis.

The summer fish inventory measurement endpoints will include an inventory of the fish community and fish health (**Table 3-1**).

Table 3-1: Measurement and Assessment Endpoints for the Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance

Measurement Endpoint	Assessment Endpoint
Fish community and abundance	Catch Per Unit Effort (CPUE), relative abundance, and fish density (fish/100 m2) for each identified species, population structure
Fish health	Length, weight, condition, age
Fish habitat	Mesohabitat unit boundaries, channel dimensions, stream morphology, cover, vegetation, substrate composition, riparian habitat

3.4.1.1 Davidson Creek Adult Kokanee and Rainbow Trout Abundance

Adult Rainbow Trout and kokanee abundance will be estimated from spawner surveys, described in **Section 3.2.3 Davidson Creek Spawner Populations (Sub-condition 3.14.2.3)**.

3.4.1.2 Rainbow Trout and Kokanee Genetic Structure and Diversity

The requirement (3.14.2.1) to monitor Rainbow Trout and kokanee genetic structure and diversity will be met by employing the genetic study methodology used in the baseline studies to the extent possible and where feasible, as described in Taylor (2012). This sampling methodology is described here, although it is recognized that adaptations may be warranted to account for advances in laboratory methods and genetic analysis techniques, based on subject matter expert guidance.

Samples of genetic material will be taken from Rainbow Trout and kokanee populations that occur in lower Davidson Creek. As described in the Project's baseline studies, two sub-populations of Rainbow Trout are understood to occur in Davidson Creek, separated by a cascade barrier in Reach 11: one in the lower reaches (i.e., downstream of the barrier) and one in the upper reaches and Lake 16 (i.e., above the barrier). This monitoring condition is specific to the effects on fish residing in Davidson Creek downstream of the mine site. Therefore, only the lower Davidson Creek sub-population of Rainbow Trout will be assessed as part of this program. Only a single identified population of kokanee occur in Davidson Creek, so this population will also be assessed.

Rainbow Trout tissue samples will be taken from stream-resident juvenile fish caught in Davidson Creek during summer electrofishing sampling (**Section 3.2.1.1 Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance**). Kokanee tissue samples will be taken from mature adult fish returning to spawn. Adult, pre-spawn kokanee will be sampled, rather than juvenile lake-resident kokanee, to ensure that fish sampled are part of the Davidson Creek spawning population, since Tatelkuz Lake likely contains fish from multiple stream-spawning populations. Migrating pre-spawn kokanee adults will be captured in lower Davidson Creek using seine nets over an area with no observed actively spawning fish. A minimum of 30 samples will be collected for each species (i.e., 30 Rainbow Trout and 30 kokanee). All fish sampled will be identified to the species level, measured for length and weight, sampled for tissue, then released back into stream site from which they were captured.

Tissue samples will be adipose and/or caudal fin clips, depending on tissue analysis volume requirements. Fin clips will be immediately placed into labelled vials containing 95% ethanol to minimize DNA degradation.

Polymerase chain reactions (PCR) of microsatellite DNA will be carried out on ten microsatellite loci of Rainbow Trout and six loci of kokanee, previously identified in the baseline genetic analysis (Taylor 2012).

Measurement and assessment endpoints have been selected with a focus on non-lethal monitoring of the fish community. The genetic measurement endpoint is a deviation from a population equilibrium (i.e., allele frequency is stable between generations; **Table 3-2**).

Table 3-2: Measurement and Assessment Endpoints for the Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance

Measurement Endpoint	Assessment Endpoint
Genetic structure	Deviation from population equilibrium

3.4.2 Juvenile Rainbow Trout Overwintering (Sub-condition 3.14.2.2)

DS Condition 3.14.2 requires "absolute abundance of overwintering Rainbow Trout juveniles". However, sampling of fish populations under ice during winter is logistically challenging and can present risks to human and fish health. Therefore, following discussion with representatives of IAAC and DFO (17 November, 2021), a technically feasible alternate assessment strategy was developed.

Juvenile Rainbow Trout overwintering surveys will include two assessment methods:

3. Mid-winter assessment of relative fish abundance using underwater cameras and evaluation of habitat characteristics at nine sites in Davidson Creek; and
4. Fall pre-overwintering and spring post-overwintering relative abundance surveys using three-pass electrofishing at five sites in Davidson Creek.

The first year of monitoring occurred in March 2022 in accordance with the methodology presented in **Section 3.2.2.1 Mid-winter Assessment**. Results of this study are available in the Blackwater Gold Project Fish and Aquatic Resources 2021 Field Survey Report (Palmer 2022a). Future years of monitoring will consider the methods and outcomes of previous years' of monitoring to optimize data collection.

3.4.2.1 Mid-winter Assessment

Winter surveys will be conducted to assess overwintering abundance and habitat use at ten sites in Davidson Creek (Site 1 to Site 10; **Figure 2**). Potential overwintering sites were identified using winter 2022 field survey information, field reconnaissance and unmanned aerial vehicle (UAV) imagery from summer 2021 surveys (**Section 3.2.3 Davidson Creek Spawner Populations (Sub-condition 3.14.2.3)**), and baseline stream habitat data. The mid-winter overwintering abundance and habitat assessment program will include measurement and assessment of juvenile Rainbow Trout overwintering abundance at each selected site using underwater cameras. Cameras and underwater lights will be placed in an overwintering deep pool habitat within each site. Stationary high-quality video will be recorded for a standardized period (e.g., 60 minutes) during daytime. The video will be reviewed to determine relative abundance using the established metric of maximum count of individuals observed simultaneously in a video frame (MaxN; Hitt et al. 2020).

At each site, recording time, camera and lighting specifications, habitat measurements, and water chemistry parameters will be recorded. MaxN values will be determined and habitat suitability will be assessed, as described in **Section 3.3.2.1 Mid-winter Assessment**. Sampling frequency is described in **Section 3.4 Frequency and Duration**. The mid-winter overwintering survey measurement endpoints will include assessment of fish abundance and habitat suitability (

Table 3-3).

Table 3-3: Measurement and Assessment Endpoints for the Mid-winter Juvenile Rainbow Trout Overwintering Assessment

Measurement Endpoint	Assessment Endpoint
Fish abundance	Maximum count of individuals observed simultaneously in a video frame (MaxN)
Habitat suitability	Flowing water is present and dissolved oxygen levels are greater than 5 mg/L

3.4.2.2 Fall Pre-overwintering and Spring Post-overwintering Assessment

Pre-and post-overwintering surveys will include three-pass electrofishing assessments of five sites in Davidson Creek (Site 5 to Site 9; **Figure 2**). The methodology will follow that previously described in **Section 3.2.1.1 Davidson Creek Young-of-Year and Juvenile Rainbow Trout Abundance** for summer Rainbow Trout assessment.

Sampling timing will vary, depending on seasonal differences in water temperature, ice cover, and discharge. Fall sampling will generally be conducted in late September before water temperatures drop below 5°C and before ice cover forms. Spring sampling will be conducted shortly after ice-off once temperatures reach 5°C and before spring freshet precludes effective electrofishing.

Notably, the five sites selected for assessment are less than the nine identified for summer YoY and juvenile sampling and overwintering assessment. This is due to the expected presence of incubating kokanee embryos in the gravel substrates of the lower reaches of Davidson Creek. Electrofishing sampling will not be conducted in potential kokanee spawning areas to minimize mortalities. Therefore, the four furthest-downstream sites (DCOH1 to DCOH 4) will not be sampled in fall or spring.

At each site, sampling effort, electrofisher specifications, and catches will be recorded. CPUE, relative abundance (number of fish per unit effort) and density metrics (number of fish per m2) will be calculated and analyzed, as described in **Section 3.3.2.2 Fall Pre-overwintering and Spring Post-overwintering Assessment**. Sampling frequency is described in **Section 3.4 Frequency and Duration**. The pre- and post-winter overwintering survey measurement endpoints will include assessment of fish abundance fish health (**Table 3-4**).

Table 3-4: Measurement and Assessment Endpoints for Fall Pre-overwintering and Spring Post-overwintering Assessment

Measurement Endpoint	Assessment Endpoint
Fish community and abundance	Catch Per Unit Effort (CPUE), relative abundance, and fish density (fish/100 m2) for each identified species, population structure
Fish health	Length, weight, condition

Davidson Creek Spawner Populations (Sub-condition 3.14.2.3)) will be completed on an annual basis for at least the first two years of Construction and the first eight years of Operations, to ensure that at least two complete kokanee cohort generations are assessed. Beyond the eight-year mark of Operations, survey frequency for Rainbow Trout could be reduced to once every two years, if no trend (changes) in fish community is observed. Long-term trend detection may require more data points for kokanee spawner surveys past the eight-year mark due to four-year spawning cohorts. Therefore, if trends analysis is not possible after Year 8 of Operations, kokanee monitoring may continue annually until statistical power increases to the point at which a trend can be detected.

The genetic sampling frequency (**Section 3.2.1.3 Rainbow Trout and Kokanee Genetic Structure and Diversity**) will be completed every four years, to allow time for potential detectable genetic drift to occur, and to coincide with the four-year life cycle of kokanee spawners.

Monitoring frequency of all FUP components for Closure and Post-Closure phases will be determined near the end of the Operations phase and will depend on monitoring results during that phase.

4. IMPLEMENTATION SCHEDULE

Follow-up Programs 3.14.1 (Parasite Pathogen Study) and 3.14.2 (Davidson Creek Populations) were initiated in 2021-2022 through field programs to select sites, refine field sampling methods, and collect baseline data prior the start of Construction. The programs will be ongoing throughout the life of the Project.

5. ADAPTIVE MANAGEMENT

The follow-up programs for Condition 3.14 described herein will evolve over time in response to the results of the monitoring, changing conditions, or development at the Project, updates to methods, and through consultation with Indigenous groups, regulators, or other stakeholders. This process of continuous improvement with changing conditions is referred to as adaptive management.

Conditions 2.5 and 2.6 in the federal DS identify requirement for follow-up programs:

"2.5 The Proponent shall, where a follow-up program is a requirement of a condition set out in this Decision Statement, have a Qualified Professional, where such a qualification exists for the subject matter of the follow-up program, determine, as part of the development of each follow-up program and in consultation with the party or parties being consulted during the development, the following information:

2.5.1 the follow-up activities that must be undertaken by a qualified individual;

2.5.2 the methodology, location, frequency, timing and duration of monitoring associated with the follow-up program;

2.5.3 the scope, content, format and frequency of reporting of the results of the follow-up program;

2.5.4 the levels of environmental change relative to baseline conditions that would require the Proponent to implement modified or additional mitigation measure(s), including instances where the Proponent may require Designated Project activities to be stopped; and

2.5.5 the technically and economically feasible mitigation measures to be implemented by the Proponent if monitoring conducted as part of the follow-up program shows that the levels of environmental change referred to in Condition 2.5.4 have been reached or exceeded.

2.6 The Proponent shall update and maintain the follow-up and adaptive management information referred to in Condition 2.5 during the implementation of each follow-up program in consultation with the party or parties being consulted during the development of each follow-up program."

Thus, an adaptive management framework has been incorporated into the follow-up programs. **Figure 3** identifies the components of the adaptive management framework.

Figure 3. Adaptive Management Framework

Plan: In collaboration with the Indigenous groups, further refine and plan Follow up Programs 3.14.1 and 3.14.2.

Do: Implement Follow-up Programs 3.14.1 and 3.14.2.

Monitor: BW Gold will review and update the follow up programs over the life of the Project. This will include:

- Review of the programs in terms of effectiveness in detecting changes with the monitoring program;

- Recommendations provided by qualified individual and Indigenous groups on the monitoring plan; and
- Engagement tracking to record input from Indigenous groups.

Adjust: BW Gold will adjust the follow-up programs (e.g., study design, field methods, data analysis methods, and reporting) based on program findings, as well as input and feedback from Indigenous groups.

5.1 Follow-Up Program Trigger Response

To determine 'the levels of environmental change relative to baseline conditions that would require the Proponent to implement modified or additional mitigation measure(s)' (Condition 2.5.4 of the Decision Statement) entails establishing levels (i.e., triggers), that, when reached, trigger a response action.

Changes in the monitoring metrics for fish populations (e.g., fish abundance, density, spawner escapement, fry emigration), may reflect natural variability and it is challenging to pinpoint whether a significant change is attributable to mine activities (namely, flow augmentation). Establishing triggers provides an early-warning system, allowing sufficient time to investigate root causes, increase monitoring, and take preventative action (i.e., implement modified or additional mitigation measures).

Monitoring metrics that serve as triggers for action and response are additionally outlined in the AEMP for the project. Triggers for temperature and hydrology have been defined in this plan as it pertains to Davidson Creek. The results of the AEMP will be considered for evaluations of the change relative to baseline described herein.

Statistical analyses, described in **Section 0 Data Analysis**, will be used to evaluate changes over time. Power analyses have been performed to support a study design that will allow for between-year comparisons and longer-term trend analysis. Between-year comparison (i.e., paired comparison between two separate annual datasets) will be completed to identify statistically differences in mean values. In addition, year over year change will be assessed qualitatively, by comparing with the baseline data.

For longer time scale (i.e., five years and onwards), non-parametric Mann-Kendall temporal trends testing will be used to determine if there are significant temporal trends in any given monitoring metric, and if so, the direction and statistical significance of temporal trend. The sensitivity of the MK trends test increases with an increasing number of time steps (i.e., consecutive years of data) and it is considered that somewhere between five and ten time-steps are a minimum requirement. For the first four years of monitoring, temporal trends analysis will not be possible, however a Trigger Action Response Plan for fish habitat endpoints has been established that is based on triggers for flow and temperature. This is outlined in the AEMP for the Project.

Table 5-1 identifies triggers and responses for adaptive management related to fish population monitoring as part of follow-up programs 3.14.2. There are no triggers proposed for follow-up program 3.14.1 (Parasite Pathogen Study), as the study found no differences in known parasites or pathogens between the two lakes.

Table 5-1.: Adaptive Management Triggers and Responses

Trigger	Response
No trend in monitoring metric (i.e., stable over time) or upward trend (e.g., increase in fish abundance)	Inform Indigenous groups No change to mitigation measures Consider reduction in the frequency of monitoring
Downward trend monitoring metric (e.g., decrease in fish abundance)	Inform Indigenous groups Identify potential causes and additional studies to test hypotheses Implement modified or additional mitigation measures

Trigger	Response
	Monitor post-implementation of the modified or additional mitigation measures and communicate results Evaluate if new mitigation or fish offsetting measures are required

In the case of negative adverse effects to fish populations that deviate from the predictions of the EA, there are limited additional mitigation measures available. Flow augmentation from the FWR/FWSS can be controlled for flow and temperature, and relative proportions of the different sources of water can be adjusted (i.e., increase the input from the diversions, reduce volume of Tatelkuz Lake water). However, BW Gold's fish habitat offsetting plan, includes habitat creation, restoration, or enhancement projects in the Davidson Creek Watershed to offset the predicted effects on fish productivity and includes measures to benefit other fish populations in the region. If effects are beyond what was predicted in the Environmental Assessment and the Fisheries Offsetting Plan (Palmer 2021b), additional contingency mitigation measures or offsetting may be required and could include:

1) Transfers of adults or eggs of similar genotypes (e.g., rainbow trout captured in Turtle Creek and rainbow trout or kokanee captured in Creek 661) into middle Davidson Creek; or
2) Tagging of suspected Davidson Creek spawners migrating through lower Chedakuz Creek (e.g., early-run kokanee) to provide insight on the fate of spawners originating from Davidson Creek.

6. REPORTING

DS Conditions 2.11, 2.12 and 2.13 set out annual reporting requirements related to the implementation of conditions in the DS. Condition 2.14 sets out information sharing requirements related to the annual reports.

DS Condition 2.11 requires:

> *"The Proponent [BW Gold] shall, commencing in the reporting year during which the Proponent begins the implementation of the conditions set out in this Decision Statement, prepare an annual report that sets out:*
>
> *2.11.1 the activities undertaken by the Proponent in the reporting year to comply with each of the conditions set out in this Decision Statement;*
> *2.11.2 how the Proponent complied with Condition 2.1;*
> *2.11.3 for conditions set out in this Decision Statement for which consultation is a requirement, how the Proponent considered any views and information that the Proponent received during or as a result of the consultation, including a rationale for how the views have, or have not, been integrated;*
> *2.11.4 the information referred to in Conditions 2.5 and 2.6 for each follow-up program;*
> *2.11.5 the results of the follow-up program requirements identified in Conditions 3.14, 3.15, 3.16, 4.5, 5.5, 6.11, 6.12, 6.13, 6.14, 8.18.6, 8.20.5, 8.21, and 8.22 if required;*
> *2.11.6 any update made to any follow-up program in the reporting year;*
> *2.11.7 any modified or additional mitigation measures implemented or proposed to be implemented by the Proponent, as determined*

under Condition 2.9 and rationale for why mitigation measures were selected pursuant to Condition 2.5.4; and

2.11.8 any change(s) to the Designated Project in the reporting year."

DS Condition 2.12 requires: *"The Proponent [BW Gold} will provide the draft annual report to Indigenous groups, no later than June 30 following the reporting year to which the annual report applies. BW Gold will consult Indigenous groups on the content and findings in the draft annual report."*

DS Condition 2.13 requires: *"The Proponent [BW Gold], in consideration of any comments received from Indigenous groups pursuant to Condition 2.12 shall revise and submit to the Agency [Impact Assessment Agency of Canada] and Indigenous groups a final annual report, including an executive summary in both official languages, no later than September 30 following the reporting year to which the annual report applies."*

DS Condition 2.14 requires: *"The Proponent [BW Gold] shall publish on the Internet, or any medium which is publicly available, the annual reports and the executive summaries referred to in Conditions 2.11 and 2.13."*

The Proponent shall keep these documents publicly available for 25 years following the end of decommissioning of the Designated Project. The Proponent shall notify the Agency and Indigenous groups of the availability of these documents within 48 hours of their publication."

Reporting in compliance with these conditions will commence when BW Gold begins to implement the follow-up programs. BW Gold will implement the follow up programs during all phases of the Project, as stipulated in the DS.

BW Gold is also committed to developing a data sharing agreement such that monitoring data can be accessed by Indigenous groups and regulators. This data sharing agreement will include both raw and interpreted data such as preliminary field memos and data packages in the fall/winter following each field season. Starting in 2023, field forms connected to a database will be used to record most monitoring field data. An example of these field forms is presented in **Figure 4**. Monitoring data for all Condition 3.14 studies will be presented to the Nations, as requested, in an annual report and saved to a Project file sharing site.

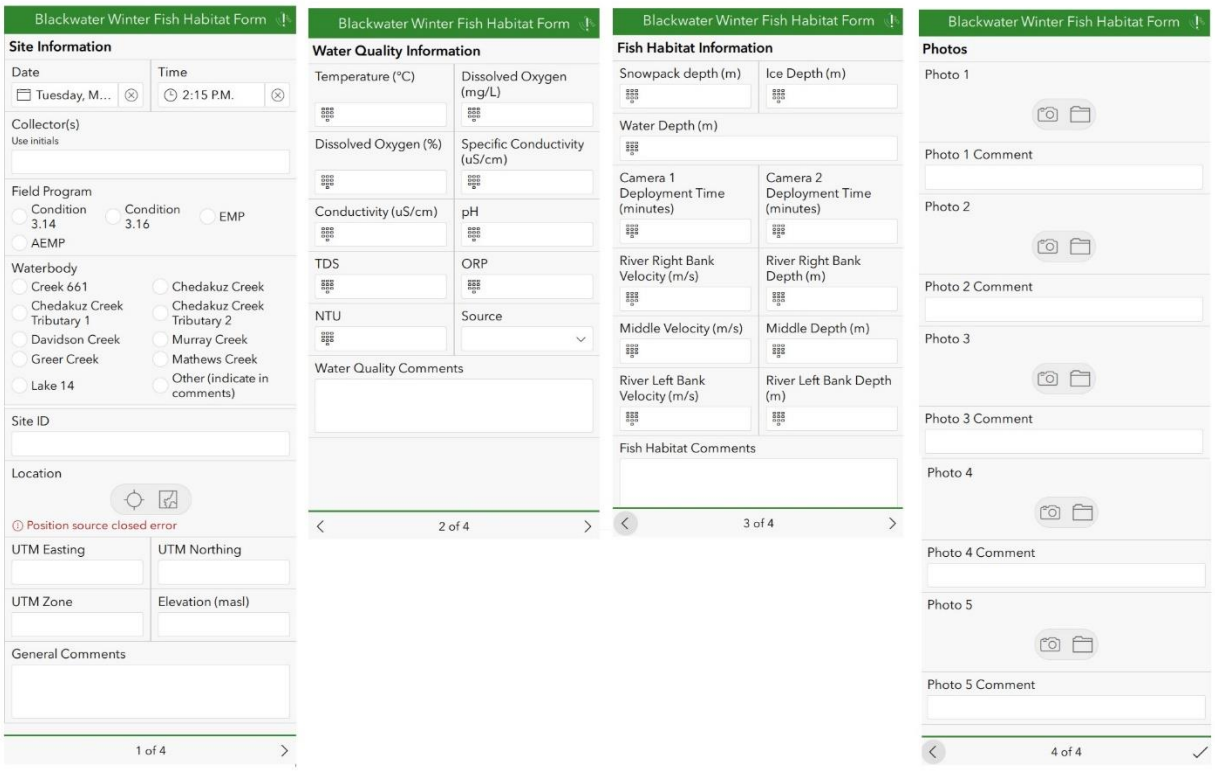

Figure 4. Screenshot of the Field Form Interface for Recording Monitoring Data.

7. SUMMARY

The follow up programs herein have been developed to fulfill DS Condition 3.14. This condition pertains to conducting an inventory of parasites and pathogens in two lakes that will be joined by a connector channel, and monitoring fish populations in Davidson Creek downstream of the Project. The follow up programs cover data collection during pre-construction (2021-2022), through to decommissioning.

Information from these programs will be used for comparison during long-term monitoring over the life of the Project to determine the accuracy and effectiveness of mitigation measures, as set out in Condition 2.9. Depending on the long-term monitoring results compared with threshold values for the monitoring metrics used, modified or additional mitigation measures may be required in conjunction with subsequent monitoring.

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APPENDIX A PARASITE AND PATHOGEN LABORATORY ANALYSIS

2022

BC Centre for Aquatic
Health Sciences - CAHS

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Date submitted: September 13, 2022

Project: Parasite/Pathogen testing from water and Rainbow Trout gill/mucus samples collected from Lake 15 and 16

[PATHOGEN SCREENING FINAL REPORT]

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I. Background

Palmer Environmental Consulting Group Inc. requested from BC Centre for Aquatic Health Sciences (BCCAHS) to conduct a study for parasite/pathogen screening in Lake 15 and Lake 16. The potential for low numbers of fish in these lakes prevented performing lethal sampling for parasites and pathogens. Therefore, it was proposed to perform a non-lethal parasite/pathogen inventory of the fish inhabiting both lakes as well as the water. The purpose of this testing is to provide an insight of presence or absence of targeted parasite/pathogens in water and on the fish. Fish gills and mucus were swabbed and water will be filtered for testing. It is important to keep in mind that these procedures will be used for inventory (presence/absence) and not for disease diagnostic purposes.
Our choice of the targeted pathogens is based on the National Aquatic Animal Health Program (NAAHP) list of reportable finfish pathogens (Table 1).

Reportable Disease	*Oncorhynchus mykiss* (Rainbow Trout)	*Oncorhynchus nerk*a	Zoned Enzootic in the Pacific Watershed
Ceratomyxosis (*Ceratomyxa shasta*)	Y	Y	Y
Epizootic haematopoietic necrosis	Y		N
Infectious haematopoietic necrosis	Y	Y	Y
Infectious pancreatic necrosis	Y		N
Infectious salmon anaemia	Y		N
Viral haemorrhagic septicaemia	Y		Y
Whirling disease (*Myxobolus cerebralis*)	Y	Y	N

Table 1: National Aquatic Animal Health Program (NAAHP) list of reportable finfish pathogens

In addition to this list of pathogens, we will be also targeting bacteria of concern for Rainbow Trout such as *Renibacterium salmoninarum*, *Yersinia ruckeri* and *Aeromonas salmonicida*.

At the BC Centre for Aquatic Health Sciences, molecular assays for Infectious haematopoietic necrosis virus, Infectious pancreatic necrosis virus, Infectious salmon anaemia virus and Viral haemorrhagic septicaemia virus are routinely used at BCCAHS for fish screening using kidney tissues. However, the assays need to be optmised for non lethal samples such as viral transport media and swabs. In this report, we will be describing the optimisation steps of the molecular assays performed at BC CAHS.

II. Real time Polymerase Chain Reaction (RT-qPCR) principle

Real time Polymerase Chain Reaction (RT-qPCR) is a molecular assay that targets genetic material of the pathogen of concern. The principle is based on designing specific primers (forward and reverse) and a probe. There are three major steps (Fig., 1):

First the extracted genetic materials (RNA for viruses or DNA for bacteria) are denaturated to separate both DNA strands by increasing the temperature to 94-98°C.

Second, the temperature is decreased to 50°C to allow the primers bind their complementary sequences.

Third, temperature is increased to 72°C and a polymerase enzyme will complete the remaining of the sequences between the forward and reverse primers.

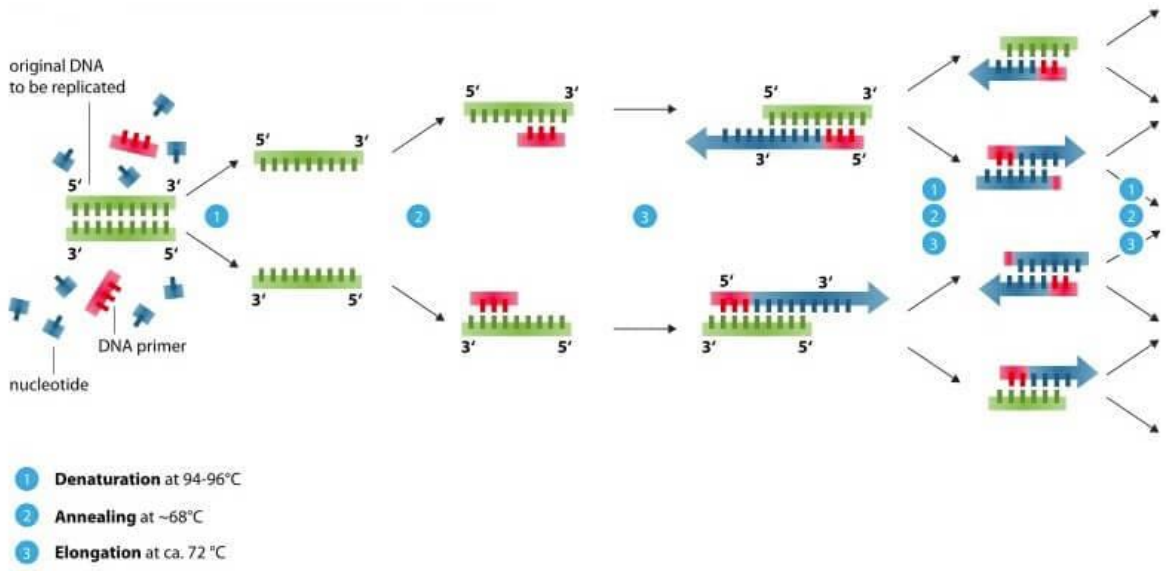

Figure 1: Polymerase Chain Reaction Principle
(source https://www.elveflow.com/microfluidic-reviews/general-microfluidics/microfluidic-pcr-qpcr-rtpcr/)

These three steps represent one cycle of the PCR. During each cycle, the amount of genetic materials increases exponentially ($2^{\text{number of cycle}}$) during the exponential phase. The cycle number when the genetic material starts to amplify is known as cycle threshold (Ct value). The Ct value is inversely related to the amount of genetic material present in the sample (ie, required Ct value is low when the quantity of genetic material is high). When all the reagents are used, the amplification achieves the plateau phase and no additional products are synthetized (Fig., 2).

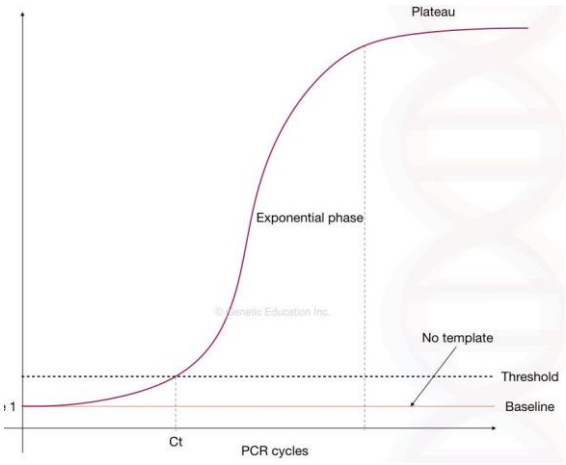

Figure 2: PCR amplification graph
(source: https://geneticeducation.co.in/real-time-pcr-principle-procedure-advantages-limitations-and-applications/)

III. Sample preparation

Three (3) bacterial isolates have been grown in their respective media for the analysis. *Yersinia ruckeri* (Yruck), *Aeromonas salmonicida* (Asal) and *Renibacterium salmoninarum* (Rsal) isolates have been used in this study. Each isolate has been diluted 5 times in 10-fold dilution. Swabs (Puritan) have been dipped in each dilution to mimic the sample swabs. DNA was extracted from each swab using the Qiagen DNeasy blood and tissue kit (Cat# 69506) and as per CAHS SOP #17 v2.1. QPCR was performed on the extracted DNA using the TaqMan qPCR kit (Applied Bisystems).

IV. Bacteria assay optimization

IVa. *Renibacterium salmoninarum*

IVa1. Swab samples

QPCR was performed on DNA extracted from the diluted samples using BCCAHS primer and probe sets for routine screening of Rsal in fish tissues. To increase the sensitivity, the DNA input was 2 µL and 5 µL for comparison. The results showed that an input DNA of **5 µL** provided a higher sensitivity compared to 2 µL (Fig., 3).

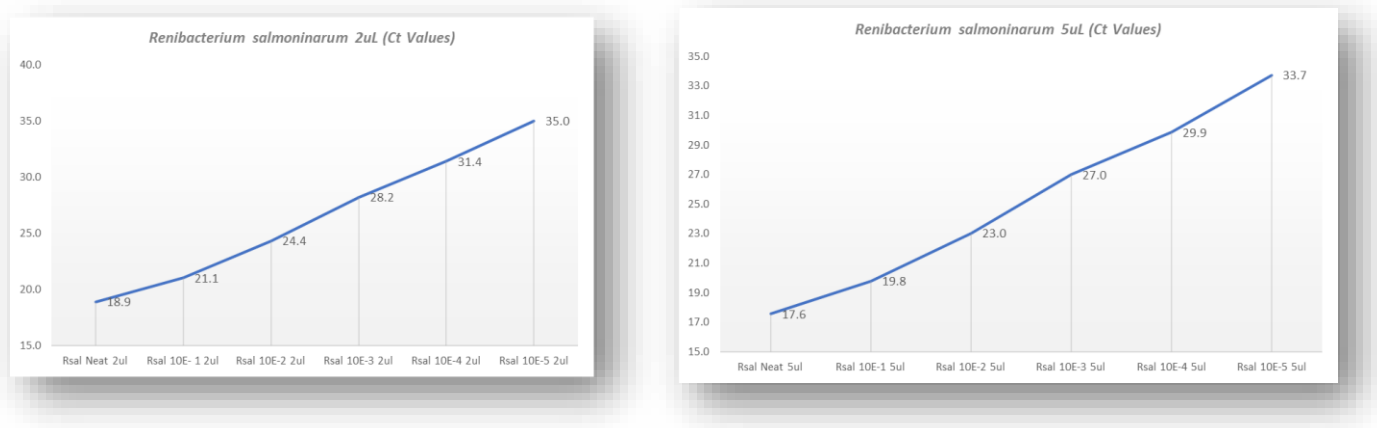

Figure 3: Comparison of qPCR results for Renibacterium salmoninarum using 2ul and 5ul DNA as input.

For optimal conditions of the qPCR assay targeting Rsal in swab, optimization of the primer and probe sets has been performed on DNA extracted from swab. Efficiency and sensitivity of the optimized assay have been also assessed for better performance and limit of amplification.

Primer optimization

DNA extracted from the swab immerged in the bacterial suspension diluted 1/10,000 from the original (neat) bacterial solution.

- Different concentrations of forward and reverse primers combination were analyzed

200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM,.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations (Table 2).

	200/200	200/400	400/200	400/400	200/600	600/200	600/600	600/400	400/600
	31.664	31.251	31.445	31.133	31.210	31.228	31.281	31.200	31.170
	31.483	31.050	31.090	31.284	31.010	31.218	31.289	31.257	30.858
	31.113	30.772	31.581	30.710	31.100	30.964	30.786	31.218	31.163
Avg	31.420	31.024	31.372	31.043	31.107	31.137	31.119	31.225	31.063
SD	0.28	0.24	0.25	0.30	0.10	0.15	0.29	0.03	0.18

Table 2: Ct values recorded for different combination of primer concentrations of assay targeting R. salmoninarum gene. Optimal concentrations of primer sets are highlighted.

Data showed that the optimal primer set combination (i.e. lower Ct values) for forward and reverse were **200nM and 400nM** respectively.

Probe optimization

Optimisation of the probe concentration was performed using the same extracted DNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM

- Recorded Ct values (Table 3)

	100nM	**125nM**	**150nM**	**175nM**	**200nM**	**225nM**	**250nM**
	31.352	30.912	30.892	30.943	30.982	30.759	30.635
	30.980	31.050	30.796	30.747	30.826	30.575	30.580
	30.973	30.809	31.189	30.815	30.689	30.688	30.622
Avg	31.102	30.924	30.959	30.835	30.832	30.674	30.612
STD	0.22	0.12	0.20	0.10	0.15	0.09	0.03

Table 3: Ct values recorded for different probe concentrations of assay targeting R. salmoninarum gene. Optimal concentration of probe is highlighted.

Data showed that the optimal concentration of the probe was **225nM** which provided the lowest Ct value and better amplification curve.

Efficiency

- DNA extracted from swab immerged into original *R. salmoninarum* bacterial solution was 10-fold serial diluted (n=6)
- qPCR was performed using the optimal conditions (200/400nM for both primer set forward and reverse respectively and 225 nM for the probe).
- The efficiency of the optimised assay was evaluated at ~**94%** (slope is -3.47) (Fig., 4). The acceptable efficiency for qPCR assays is between 90% and 110%.

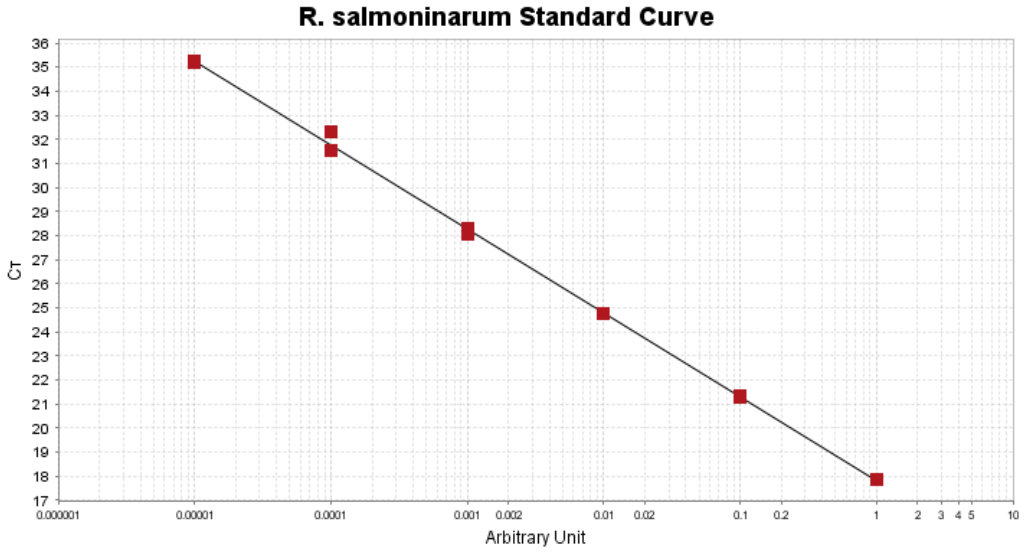

Figure 4: Standard curve of the assay targeting R. salmoninarum DNA extracted from swab

Sensitivity

- Sample from 10E-4 was used for the evaluation of the assay sensitivity
- Concentration of the probe was 225 nM and the concentrations of the sets of primers, forward and reverse, were 200 nM and 400 nM respectively.
- 2-fold serial dilution (n=6) was performed using sample from 10E-4
- 10 replicates for each dilution were performed to define the sensitivity of the assay
- Data showed that undetermined (U: no amplification) is recorded in 1 replicate out of 10 at 2E-2 at an average Ct value of 33.6. Therefore, the threshold is set at Ct value of **34** (Table 4)

	Neat (10E-4)	2E(-1)	2E(-2)	2E(-3)	2E(-4)	2E(-5)
	32.8	33.2	34.1	U	U	U
	33.4	33.9	33.7	34.6	35.5	U
	32.9	33.8	U	36.6	U	U
	32.3	32.5	34.6	U	U	35.6
	32.4	34.7	35.4	36.1	U	U
	32.6	33.8	36.5	U	U	U
	31.9	32.9	33.5	35.5	35.5	U
	32.0	33.4	35.3	35.6	35.6	U
	33.2	33.6	33.8	35.5	U	U
	33.0	34.4	36.4	U	U	
Avg	**32.6**	**33.6**	**34.8**	**35.7**	**35.5**	**35.6**
SD	**0.5**	**0.7**	**1.1**	**0.7**	**0.1**	

Table 4: Results of sensitivity assessment for assay targeting R. salmoninarum gene. Ten replicates for each dilution have been performed.

IVa2. Water samples

Primer optimization

DNA extracted from the water spiked with *R. salmoninarum* and filtered through sterivex filters. DNA was diluted 1/100.

- Different concentrations of forward and reverse primers combination were analyzed

200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations
- 5µl of extracted DNA was added to each reaction (Table 5)

	200/200	200/400	400/200	400/400	200/600	600/200	600/600	600/400	400/600
	29.31	29.03	**29.06**	29.11	29.11	29.18	29.10	29.24	29.06
	29.13	29.13	**28.98**	29.11	29.11	29.18	29.10	29.24	29.06
Avg	29.22	29.08	29.02	29.11	29.11	29.18	29.10	29.24	29.06
SD	0.13	0.08	0.06	0.00	0.00	0.00	0.00	0.00	0.00

Table 5: Ct values recorded for different combination of primer concentrations of assay targeting R. salmoninarum gene. Optimal concentrations of primer sets are highlighted.

Data showed that the optimal primer set combination (lower Ct values) for forward and reverse were **400nM and 200nM** respectively.

Probe optimization

Optimisation of the probe concentration was performed using the same extracted DNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM

Recorded Ct values (Table 6)

	100nM	125nM	150nM	175nM	200nM	225nM	250nM
	29.19	29.02	28.96	28.86	28.60	28.59	**28.00**
	29.02	29.20	28.90	28.78	28.66	28.65	**28.21**
Avg	29.11	29.11	28.93	28.82	28.63	28.62	28.10
STD	0.12	0.12	0.04	0.06	0.05	0.04	0.15

Table 6: Ct values recorded for different probe concentrations of assay targeting R. salmoninarum gene. Optimal concentration of probe is highlighted.

Data showed that the optimal concentration of the probe was **250nM** which provided the lowest Ct value and better amplification curve.

Efficiency

- DNA extracted from spiked water with *R. salmoninarum* was 5-fold serial diluted (n=5)
- qPCR was performed using the optimal conditions (400/200nM for both primer set forward and reverse respectively and 250 nM for the probe).
- The efficiency of the optimised assay was evaluated at ~**94%** (slope is -3.47) (Fig., 5). The acceptable efficiency for qPCR assays is between 90% and 110%.

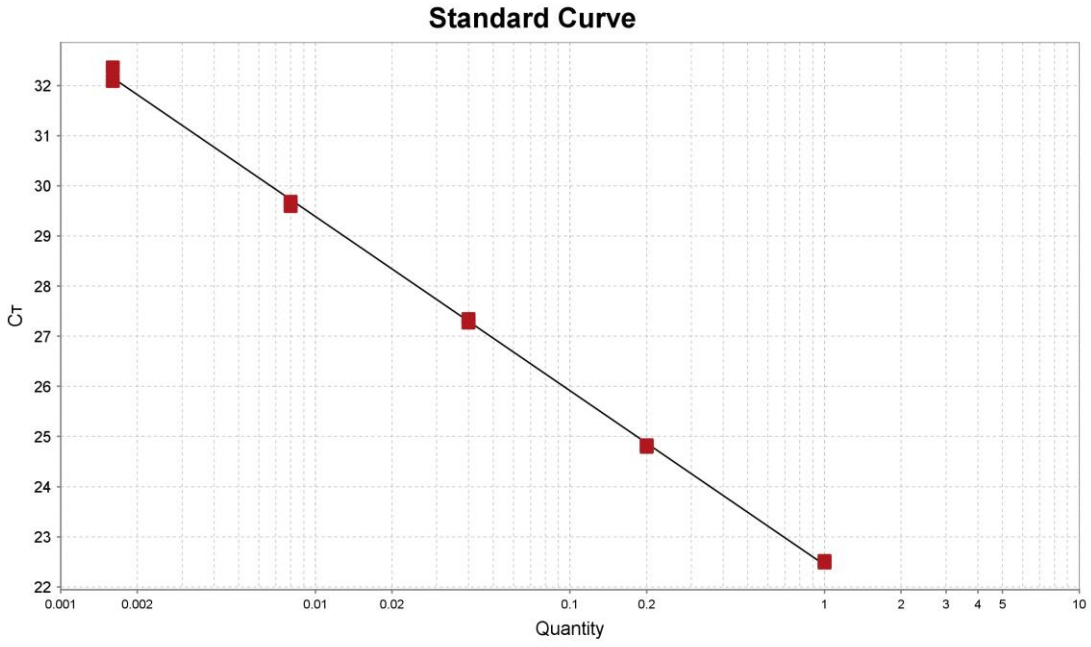

Figure 5: Standard curve of the assay targeting R. salmoninarum DNA extracted from filtered water

Sensitivity

- Sample from standard curve (1/125) diluted 1/10 was used for the evaluation of the assay sensitivity
- Concentration of the probe was 250 nM and the concentration of the sets of primers, forward and reverse, was 400 nM and 200 nM respectively.
- 2 fold serial dilution (n=4) was performed using sample from 1/125 diluted 1/10
- 10 replicates for each dilution were performed to define the sensitivity of the assay
- Data showed that undetermined (U: no amplification) is recorded in 2 replicates out of 10 2-fold twice (2E-3) at an average Ct value of 36.1. Therefore, the threshold is set at Ct value of **35-36** (Table 7).

	Neat	2E-1	2E-2	2E-3
	32.5	33.4	34.8	U
	32.3	33.2	34.2	U
	32.4	33.3	34.4	35.5
	32.4	33.8	34.4	36.1
	32.1	34.1	34.9	35.2
	32.4	34.0	34.7	36.3
	32.2	33.5	36.2	34.6
	32.2	33.4	34.8	35.8
	32.6	33.9	34.6	36.8
	32.1	33.9	34.4	35.3
Avg	32.3	33.6	34.7	36.1
SD	0.2	0.3	0.6	35.1

Table 7: Results of sensitivity assessment for assay targeting R. salmoninarum gene. Ten replicates for each dilution have been performed.

IVb. *Aeromonas salmonicida*

IVb1. Swab samples

QPCR was performed on DNA extracted from the diluted samples using BCCAHS routine screening assay of *A. salmonicida* in fish tissues. To increase the sensitivity, the DNA input was 2 µL and 5 µL for comparison. The results showed that an input DNA of **5 µL** provided a higher sensitivity compared to 2 µL (Fig., 6).

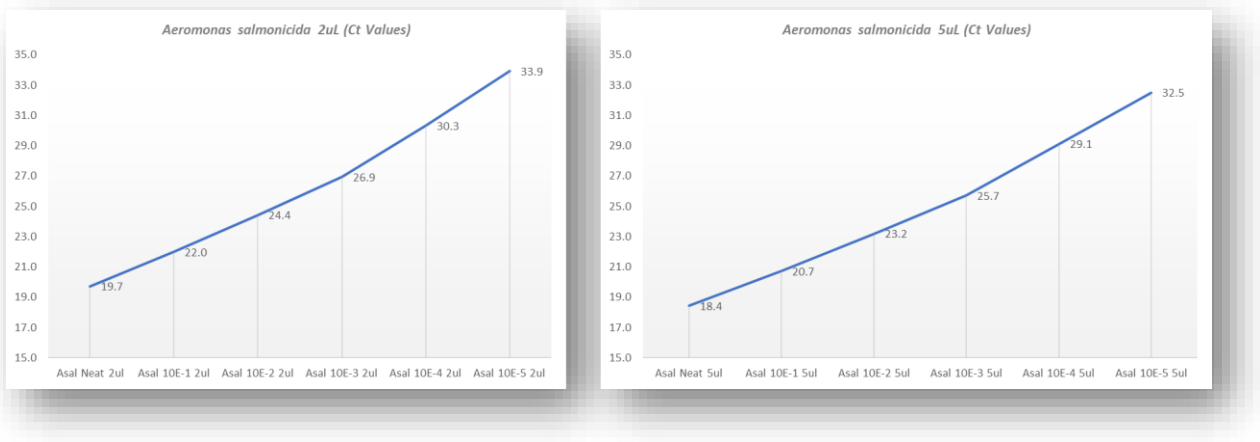

Figure 6: Comparison of qPCR results for Aeromonas salmonicida using 2ul and 5ul DNA extracted from swab as input.

For optimal conditions of the qPCR assay targeting *A. salmonicida* in swab, we have performed optimization of the primer and probe sets. Efficiency and sensitivity of the optimized assay have been also evaluated for better performance and limit of amplification.

Primer optimization

DNA extracted from the swab immerged in the bacterial suspension diluted 1/1,000 from the original (neat) bacterial solution.

- Different concentrations of forward and reverse primers combination were analyzed

200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM,.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations (Table 8)

	200/200	200/400	400/200	400/400	200/600	600/200	600/600	600/400	400/600
	28.334	28.103	28.058	28.042	28.416	27.917	27.987	27.990	28.012
	28.526	27.991	28.060	27.982	28.112	28.027	28.087	28.173	28.004
Avg	**28.430**	**28.047**	**28.059**	**28.012**	**28.264**	**27.972**	**28.037**	**28.082**	**28.008**
SD	**0.14**	**0.08**	**0.00**	**0.04**	**0.21**	**0.08**	**0.07**	**0.13**	**0.01**

Table 8: Ct values recorded for different combination of primer concentrations of assay targeting A. salmonicida gene. Optimal concentrations of primer sets are highlighted.

Data showed that the optimal primer set combination (lower Ct values) for forward and reverse were **600nM and 200nM** respectively.

Probe optimization

Optimisation of the probe concentration was performed using the same extracted DNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM

- Recorded Ct values (Table 9)

	100nM	125nM	150nM	175nM	200nM	225nM	250nM
	25.00	24.78	24.74	25.00	24.60	24.54	24.51
	25.03	24.74	24.69	25.03	24.68	24.57	24.48
Avg	**25.02**	**24.76**	**24.71**	**25.02**	**24.64**	**24.56**	**24.49**
STD	**0.01**	**0.03**	**0.03**	**0.02**	**0.05**	**0.02**	**0.02**

Table 9: Ct values recorded for different probe concentrations of assay targeting R. salmoninarum gene. Optimal concentration of probe is highlighted

Data showed that the optimal concentration of the probe was **250nM** which provided the lowest Ct value and better amplification curve.

Efficiency

- DNA extracted from swab immerged into the original *A. salmonicida* bacterial solution was 10-fold serial dilution (n=6)

- qPCR performed using the optimal conditions (600/200nM for both primers forward and reverse respectively and 250 nM for the probe)

- The efficiency of the optimised assay was evaluated at **~97%** (slope is -3.39) (Fig., 7).

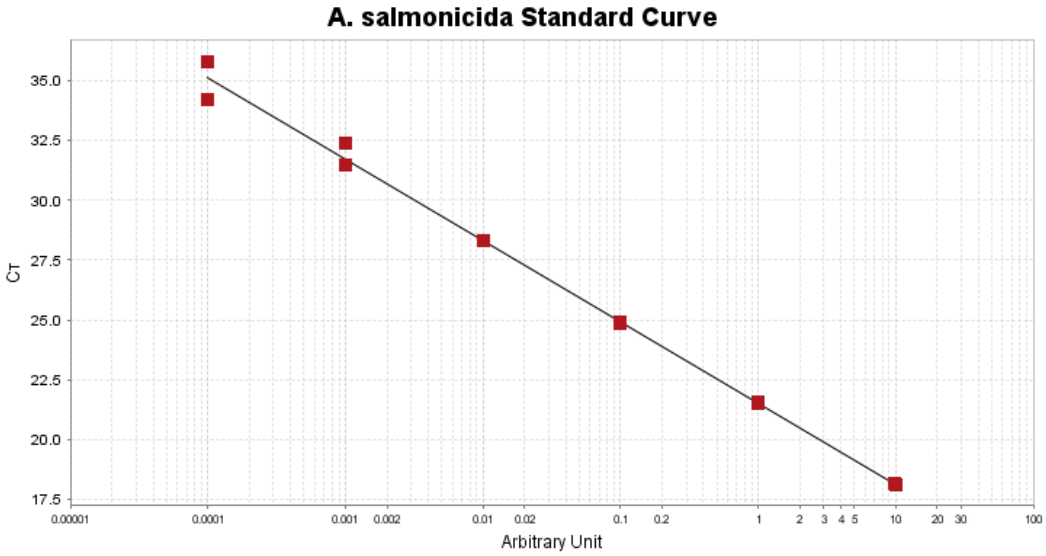

Figure 7: Standard curve of the assay targeting Aeromonas salmonicida DNA extracted from swab

Sensitivity

- Sample from 10E-4 was used for the evaluation of the assay sensitivity
- Concentration of the probe was 250 nM and the concentration of the sets of primers, forward and reverse, was 600 nM and 200 nM respectively.
- 2-fold serial dilution (n=6) was performed using sample from 10E-5
- 10 replicates for each dilution were performed to define the sensitivity of the assay
- Data showed that undetermined (U: no amplification) is recorded in 1 replicate out of 10 at 10E-5 at an average Ct value of 33.7. Therefore, the threshold is set at Ct value of **34** (Table 10).

	10E-5	2E-1	2E-2	2E-3	2E-4
	32.149	34.0	U	U	U
	33.251	U	34.8	U	U
	35.4	34.0	35.9	U	35.0
	U	34.3	U	34.9	34.9
	33.0	33.9	U	34.9	U
	34.2	34.0	U	37.9	U
	33.6	34.9	U	U	35.0
	34.1	U	35.0	U	U
	33.8	34.9	33.9	U	34.9
	33.7	33.7	U	U	35.0
Avg	33.7	34.2	34.9	35.9	35.0
SD	0.9	0.5	0.8	1.7	0.0

Table 10: Results of sensitivity assessment for assay targeting A. salmonicida gene. Ten replicates for each dilution have been performed.

IVb2. Water samples

Primer optimization

DNA extracted from the water spiked with *A. salmonicida* and filtered through sterivex filters. DNA was diluted 1/100.

- Different concentrations of forward and reverse primers combination were analyzed

200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations
- 5µl of extracted DNA was added to each reaction (Table 11)

	200/200	200/400	400/200	400/400	200/600	600/200	600/600	600/400	400/600
	31.78	31.76	31.39	31.85	31.24	31.57	31.66	31.39	31.41
	31.68	31.46	31.52	31.45	31.28	31.35	31.64	31.32	31.58
Avg	31.73	31.61	31.46	31.65	31.26	31.46	31.65	31.35	31.49
SD	0.08	0.21	0.09	0.28	0.03	0.15	0.01	0.04	0.12

Table 11: Ct values recorded for different combination of primer concentrations of assay targeting A. salmonicida gene. Optimal concentrations of primer sets are highlighted.

Data showed that the optimal primer set combination (lower Ct values) for forward and reverse were **200nM and 600nM** respectively.

Probe optimization

Optimisation of the probe concentration was performed using the same extracted DNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM

Recorded Ct values (Table 12)

	100nM	**125nM**	**150nM**	**175nM**	**200nM**	**225nM**	**250nM**
	31.59	31.48	31.11	31.18	30.99	30.88	31.04
	31.17	31.68	31.42	31.32	30.99	30.88	31.04
Avg	31.38	31.58	31.26	31.25	30.99	30.88	31.04
STD	0.30	0.14	0.22	0.10	0.00	0.00	0.00

Table 12: Ct values recorded for different probe concentrations of assay targeting A. salmonicida gene. Optimal concentration of probe is highlighted

Data showed that the optimal concentration of the probe was **225nM** which provided the lowest Ct value and better amplification curve.

Efficiency

- Extracted DNA extracted was 5-fold serial dilution (n=6)

- qPCR performed using the optimal conditions (200/600nM for both primers forward and reverse respectively and 225 nM for the probe)

- The efficiency of the optimised assay was evaluated at **~90%** (slope is -3.58) (Fig., 8).

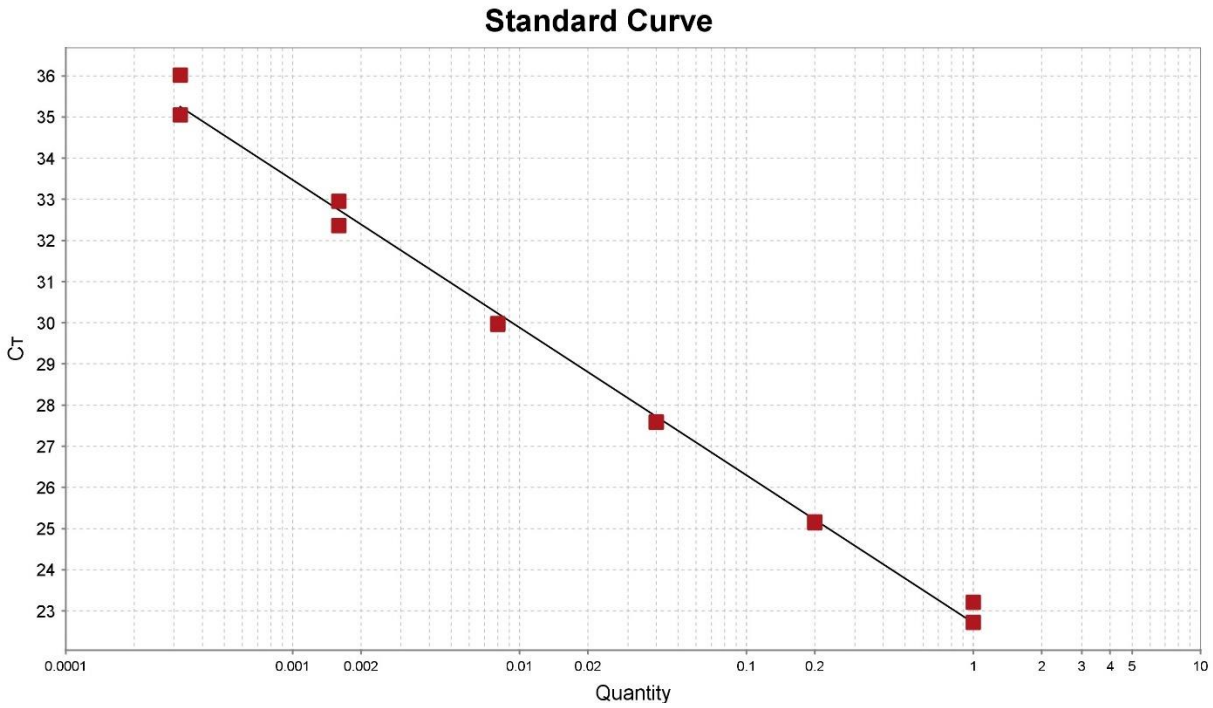

Figure 8: Standard curve of the assay targeting Aeromonas salmonicida DNA extracted from filtered water

Sensitivity

- Sample from standard 1/3125 diluted 3 times was used for the evaluation of the assay sensitivity
- Concentration of the probe was 225 nM and the concentration of the sets of primers, forward and reverse, was 600 nM and 200 nM respectively.
- 2-fold serial dilution (n=6) was performed using sample from standard 1/3125 diluted 3 times
- 10 replicates for each dilution were performed to define the sensitivity of the assay
- Data showed that undetermined (U: no amplification) is recorded in 2 replicate out of 10 at sample 2E-2 with an average Ct value of 34. Therefore, the threshold is set at Ct value of **34** (Table 13).

	1/3125	2E-1	2E-2	2E-3
	31.2	32.6	U	U
	31.3	31.9	U	U
	30.5	33.7	36.3	U
	31.3	31.6	32.9	U
	32.0	34.2	33.5	U
	31.2	31.6	34.1	U
	30.5	33.4	33.2	34.2
	30.8	32.6	34.2	U
	31.2	32.8	34.3	34.5
	32.2	35.4	33.4	33.2
Avg	31.2	33.0	34.0	33.3
SD	0.5	1.2	1.1	0.7

Table 13: Results of sensitivity assessment for assay targeting A. salmonicida gene. Ten replicates for each dilution have been performed.

IVc. *Yersinia ruckeri*

IVc1. Water samples

QPCR was performed on DNA extracted from the diluted samples using BCCAHS routine screening assay of *Y. ruckeri* in fish tissues. To increase the sensitivity, the DNA input was 2 µL and 5 µL for comparison. Overall, the results showed that an input DNA of **5 µL** provided a higher sensitivity compared to 2 µL (Fig., 9).

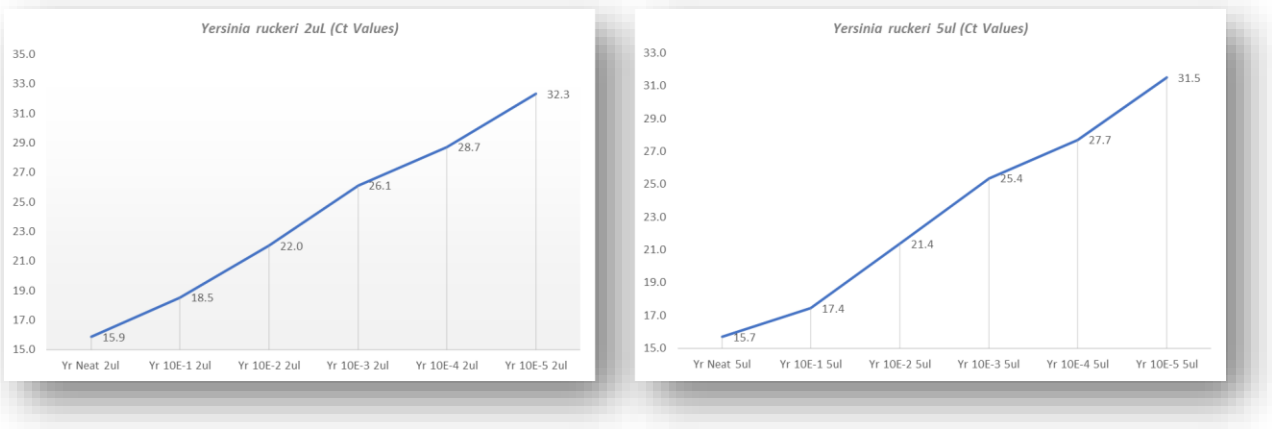

Figure 9: Comparison of qPCR results for Yersinia ruckeri using 2ul and 5ul DNA extracted from swab as input.

For optimal conditions of the qPCR assay targeting *Y. ruckeri* in swab, the primer and probe sets have been optimised using DNA extracted from spiked swab. Efficiency and sensitivity of the optimized assay have been also evaluated for better performance and limit of amplification.

Primer optimization

DNA extracted from the swab immerged in the bacterial suspension diluted 1/1,000 from the original (neat) bacterial solution.

- Different concentrations of forward and reverse primers combination were analyzed

200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations (Table 14)

	200/200	200/400	400/200	400/400	200/600	600/200	600/600	600/400	400/600
	28.278	26.500	28.242	26.210	25.850	27.814	25.759	26.296	25.866
	28.149	26.452	27.977	26.189	25.879	27.693	25.788	26.270	25.868
Avg	**28.213**	**26.476**	**28.110**	**26.200**	**25.864**	**27.754**	**25.774**	**26.283**	**25.867**
SD	**0.09**	**0.03**	**0.19**	**0.02**	**0.02**	**0.09**	**0.02**	**0.02**	**0.00**

Table 14: Ct values recorded for different combination of primer concentrations of assay targeting Y. ruckeri gene. Optimal concentrations of primer sets are highlighted.

Data showed that the optimal primer set combination (lower Ct values) for forward and reverse were **600nM and 600nM** respectively.

Probe optimization

Optimisation of the probe concentration was performed using the same extracted DNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM
- Recorded Ct values (Table 15)

	100nM	125nM	150nM	175nM	200nM	225nM	250nM
	26.315	26.169	26.143	26.074	26.396	25.884	25.927
	26.252	26.150	26.199	25.978	25.922	26.027	25.921
Avg	**26.283**	**26.160**	**26.171**	**26.026**	**26.159**	**25.955**	**25.924**
STD	**0.04**	**0.01**	**0.04**	**0.07**	**0.33**	**0.10**	**0.00**

Table 15: Ct values recorded for different probe concentrations of assay targeting Y. ruckeri gene. Optimal concentration of probe is highlighted

Data showed that the optimal concentration of the probe was **250nM** which provided the lowest Ct value and better amplification curve.

Efficiency

- DNA extracted from swap immerged into original *Y. ruckeri* bacterial solution was 10-fold serial diluted (n=6) and used to assess the efficiency.

- qPCR performed using the optimal conditions (600/600nM for both primers forward and reverse respectively and 250 nM for the probe)
- The efficiency of the optimised assay was evaluated at **~96%** (slope is -3.42) (Fig., 10).

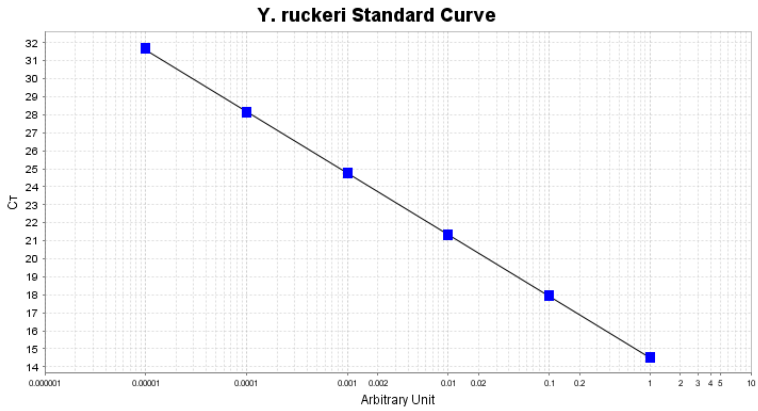

Figure 10: Standard curve of the assay targeting Yersinia ruckeri DNA extracted from swab

Sensitivity

- Sample from 10E-4 was used for the evaluation of the assay sensitivity
- Concentration of the probe was 250 nM and the concentration of the sets of primers, forward and reverse, was 600 nM and 600 nM respectively.
- 2 fold serial dilution (n=6) was performed using sample from 1/125
- 10 replicates for each dilution were performed to define the sensitivity of the assay
- Data showed that undetermined (U: no amplification) is recorded in 4 replicates out of 10 at 10E-5 diluted 2-fold twice (2E-2) at an average Ct value of 34.7. Therefore, the threshold is set at Ct value of **34** (Table 16).

	10E-6	2E-1	2E-2	2E-3	2E-4	2E-5
	32.8	33.3	U	U	U	35.3
	34.6	35.8	34.8	U	U	U
	31.9	35.0	U	U	U	U
	34.0	32.9	34.8	U	U	U
	33.0	34.9	35.9	34.4	U	U
	35.1	34.1	34.0	34.9	U	U
	33.2	34.3	U	U	U	U
	32.6	33.3	34.9	U	U	U
	34.9	32.9	U	34.8	U	U
	32.5	33.2	33.9	U	U	U
Avg	**33.5**	**34.0**	**34.7**	**34.7**	**U**	**35.3**
SD	**1.1**	**1.0**	**0.7**	**0.3**		

Table 16: Results of sensitivity assessment for assay targeting Y. ruckeri gene. Ten replicates for each dilution have been performed.

IVc2. Water samples

Primer optimization

DNA extracted from the water spiked with *Y. ruckeri* and filtered through sterivex filters. DNA was diluted 1/100.

- Different concentrations of forward and reverse primers combination were analyzed

  200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations
- 5µl of extracted DNA was added to each reaction

Data showed that the optimal primer set combination (lower Ct values) for forward and reverse were **600nM and 600nM** respectively (Table 17).

	200/200	200/400	400/200	400/400	200/600	600/200	600/600	600/400	400/600
	31.05	29.66	30.39	29.58	30.70	29.69	**29.39**	31.69	29.14
	31.27	29.77	30.33	29.72	31.77	29.93	**29.52**	31.62	29.82
Avg	31.16	29.72	30.36	29.65	31.24	29.81	29.46	31.65	29.48
SD	0.16	0.08	0.04	0.10	0.76	0.17	0.09	0.05	0.48

Table 17: Ct values recorded for different combination of primer concentrations of assay targeting Y. ruckeri gene. Optimal concentrations of primer sets are highlighted

Probe optimization

Optimisation of the probe concentration was performed using the same extracted DNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM

Recorded Ct values (Table 18)

	100nM	**125nM**	**150nM**	**175nM**	**200nM**	**225nM**	**250nM**
	29.65	29.80	29.97	29.32	29.23	30.12	**29.37**
	29.68	29.98	30.30	29.43	29.58	29.28	**28.99**
Avg	29.66	29.89	30.14	29.37	29.40	29.70	29.18
STD	0.02	0.13	0.23	0.08	0.24	0.59	0.27

Table 18: Ct values recorded for different probe concentrations of assay targeting Y. ruckeri gene. Optimal concentration of probe is highlighted

Data showed that the optimal concentration of the probe was **250nM** which provided the lowest Ct value and better amplification curve.

Efficiency

- DNA extracted from spiked water with *Y. ruckeri* was 5-fold serial diluted (n=5)
- qPCR was performed using the optimal conditions (600/600nM for both primers set forward and reverse respectively and 250 nM for the probe).
- The efficiency of the optimised assay was evaluated at ~**98%** (slope is -3.34) (Fig., 11).

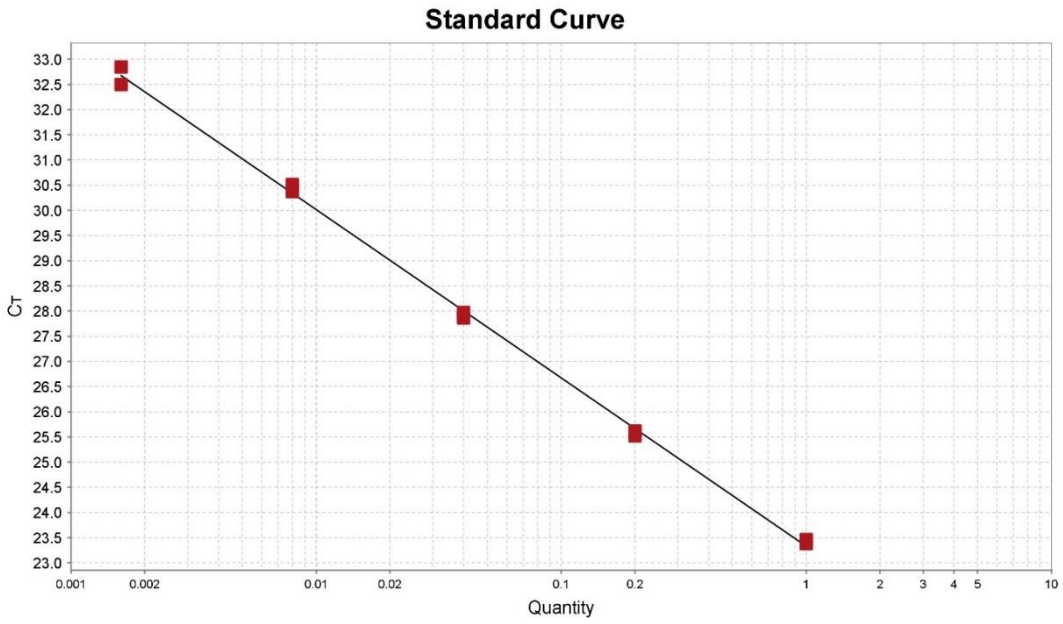

Figure 11: Standard curve of the assay targeting Yersinia ruckeri DNA extracted from sipked filtered water

Sensitivity

- Sample from standard curve (1/125) diluted 1/10 was used for the evaluation of the assay sensitivity
- Concentration of the probe was 250 nM and the concentration of the sets of primers, forward and reverse, was 600 nM and 600 nM respectively.
- 2 fold serial dilution (n=4) was performed using sample from 1/125 diluted 1/10
- 10 replicates for each dilution were performed to define the sensitivity of the assay
- Data showed that undetermined (U: no amplification) is recorded in 2 replicates out of 10 2-fold twice (2E-2) at an average Ct value of 35. Therefore, the threshold is set at Ct value of **34-35** (Table 19).

	Neat	2E-1	2E-2	2E-3
	34.220	33.092	U	U
	33.949	33.259	34.706	U
	33.198	34.762	34.782	34.354
	33.554	33.855	36.303	34.732
	33.420	34.092	34.594	36.272
	33.370	33.713	35.381	U
	33.262	34.571	35.594	35.519
	33.435	34.306	U	36.330
	32.883	33.749	33.967	U
	33.216	36.400	34.447	U
Avg	33.5	34.2	35.0	35.4
SD	0.4	0.9	0.7	0.9

Table 19: Results of sensitivity assessment for assay targeting Y. ruckeri gene. Ten replicates for each dilution have been performed.

V. Viruses assay optimisation

During sampling, fish mucus and gills were swabbed using the swabs manufactured by Launchork CDMO (Quebec, Canada). Swabs were stored in Viral Transport Media and kept frozen during transportation (Fig., 12).

Figure 12: swabs manufactured by Launchwork CDMO used in this study

To ensure that the assay can detect virus genetic material in the collected samples, swabs were immerged in cell culture supernatant containing VHSv and IHNv. As cell culture infected with ISAv and IPNv are not available at BC CAHS, swabs were immerged in solution with ISAv and IPNv genetic materials. Swabs were stored in VTM and kept frozen until analysis.

RNA was extracted from the different solutions and RT-qPCR performed according to BC CAHS routine screening.

Results showed that the assay can detect VHSv, IHNv, ISAv but not IPNv in the spiked solutions. Testing was performed to compare Ct values from supernatant and swab immerged into the supernatant or spiked solution with genetic materials. For VHSv, there was a difference of Ct values ranging between 5 for high virus concentration and 1 for lower concentrations (Fig., 13). For IHNv, the comparison showed a difference of Ct values between 6 and 3 (Fig., 14).

Figure 13: Comparison of RT-qPCR results targeting VHSv RNA extracted from supernatant and spiked swab in VTM

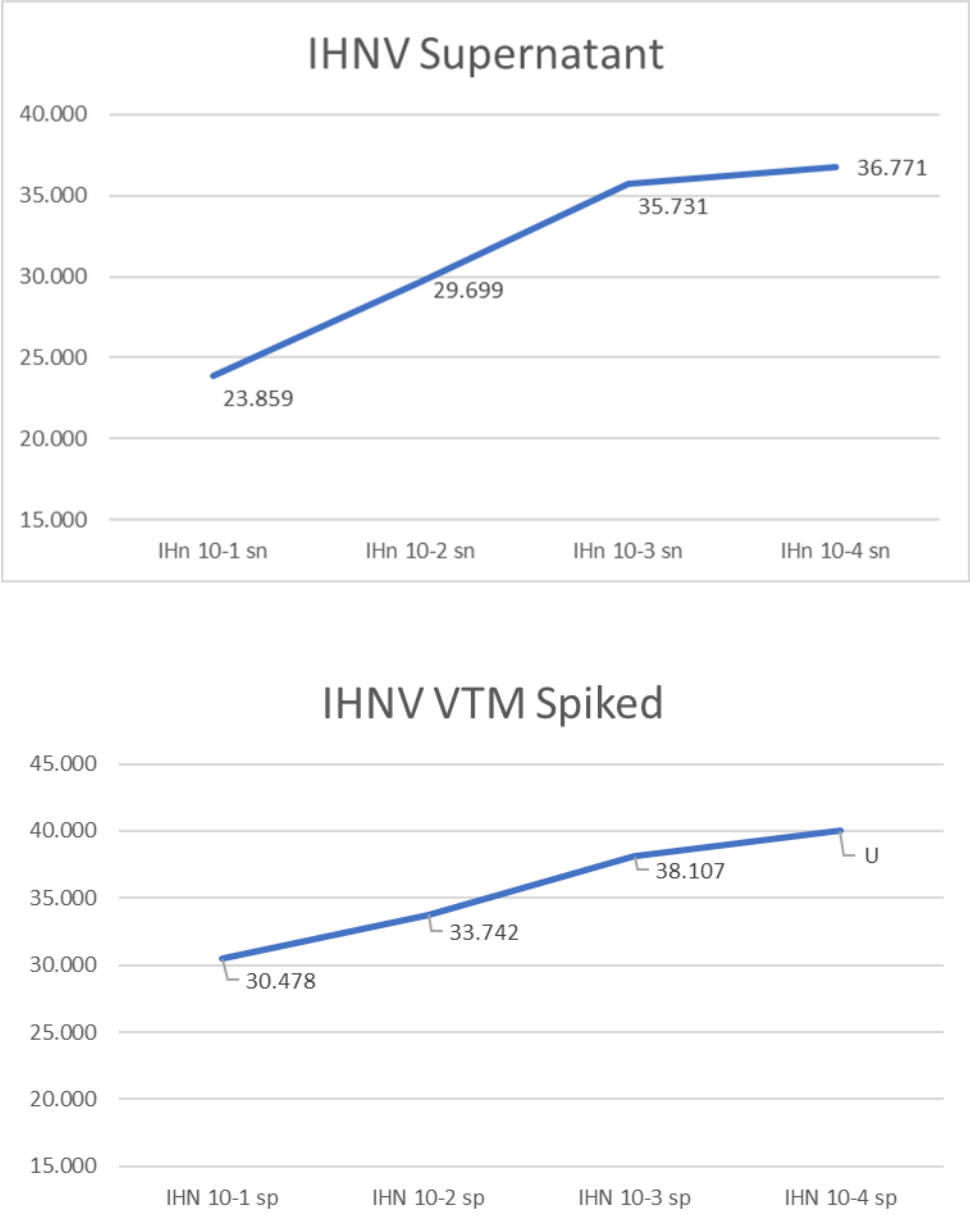

Figure 14: Comparison of RT-qPCR results targeting IHNv RNA extracted from supernatant and spiked swab in VTM

VTM spiked with ISAv genetic material diluted 100,000 times (ISA 10E-5, Table 20), ISAv genetic material was detected at Ct value ~34 (34.9 and 34.1). However, VTM spiked with ISAv genetic material diluted 1,000,000 times (ISA 10E-6), no amplification has been recorded (Table 20). This would mean that the limit of detection by the assay in VTM spiked with ISAv genetic material diluted between 10^5 and 10^6 time from the original sample (neat).

Well	Well Position	Sample Name	Target Name	CT
9	A9	ISA 10-5	ISA FAM	34.903
10	A10	ISA 10-5	ISA FAM	34.149
21	B9	ISA 10-6	ISA FAM	Undetermined
22	B10	ISA 10-6	ISA FAM	Undetermined

Well	Well Position	Sample Name	Target Name	CT
81	G9	IPN 10-3	IPN FAM	Undetermined
82	G10	IPN 10-3	IPN FAM	Undetermined
93	H9	IPN 10-4	IPN FAM	Undetermined
94	H10	IPN 10-4	IPN FAM	Undetermined

Table 20: Ct values of ISAv and IPNv genetic materials spiked in viral transport media. VTM was spiked with ISAv genetic material diluted 10^5 and 10^6 times and with IPNv genetic material diluted 10^3 and 10^4 times.

VTM was spiked with IPNV genetic materials diluted 1,000 and 10,000 times from the original sample (neat). However, our assay did not amplify any IPNv genetic material from both spiked samples (Table 20).

To increase the sensitivity of the detection, we investigated the usage of centrifugal filters to concentrate the virus (VHSv and IHNv). VHSv and IHNv concentration was performed by ultrafiltration using Amico Ultra Centrifugal Filtration Units manufactured Millipore/Sigma (ON, Canada) (Fig., 15). The expected results are to concentrate the virus by 100 times which should corresponds to a gain of sensitivity by 6-7 Ct values.

Figure 15: Centrifugal filter units used to concentrate virus

Comparative analysis has been performed between samples tested with and without ultrafiltration (UFC) for VHSv and IHNv. Results showed that UFC increased the sensitivity of the testing by 1 to 2 Ct values (Table 21). However, this increase in sensitivity was much lower than expected (6-7 Ct values).

	Sample Name	CT	Ct Mean	Sample Name	CT	Ct Mean
VHSv	UFC 10E-4 VHS	32.5	32.9	VHS 10E-4	35.3	34.8
	UFC 10E-4 VHS	33.3		VHS 10E-4	34.3	
	UFC 10E-3 VHS	31.4	31.4	VHS 10E-3	31.6	31.4
	UFC 10E-3 VHS	31.5		VHS 10E-3	31.3	
IHNv	UFC 10E-4 IHN	33.9	34.2	IHN 10E-4	36.6	36.6
	UFC 10E-4 IHN	34.4		IHN 10E-4	U	
	UFC 10E-3 IHN	35.5	35.5	IHN 10E-3	36.5	36.5
	UFC 10E-3 IHN	U		IHN 10E-3	U	

Table 21: Ct values of VHSv and IHNv spiked in viral transport media. VTM was spiked with IHNv and VHSv diluted 10^3 and 10^4 times and treated with or without ultrafiltration (UFC).

Further analysis was performed to test if increasing the volume of sample from 100ul to 200ul may improve the sensitivity. Results showed that the increase of the volume has a slight improvement in the sensitivity by 1 Ct value mainly for low load of VHSv virus (10E-4) (Table 22). It is noteworthy that IPNv was detected (IPN 10E-2 200ul) when the volume was increased from 2 to 4 ul (Table 23). In general, the increase of volume improves the sensitivity of the detection for low virus load.

	Sample Name	CT	Ct Mean	Sample Name	CT	Ct Mean
VHSv	VHS 10E-4 100ul	35.3	34.8	VHS 10E-4 200ul	33.1	32.8
	VHS 10E-4 100ul	34.3		VHS 10E-4 200ul	32.5	
	VHS 10E-3 100ul	31.6	31.4	VHS 10E-3 200ul	34.0	33.7
	VHS 10E-3 100ul	31.3		VHS 10E-3 200ul	33.5	
IHNv	IHN 10E-4 100ul	36.6	36.6	IHN 10E-4 200ul	U	36.8
	IHN 10E-4 100ul	U		IHN 10E-4 200ul	36.8	
	IHN 10E-3 100ul	36.5	36.5	IHN 10E-3 200ul	37.0	35.9
	IHN 10E-3 100ul	U		IHN 10E-3 200ul	34.9	
ISAv	ISA 10E-5 100ul	36.0	36.8	ISA 10E-5 200ul	35.1	35.3
	ISA 10E-5 100ul	37.6		ISA 10E-5 200ul	35.4	
	ISA 10E-4 100ul	30.2	30.3	ISA 10E-4 200ul	31.2	31.5
	ISA 10E-4 100ul	30.5		ISA 10E-4 200ul	31.7	
IPNv	IPN 10E-3 100ul	U	U	IPN 10E-3 200ul	U	U
	IPN 10E-3 100ul	U		IPN 10E-3 200ul	U	
	IPN 10E-2 100ul	U	U	IPN 10E-2 200ul	35.8	35.3
	IPN 10E-2 100ul	U		IPN 10E-2 200ul	34.8	

Table 22: Ct values of VHSv, IHNv, ISAv and IPNv spiked in viral transport media. VTM was spiked with IHNv and VHSv diluted 103 and 104 times and treated with or without ultrafiltration (UFC) as well as ISAv genetic material diluted 10^4 and 10^5 times

and with IPNv genetic material diluted 10^2 and 10^2 times. Ct values are compared when using 100ul or 200ul of starting materials for testing.

Finally, we investigated if combining increase in volume of the starting sample as well as increase the volume of the extracted RNA from 2 to 4ul may improve the detection sensitivity. Comparative testing for VHSv showed that increasing the volume in both starting material and RNA improved the sensitivity by an average 1 Ct value.

Sample Name	CT	Ct Mean
VHS 10E-4 200ul 2ul	33.1	32.8
VHS 10E-4 200ul 2ul	32.5	
VHS 10E-4 200ul 4ul	31.4	31.8
VHS 10E-4 200ul 4ul	32.3	
VHS 10E-3 200ul 2ul	34.0	33.7
VHS 10E-3 200ul 2ul	33.5	
VHS 10E-3 200ul 4ul	32.5	32.6
VHS 10E-3 200ul 4ul	32.7	

Table 23: Ct values of VHSv spiked in viral transport media. VTM was spiked with VHSv diluted 10^3 and 10^4 times . Ct values are compared when using 2ul or 4ul of extracted RNA.

For further assay optimisation, the volume of starting materials will be **200 ul** and the volume of RNA for RT-qPCR will be **4ul**.

Va. Infectious pancreatic necrosis virus (IPNv)

Primer optimization

RNA was extracted from the swab immerged in solution spiked with IPN genetic material. Different concentrations of forward and reverse primers combination were analyzed

200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations (Table 24)

	200/200	200/400	400/200	400/400	200/600	600/200	600/600	600/400	400/600
	20.62	21.05	20.88	20.73	20.61	20.53	21.85	22.03	21.23
	20.91	21.28	20.88	20.66	20.60	20.70	21.90	22.41	21.24
Avg	**20.76**	**21.17**	**20.88**	**20.69**	**20.60**	**20.62**	**21.87**	**22.22**	**21.23**
SD	0.20	0.16	0.00	0.05	0.01	0.12	0.03	0.27	0.00

Table 24: Ct values recorded for different combination of primer concentrations of assay targeting IPNv gene. Optimal concentrations of primer sets are highlighted

Data showed that the optimal primer set combination (lower Ct values) for forward and reverse were **200nM and 600nM** respectively.

Probe optimization

Optimisation of the probe concentration was performed using the same extracted RNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM
- Recorded Ct values (Table 25)

	100	125	150	175	200	225	250
	20.66	20.39	20.00	19.99	19.97	19.78	19.84
	20.54	20.85	20.09	19.91	20.04	19.71	19.85
Avg	20.60	20.62	20.04	19.95	20.00	19.75	19.84
SD	0.09	0.32	0.06	0.05	0.04	0.05	0.01

Table 25: Ct values recorded for different probe concentrations of assay targeting IPNv gene. Optimal concentration of probe is highlighted

Data showed that the optimal concentration of the probe was **225nM** which provided the lowest Ct value and better amplification curve.

Efficiency

- RNA extracted from swap immerged into spiked IPN genetic material into supernatant solution was 10 fold serial diluted (n=6) and used to assess the efficiency.

- RT qPCR performed using the optimal conditions (200/600nM for both primers forward and reverse respectively and 225 nM for the probe)

- The efficiency of the optimised assay was evaluated at **~92%** (slope is -3.53) (Fig., 16).

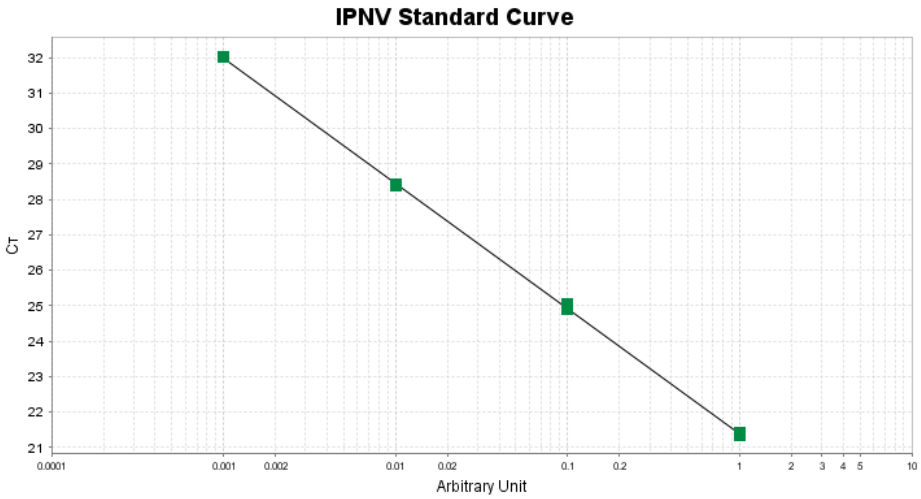

Figure 16: Standard curve of the assay targeting infectious pancreatic necrosis genetic material extracted from viral transport media

Sensitivity

- Sample from 10E-3 was used for the evaluation of the assay sensitivity
- Concentration of the probe was 225 nM and the concentration of the sets of primers, forward and reverse, was 200 nM and 600 nM respectively.
- 2 fold serial dilution (n=6) was performed using sample from 10E-4
- 10 replicates for each dilution were performed to define the sensitivity of the assay
- Data showed that undetermined (U: no amplification) is recorded in 4 replicates out of 10 at 10E-4 diluted 2-fold (2E-1) at an average Ct value of 34.7. Therefore, the threshold is set at Ct value of **33-34** (Table 26).

	10E-4	2E-1	2E-2	2E-3
	32.6	32.4	36.4	U
	32.4	37.0	34.6	U
	33.6	34.6	36.4	U
	32.9	34.9	36.4	36.1
	33.2	35.8	35.2	37.6
	33.6	35.3	U	35.9
	32.6	33.1	U	U
	32.3	34.5	34.0	U
	32.8	U	U	37.4
	32.5	32.5	35.4	31.9
Avg	**32.9**	**34.5**	**35.5**	**35.8**
SD	**0.5**	**1.5**	**1.0**	**2.3**

Table 26: Results of sensitivity assessment for assay targeting IPNv gene. Ten replicates for each dilution have been performed.

Vb. Viral haemorrhagic septicaemia virus (VHSv)

Primer optimization

RNA was extracted from the swab immerged in VHS viral supernatant.
Different concentrations of forward and reverse primers combination were analyzed

200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations (Table 27)

	200/200	200/400	400/200	400/400	200/600	600/200	600/600	600/400	400/600
	21.26	21.47	20.76	21.87	21.36	21.19	21.97	22.14	21.94
	21.41	21.41	20.75	21.79	21.40	21.07	21.94	22.07	21.90
Avg	**21.34**	**21.44**	**20.76**	**21.83**	**21.38**	**21.13**	**21.95**	**22.10**	21.08
SD	0.10	0.04	0.00	0.05	0.03	0.09	0.02	0.05	0.03

Table 27: Ct values recorded for different combination of primer concentrations of assay targeting VHSv gene. Optimal concentrations of primer sets are highlighted

Data showed that the optimal primer set combination (lower Ct values) for forward and reverse were **400nM and 200nM** respectively.

Probe optimization

Optimisation of the probe concentration was performed using the same extracted RNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM
- Recorded Ct values (Table 28)

	100	125	150	175	200	225	250
	22.21	22.07	21.90	21.80	21.62	21.60	21.64
	22.17	22.06	21.84	21.79	21.63	21.57	21.56
Avg	22.19	22.07	21.87	21.79	21.63	21.59	21.60
SD	0.03	0.01	0.04	0.01	0.00	0.02	0.05

Table 28: Ct values recorded for different probe concentrations of assay targeting VHSv gene. Optimal concentration of probe is highlighted

Data showed that the optimal concentration of the probe was **225nM** which provided the lowest Ct value and better amplification curve.

Efficiency

- A solution combining RNA extracted from swap immerged into spiked VHS viral supernatant solution as well as spiked amplicon from dilution 10^{-7} was 10 fold serial diluted (n=6) and used to assess the efficiency.

- RT qPCR performed using the optimal conditions (400/200nM for both primers forward and reverse respectively and 225 nM for the probe)

- The efficiency of the optimised assay was evaluated at **~96%** (slope is -3.41) (Fig., 17).

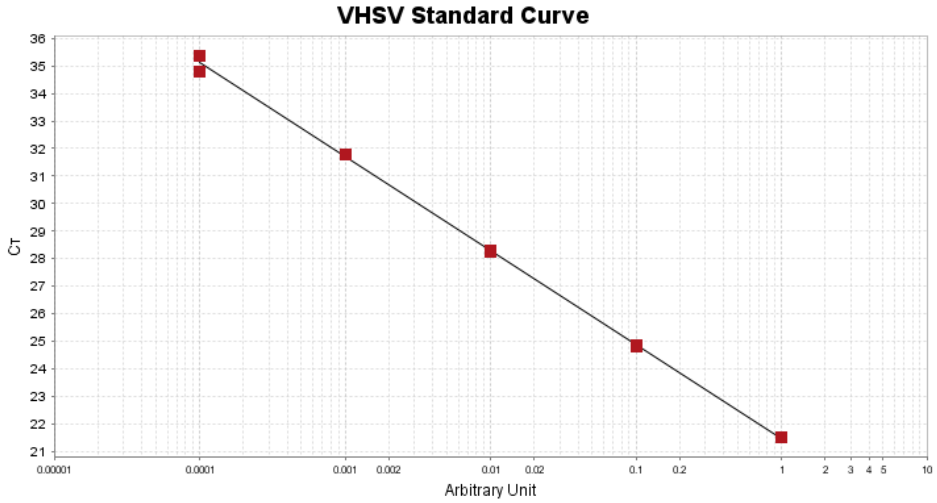

Figure 17: Standard curve of the assay targeting Viral haemorrhagic septicaemia RNA extracted from viral transport media

Sensitivity

- Sample from 10E-3 diluted 5 times was used for the evaluation of the assay sensitivity
- Concentration of the probe was 225 nM and the concentration of the sets of primers, forward and reverse, was 400 nM and 200 nM respectively.
- 2 fold serial dilution (n=4) was performed using sample from 10E-3 diluted 5 times
- 10 replicates for each dilution were performed to define the sensitivity of the assay
- Data showed that undetermined (U: no amplification) is recorded in 4 replicates out of 10 at 10E-3 diluted 5 times diluted 2 fold three times (2E-3) at an average Ct value of 34.8. Therefore, the threshold is set at Ct value of **34** (Table 29).

	10E-3 - dil 1/5	2E-1	2E-2	2E-3	2E-4
	32.8	32.7	34.6	34.6	35.0
	33.6	33.1	34.6	35.5	34.6
	32.1	33.2	32.9	34.7	35.5
	32.0	33.8	33.9	U	35.3
	32.0	33.3	34.2	34.8	U
	33.3	33.0	33.7	34.6	35.2
	33.2	33.0	33.3	35.3	34.2
	32.4	32.6	34.1	34.5	U
	32.2	33.2	35.2	34.3	34.1
	32.7	32.3	34.7	U	35.2
Avg	**32.6**	**33.0**	**34.1**	**34.8**	**34.9**
SD	**0.6**	**0.4**	**0.7**	**0.4**	**0.5**

Table 29: Results of sensitivity assessment for assay targeting VHSv gene. Ten replicates for each dilution have been performed.

Vc. Infectious salmon anaemia virus (ISAv)

Primer optimization

RNA was extracted from the swab immerged in solution spiked with ISA genetic material. Different concentrations of forward and reverse primers combination were analyzed

200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations (Table 30)

	200/200	200/400	400/200	400/400	200/600	600/200	600/600	600/400	400/600
	22.07	22.20	22.35	22.14	22.32	22.18	22.98	23.33	22.11
	22.23	22.14	22.33	22.18	22.28	22.05	23.02	23.31	22.33
Avg	**22.15**	**22.17**	**22.34**	**22.16**	**22.30**	**22.12**	**23.00**	**23.32**	**22.22**
SD	0.11	0.04	0.02	0.03	0.03	0.09	0.03	0.01	0.16

Table 30: Ct values recorded for different combination of primer concentrations of assay targeting ISAv gene. Optimal concentrations of primer sets are highlighted

Data showed that the optimal primer set combination (lower Ct values) for forward and reverse were **600nM and 200nM** respectively.

Probe optimization

Optimisation of the probe concentration was performed using the same extracted RNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM
- Recorded Ct values (Table 31)

	100	125	150	175	200	225	250
	23.26	23.08	23.01	22.77	22.72	22.59	22.36
	23.22	23.06	23.04	22.98	22.66	22.53	22.36
Avg	23.24	23.07	23.02	22.88	22.69	22.56	22.36
SD	0.02	0.01	0.02	0.15	0.04	0.04	0.00

Table 31: Ct values recorded for different probe concentrations of assay targeting ISAv gene. Optimal concentration of probe is highlighted

Data showed that the optimal concentration of the probe was **250nM** which provided the lowest Ct value and better amplification curve.

Efficiency

- RNA extracted from swap immerged into spiked ISA genetic material into solution was 10 fold serial diluted (n=6) and used to assess the efficiency.

- RT qPCR performed using the optimal conditions (600/200nM for both primers forward and reverse respectively and 250 nM for the probe)

- The efficiency of the optimised assay was evaluated at ~**93%** (slope is -3.50) (Fig., 18).

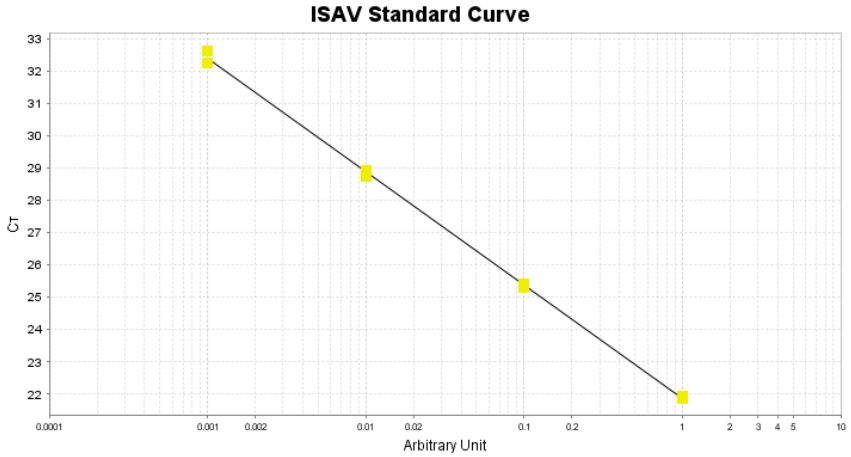

Figure 18: Standard curve of the assay targeting infectious salmon anaemia virus extracted from viral transport media

Sensitivity

- Sample from 10E-3 diluted 2 fold was used for the evaluation of the assay sensitivity
- Concentration of the probe was 250 nM and the concentration of the sets of primers, forward and reverse, was 600 nM and 200 nM respectively.
- 2 fold serial dilution (n=4) was performed using sample from 10E-3 diluted 2 fold
- 10 replicates for each dilution were performed to define the sensitivity of the assay
- Data showed that undetermined (U: no amplification) is recorded in 2 replicates out of 10 at 10E-3 diluted 2 fold three times (2E-3) at an average Ct value of 35.4. Therefore, the threshold is set at Ct value of **35** (Table 32).

	Neat	2E-1	2E-2	2E-3
	32.5	32.2	34.9	34.9
	31.6	33.6	36.1	35.9
	31.3	32.3	33.2	33.7
	31.4	32.5	33.9	37.9
	31.8	32.9	34.4	34.9
	31.7	33.6	36.0	34.4
	32.4	33.2	33.9	35.9
	31.3	32.6	33.2	U
	31.7	33.2	33.4	35.8
	32.7	32.7	33.5	U
Avg	**31.9**	**32.9**	**34.3**	**35.4**
SD	**0.5**	**0.5**	**1.1**	**1.3**

Table 32: Results of sensitivity assessment for assay targeting ISAv gene. Ten replicates for each dilution have been performed.

Vd. Infectious hematopoietic necrosis virus (IHNv)

Primer optimization

RNA was extracted from the swab immerged in solution spiked with IHN genetic material. Different concentrations of forward and reverse primers combination were analyzed

200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations (Table 33)

	200/200	400/200	600/200	200/400	400/400	600/400	200/600	400/600	600/600
	21.1	20.9	21.2	21.4	23.0	22.0	21.9	22.0	21.4
	22.3	21.6	22.4	22.8	21.8	22.4	21.0	21.6	21.7
Avg	**21.7**	**21.2**	**21.8**	**22.1**	**22.4**	**22.2**	**21.5**	**21.8**	**21.5**
SD	**0.8**	**0.5**	**0.8**	**1.0**	**0.8**	**0.3**	**0.6**	**0.3**	**0.2**

Table 33: Ct values recorded for different combination of primer concentrations of assay targeting IHNv gene. Optimal concentrations of primer sets are highlighted

Data showed that the optimal primer set combination (lower Ct values) for forward and reverse were **400nM and 200nM** respectively.

Probe optimization

Optimisation of the probe concentration was performed using the same extracted RNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM
- Recorded Ct values (Table 34)

	100	125	150	175	200	225	250
	31.19	30.36	30.99	32.29	33.35	33.12	33.74
	30.95	30.36	31.34	32.83	33.50	33.09	33.76
Avg	31.07	30.36	31.16	32.56	33.43	33.10	33.75
SD	0.16	0.00	0.25	0.38	0.10	0.02	0.01

Table 34: Ct values recorded for different probe concentrations of assay targeting IHNv gene. Optimal concentration of probe is highlighted

Data showed that the optimal concentration of the probe was **125nM** which provided the lowest Ct value and better amplification curve.

Efficiency

- RNA extracted from swap immerged into spiked ISA genetic material into solution was 10 fold serial diluted (n=6) and used to assess the efficiency.

- RT qPCR performed using the optimal conditions (400/200nM for both primers forward and reverse respectively and 125 nM for the probe)

The efficiency of the optimised assay was evaluated at ~**94%** (slope is -3.46) (Fig., 19)

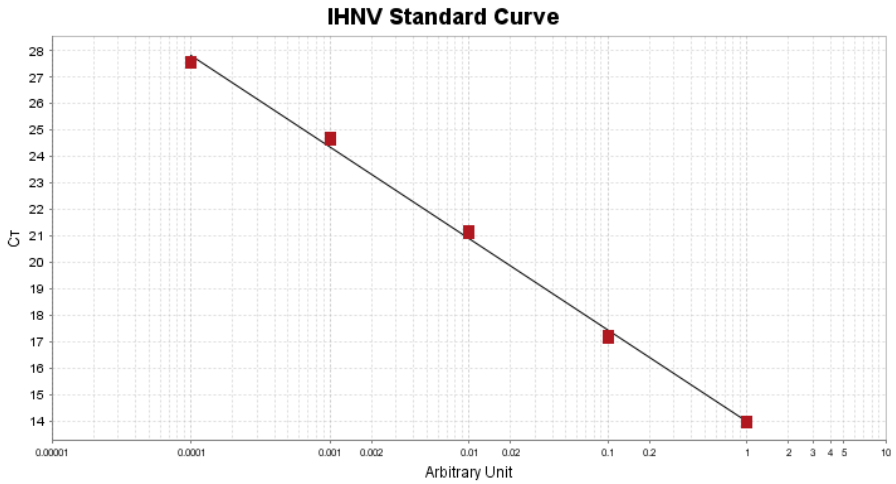

Figure 19: Standard curve of the assay targeting Infectious hematopoietic necrosis RNA extracted from viral transport media

Sensitivity

- Sample from 10E-2 diluted 5-fold was used for the evaluation of the assay sensitivity
- Concentration of the probe was 125 nM and the concentration of the sets of primers, forward and reverse, was 400 nM and 200 nM respectively.
- 2 fold serial dilution (n=4) was performed using sample from 10E-2 diluted 5 fold
- 10 replicates for each dilution were performed to define the sensitivity of the assay
- Data showed that undetermined (U: no amplification) is recorded in 1 replicates out of 10 at 10E-2 diluted 5 fold two times (2E-2) at an average Ct value of 37. Therefore, the threshold is set at Ct value of **35-36** (Table 35).

	Neat	2E-1	2E-2	2E-3
	34.8	35.5	U	37.5
	34.5	36.1	35.5	37.4
	35.2	34.6	36.1	U
	34.6	36.1	36.7	39.8
	33.8	34.8	37.6	U
	35.2	37.5	36.7	U
	34.4	35.1	37.4	36.6
	34.7	35.7	37.5	U
	34.3	35.4	37.5	37.4
	35.0	35.4	37.8	37.5
Avg	34.7	35.6	37.0	37.7
SD	0.4	0.8	0.8	1.1

Table 35: Results of sensitivity assessment for assay targeting IHNv gene. Ten replicates for each dilution have been performed.

Ve. Epizootic hematopoietic necrosis virus (EHNv)

Ve1. OIE assay optimization

Optimization

For EHNv identification, a PCR protocol suggested as a diagnostic test for EHNv from the OIE (World Organisation of Animal Health) Manual of Diagnostic Tests for Aquatic Animals was performed with minor modifications. No qPCR tests were available in the OIE Manual for testing EHNv.

In this assay, two primers, a reverse primer (5'-AAA-GAC-CCG-TTT-TGC-AGC-AAA-C-3') and forward primer (5'-CGC AGT CAA GGC CTT GAT GT-3') are used for amplification of a 580bp region of the MCP sequence of EHNv. The PCR mixture is amplified in an automatic thermal cycler programmed for 35 cycles at 95°C for 60 seconds, 55°C for 60 seconds, and 72°C for 60 seconds, and held at 72°C for 15 minutes. Amplified DNA (580 bp) is analysed by agarose gel electrophoresis.

To verify this assay with EHNv amplicon, dilutions were amplified using the PCR conditions specified and visualized by agarose gel electrophoresis.

- Five 10-fold dilutions were prepared from a 0.1ng/µl stock of amplicon (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5})
- 5µL of amplicon dilutions were added to the PCR reaction
- Forward and reverse primers were added at a concentration of 1µM each in accordance with the OIE guidelines.

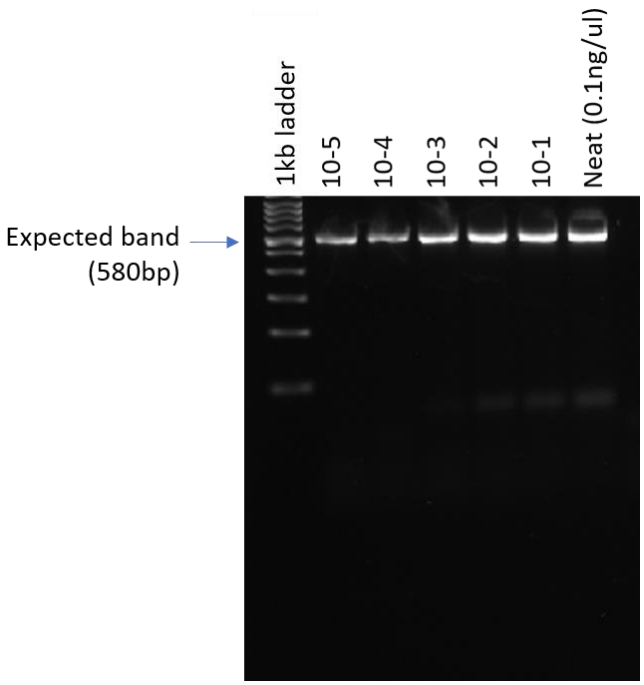

Figure 20: 1.5% agarose gel with PCR products from EHNv

All dilutions showed a strong band at the expected size (580bp) in a 1.5% agarose gel after PCR with EHNv primers.

To test whether the assay resulted in loss of sensitivity when performed after DNA extraction, EHNv was spiked into VTM and a DNA extraction was performed using a MagMAX™ DNA Multi-Sample Ultra 2.0 Kit on the KingFisher™ Flex. As cell culture infected with EHNv is not available at BC CAHS, swabs were immersed in solutions of EHNv amplicon dilutions and stored in VTM and kept frozen until analysis. Dilutions of 10^{-2}-10^{-8} were tested, and PCR and gel electrophoresis were performed according to OIE guidelines.

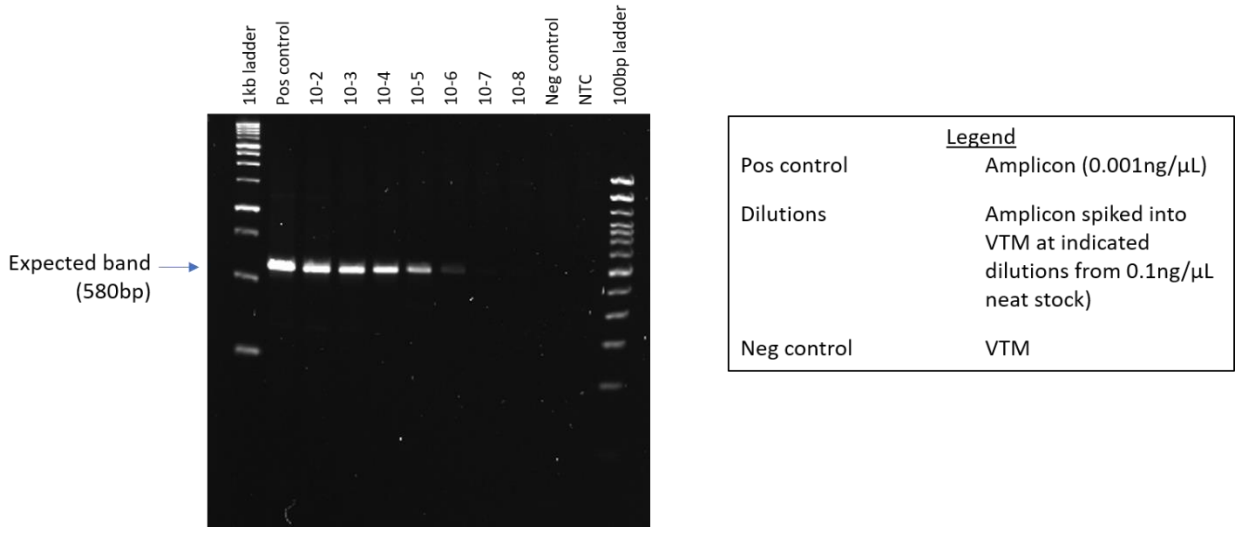

Figure 21: 1.5% agarose gel with PCR products from EHNv spiked VTM after DNA extraction

Results indicated that the assay can detect EHNv at dilutions of 10^{-2} – 10^{-5}, which were visible as strong bands on the agarose gel. Therefore, the limit of detection of EHNv is between 10^{-6} and 10^{-7}.

Sample testing

To verify that this assay worked with swab samples submerged in VTM, a subset of samples were tested using this assay, using the same MagMAX™ DNA Multi-Sample Ultra 2.0 Kit on the KingFisher™ Flex.

- Sample input volume was 400µL, elution volume 100µL
- Negative control was viral transport media alone and positive control was a spiked swab (swab dipped in amplicon dilutions 10^{-3}-10^{-5}) in viral transport media
- PCR products visualized on 2% agarose gel

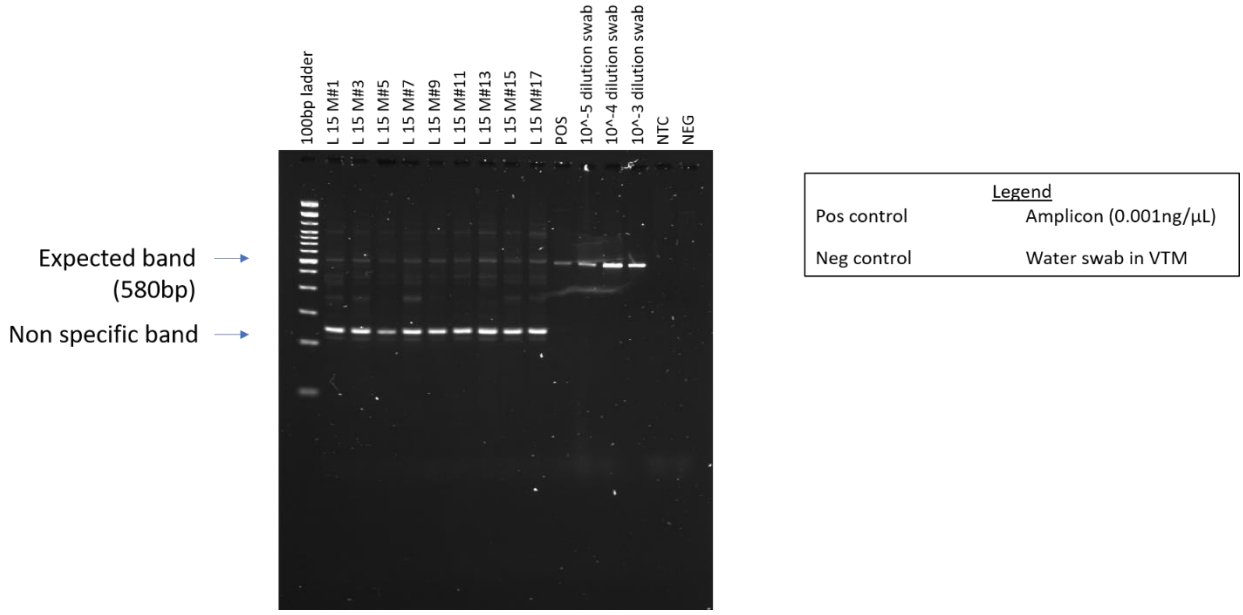

Figure 22: 2% agarose gel with PCR products from a subset of samples (L15 M#1-17) and controls showing non-specific bands

Results from the PCR and gel electrophoresis demonstrated that the samples had non-specific amplification of a smaller fragment of DNA than the amplicon size (~250bp). Given the presence of non-specific bands in our swab samples but not our NTC and negative control, we can conclude that the EHNv primers used for this assay are not specific enough for identification of EHNv.

Ve2. qPCR assay

Optimization

As the OIE assay was not able to discriminate EHNv in swab samples stored in VTM, we moved to a quantitative PCR method using primers targeting the MCP region of the EHNv genome as described in (Jaramillo, et al., 2012). In comparison to the OIE PCR method, this method incorporates a Sybr Green dye that binds to the double stranded DNA during amplification. The analysis is conducted in real time using fluorescence amplification and meting curve. Forward primer 5' GACTGACCAACGCCAGCCTTAACG3' and reverse primer 5'GCGGTGGTGTACCCAGAGTTGTCG3' were used, generating a 94bp amplicon. The QuantiNova SYBR Green Master Mix kit (Qiagen®) was used for master mix preparation.

- Primer concentrations were set at 0.7µM as final concentration as per SYBR Green kit
- 10-fold serial dilution (n=6) were performed to define the sensitivity of the assay

- Melt curve analysis was recorded after amplification to distinguish between amplification of the EHNv amplicon region and non-specific amplification
- Agarose gel electrophoresis was used to verify products
- 4µL of extracted DNA was added to each reaction

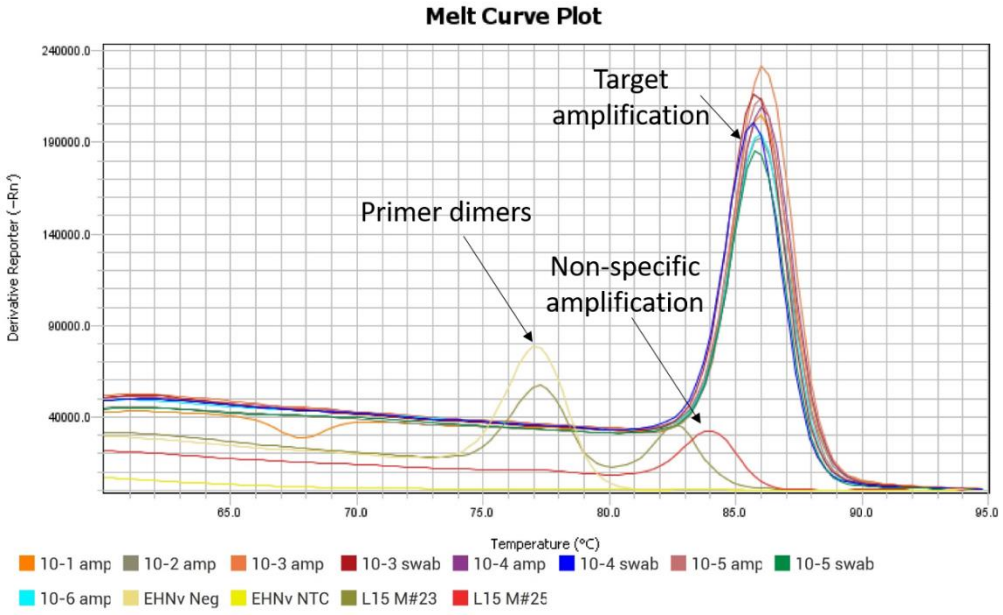

Figure 23: SYBR Green qPCR Melt curve showing amplification of EHNv amplicon and non-specific amplification in negative controls and samples

Sample	10^{-1} amp	10^{-2} amp	10^{-3} amp	10^{-4} amp	10^{-5} amp	10^{-6} amp
Ct	10.30	14.49	18.63	20.75	26.40	28.97
Tm	86.03	85.89	86.03	86.03	85.88	85.88
Melt Peak Height	2.05E+05	1.93E+05	2.31E+05	2.09E+05	2.14E+05	1.95E+05

Table 36: Recorded Ct and Tm values for amplicon dilution range

- Data showed that the amplicon dilutions all demonstrated an average melt curve Tm of 85.96 ± 0.08 with a similar peak height (Fig., 23 and Table 36)
- The EHNv amplicon peaks were readily distinguished from the primer dimers (~Tm = 77) and non-specific amplification (~Tm = 82-84) (Fig., 23)

- Data showed that the swab dilutions all demonstrated an average melt curve Tm of 85.81 ± 0.10 with a similar peak height (Table 37)
- Data showed that the optimal concentration of the positive control amplicon was 10^{-4} (swab in VTM), which provided the best Ct value and melt curve, and gave a strong band on agarose gel electrophoresis (Fig., 24)

• Sample	10^{-3} swab	10^{-4} swab	10^{-5} swab
Ct	22.78	25.75	30.046
Tm	85.84	85.70	85.89
Melt Peak Height	2.16E+05	2.01E+05	1.86E+05

Table 37: Recorded Ct and Tm values for amplicon swab dilution range

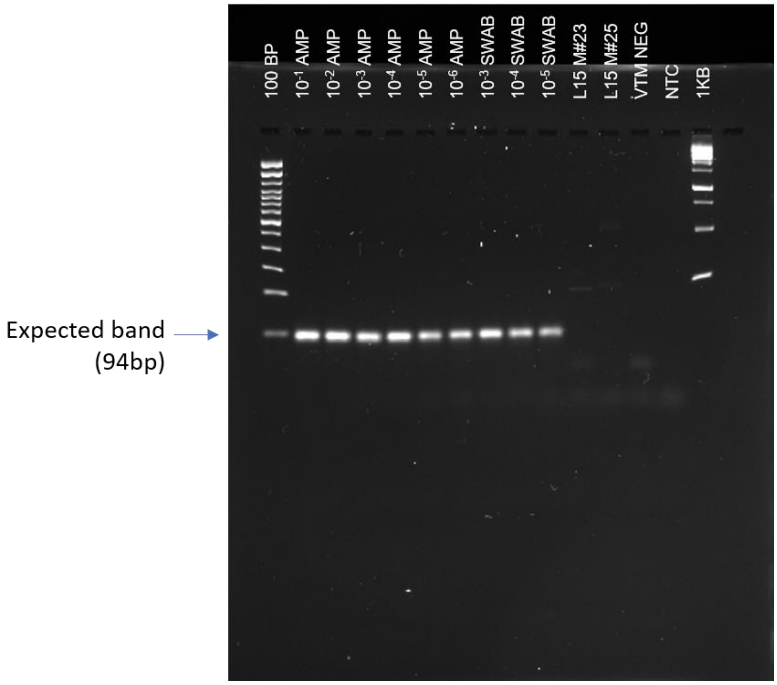

Figure 24: 2% agarose gel visualizing SYBR Green qPCR products, verifying the presence of EHNv in amplicon samples, and the absence of products in samples and negative controls

Data showed that confirmation of SYBR Green qPCR results with agarose gel electrophoresis is sufficiently sensitive to detect the presence of EHNv in swab samples.

Reference

Jaramillo, D., Tweedie, A., Becker, J. A., Hyatt, A., Crameri, S., & Whittington, R. J. (2012). A validated quantitative polymerase chain reaction assay for the detection of ranaviruses (Family Iridoviridae) in fish tissue and cell cultures, using EHNV as a model. *Aquaculture, 356–357*, 186-192. doi:10.1016/j.aquaculture.2012.05.017

VI. Parasite assay optimisation

VIa. *Ceratomyxa shasta* (*C. shasta*)

Va1. Swab samples

Primer optimization

DNA extracted from the swab immerged in the suspension containing amplicon of *C. shasta* purchased from IDT and diluted 10,000 times.

- Different concentrations of forward and reverse primers combination were analyzed

200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM,.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations (Table 38)

	200/200	200/400	400/200	400/400	200/600	600/200	600/600	600/400	400/600
	20.3	19.0	20.5	18.9	18.6	20.5	18.5	19.1	18.5
	20.4	19.0	20.5	18.9	18.6	20.5	18.5	19.0	18.5
Avg	**20.3**	**19.0**	**20.5**	**18.9**	**18.6**	**20.5**	**18.5**	**19.0**	**18.5**
SD	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**

Table 38: Ct values recorded for different combination of primer concentrations of assay targeting C. shasta gene. Optimal concentrations of primer sets are highlighted

Data showed that the optimal primer set combination (lower Ct values) for forward and reverse were **400nM and 600nM** respectively.

Probe optimization

Optimisation of the probe concentration was performed using the same extracted DNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM

- Recorded Ct values (Table 39)

	100nM	125nM	150nM	175nM	200nM	225nM	250nM
	18.5	18.4	18.2	18.1	18.2	18.2	18.1
	18.5	18.5	18.3	18.1	18.2	18.3	18.2
Avg	**18.52**	**18.44**	**18.24**	**18.14**	**18.22**	**18.25**	**18.19**
SD	**0.001**	**0.039**	**0.039**	**0.003**	**0.008**	**0.005**	**0.062**

Table 39: Ct values recorded for different probe concentrations of assay targeting C. shasta gene. Optimal concentration of probe is highlighted

Data showed that the optimal concentration of the probe was **225nM** which provided one of the lowest Ct value and better amplification curve (higher saturation).

Efficiency

- DNA extracted from swap immerged in the suspension containing amplicon of *C. shasta* purchased from IDT was 10-fold serial diluted (n=6) and used to assess the efficiency.

- qPCR performed using the optimal conditions (400/600nM for both primers forward and reverse respectively and 225 nM for the probe)

The efficiency of the optimised assay was evaluated at **~90%** (slope is -3.59) (Fig., 25).

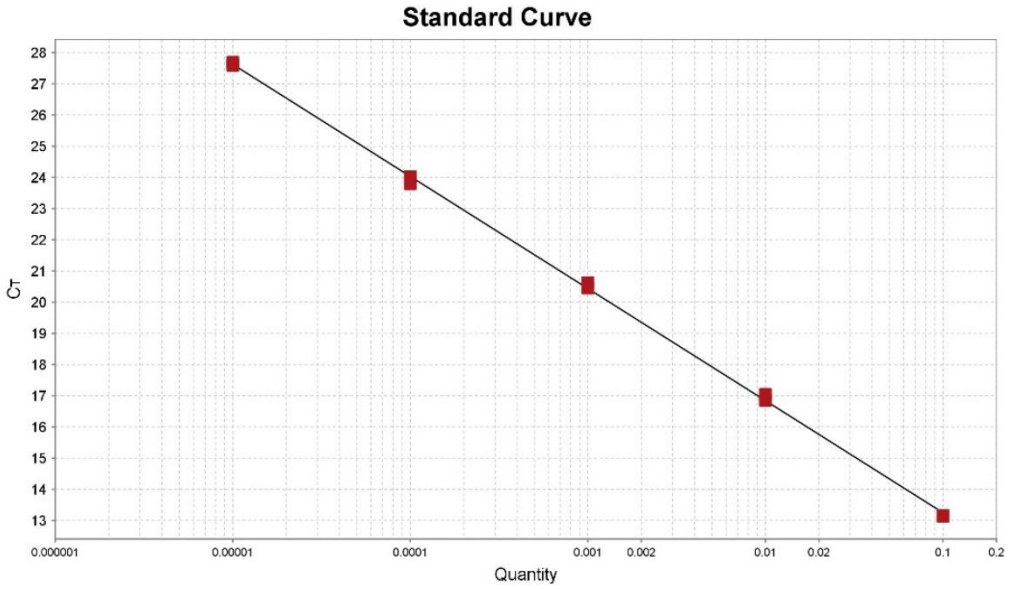

Figure 25: Standard curve of the assay targeting C. shasta DNA amplicon extracted from spiked swab

Sensitivity

- Sample from 10E-7 from efficiency curve was used for the evaluation of the assay sensitivity
- Concentration of the probe was 225 nM and the concentration of the sets of primers, forward and reverse, was 400 nM and 600 nM respectively.
- 2 fold serial dilution (n=6) was performed using sample from 10E-7
- 10 replicates for each dilution were performed to define the sensitivity of the assay

Data showed that undetermined (U: no amplification) is recorded in 5 replicates out of 10 at 2E-1 at an average Ct value of 35. Therefore, the threshold is set at Ct value of **34-35** (Table 40).

	10E-7	2E-1	2E-2	2E-3	2E-4	2E-5
	33.155	U	U	U	U	U
	33.822	U	U	35.227	U	U
	32.932	U	33.924	U	36.159	U
	32.979	35.936	36.301	U	U	U
	33.509	U	U	U	U	U
	33.419	U	34.816	U	U	U
	34.955	34.363	U	U	36.206	U
	34.176	34.837	U	U	U	U
	33.861	35.984	U	U	U	U
	34.379	34.017	U	U	U	U
Avg	33.7	35.0	35.0	35.2	36.2	U
SD	0.62	0.80	0.98	0.00	0.02	U

Table 40: Results of sensitivity assessment for assay targeting C. shasta gene. Ten replicates for each dilution have been performed.

Va2. Water samples

Primer optimization

DNA extracted from sterivex filtered water spiked with amplicon of *C. shasta.*

Different concentrations of forward and reverse primers combination were analyzed

200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations (Table 41)

	200/200	200/400	400/200	400/400	200/600	600/200	600/600	600/400	400/600
	36.48	36.67	38.61	36.01	33.72	37.44	33.81	39.56	U
	37.37	36.97	37.23	33.53	33.62	38.12	34.47	U	36.28
Avg	36.93	36.82	37.92	34.77	33.67	37.78	34.14	39.56	36.28
SD	0.63	0.21	0.98	1.76	0.07	0.48	0.46	NA	NA

Table 41: Ct values recorded for different combination of primer concentrations of assay targeting C. shasta gene. Optimal concentrations of primer sets are highlighted

Data showed that the optimal primer set combination (lower Ct values) for forward and reverse were **200nM and 600nM** respectively.

Probe optimization

Optimisation of the probe concentration was performed using the same extracted DNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM

- Recorded Ct values (Table 42)

	100nM	125nM	150nM	175nM	200nM	225nM	250nM
	11.07	11.32	10.92	10.95	10.83	10.89	10.93
	10.97	11.18	10.95	10.83	10.69	10.75	10.82
Avg	11.02	11.25	10.94	10.89	10.76	10.82	10.87
STD	0.07	0.10	0.02	0.09	0.10	0.10	0.08

Table 42: Ct values recorded for different probe concentrations of assay targeting C. shasta gene. Optimal concentration of probe is highlighted

Data showed that the optimal concentration of the probe was **200nM** which provided one of the lowest Ct value.

Efficiency

- DNA was 5-fold serial diluted (n=6) and used to assess the efficiency.

- qPCR performed using the optimal conditions (200/600nM for both primers forward and reverse respectively and 200 nM for the probe)

The efficiency of the optimised assay was evaluated at **~92%** (slope is -3.54) (Fig., 26).

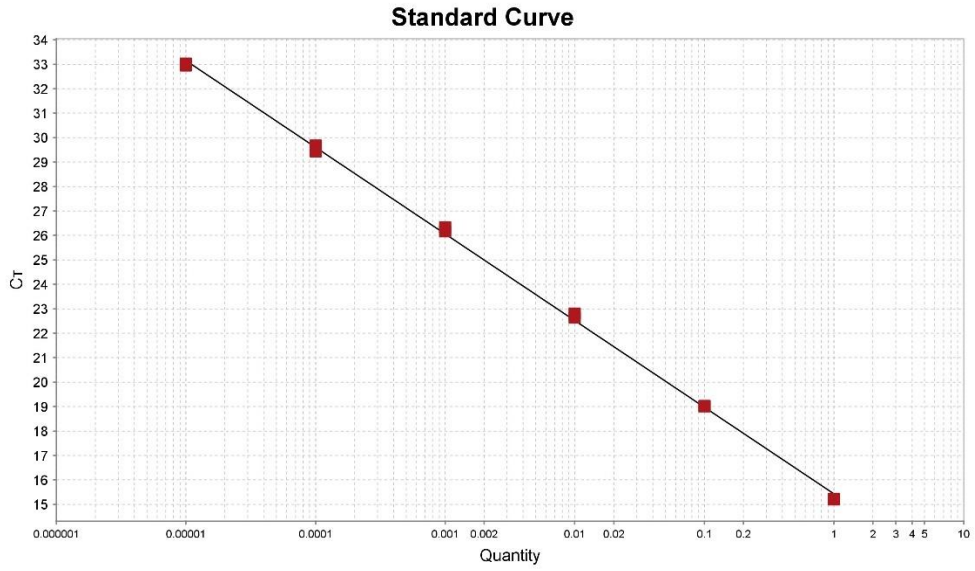

Figure 26: Standard curve of the assay targeting C. shasta DNA amplicon extracted from spiked filtered water

Sensitivity

- Sample from 10E-1 diluted 1/3 was used for the evaluation of the assay sensitivity
- Concentration of the probe was 200 nM and the concentration of the sets of primers, forward and reverse, was 200 nM and 600 nM respectively.
- 2 fold serial dilution was performed 8 times
- 10 replicates for each dilution were performed to define the sensitivity of the assay

Data showed that undetermined (U: no amplification) is recorded in 1 replicate out of 10 at 2E-5 at an average Ct value of 36.7. Therefore, the threshold is set at Ct value of **34** (Table 43).

	2E-4	2E-5	2E-6	2E-7
	33.1	33.4	36.2	U
	33.4	35.3	35.4	35.7
	34.7	U	34.6	33.8
	35.7	33.6	34.6	U
	33.2	34.6	34.5	U
	33.7	34.0	34.5	35.5
	33.3	34.6	U	35.0
	32.5	35.7	U	U
	34.0	33.2	36.1	U
	33.2	33.6	35.5	U
Avg	33.7	34.2	35.2	36.1
SD	0.9	0.9	0.7	35.1

Table 43: Results of sensitivity assessment for assay targeting C. shasta gene. Ten replicates for each dilution have been performed.

VIb. *Myxobolus cerebralis* (*M. cerebralis*)

VIb1. Swab samples

Primer optimization

DNA extracted from the swab immerged in the suspension containing amplicon of *M. cerebralis* purchased from IDT and diluted 10,000 000 times.

- Different concentrations of forward and reverse primers combination were analyzed

200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations (Table 44)

	200/200	200/400	400/200	400/400	200/600	600/200	600/600	600/400	400/600
	25.705	25.601	25.879	25.415	25.552	25.775	25.766	25.824	25.715
	25.631	25.774	25.757	25.460	25.664	25.976	25.950	25.869	25.446
Avg	**25.67**	**25.69**	**25.82**	**25.44**	**25.61**	**25.88**	**25.86**	**25.85**	**25.58**
SD	**0.037**	**0.087**	**0.061**	**0.023**	**0.056**	**0.101**	**0.092**	**0.022**	**0.134**

Table 44: Ct values recorded for different combination of primer concentrations of assay targeting M. cerebralis gene. Optimal concentrations of primer sets are highlighted

Data showed that the optimal primer set combination (lower Ct values) for forward and reverse were **400nM and 400nM** respectively.

Probe optimization

Optimisation of the probe concentration was performed using the same extracted DNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM

- Recorded Ct values (Table 45)

	100nM	125nM	150nM	175nM	200nM	225nM	250nM
	27.019	26.246	25.864	25.757	25.075	24.919	24.704
	26.970	26.396	25.839	25.832	24.885	24.983	24.816
Avg	**26.99**	**26.32**	**25.85**	**25.79**	**24.98**	**24.95**	**24.76**
SD	**0.025**	**0.075**	**0.013**	**0.038**	**0.095**	**0.032**	**0.056**

Table 45: Ct values recorded for different probe concentrations of assay targeting M. cerebralis gene. Optimal concentration of probe is highlighted

Data showed that the optimal concentration of the probe was **225nM** which provided one of the lowest Ct value and better amplification curve (higher saturation).

Efficiency

- DNA extracted from swap immerged in the suspension containing amplicon of *M. cerebralis* purchased from IDT was 5-fold serial diluted (n=6) and used to assess the efficiency.

- qPCR performed using the optimal conditions (400/400nM for both primers forward and reverse respectively and 225 nM for the probe).

- The efficiency of the optimised assay was evaluated at **~93%** (slope is -3.42) (Fig., 27).

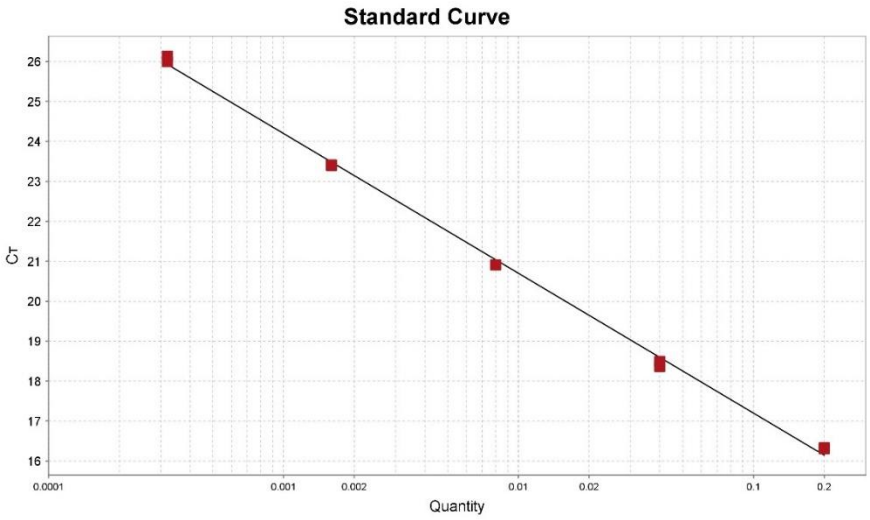

Figure 27: Standard curve of the assay targeting M. cerebralis DNA amplicon extracted from spiked swab

Sensitivity

- Sample from 10E-1 diluted 1/50 was used for the evaluation of the assay sensitivity

- Concentration of the probe was 225 nM and the concentration of the sets of primers, forward and reverse, was 400 nM and 400 nM respectively.
- 2 fold serial dilution was performed 8 times
- 10 replicates for each dilution were performed to define the sensitivity of the assay

Data showed that undetermined (U: no amplification) is recorded in 4 replicates out of 10 2E-6 at an average Ct value of 36.7. Therefore, the threshold is set at Ct value of **35-36** (Table 46).

	Neat	2E-1	2E-2	2E-3	2E-4	2E-5	2E-6	2E-7	2E-8
	30.3	31.0	31.9	33.1	34.0	34.8	35.9	U	U
	29.9	30.9	32.0	32.9	33.1	33.9	37.7	35.7	U
	30.0	30.9	31.4	32.2	33.9	34.7	37.6	37.5	U
	29.9	30.8	31.7	33.0	35.4	34.6	37.5	36.2	U
	30.1	30.8	31.8	33.0	34.0	35.7	U	37.5	U
	30.1	30.7	31.8	33.0	33.8	35.2	36.2	U	37.1
	30.3	31.0	31.2	33.0	33.9	35.0	U	37.6	U
	30.1	31.0	31.8	32.6	34.7	35.0	35.4	36.0	35.5
	30.0	30.7	32.0	33.4	34.4	35.1	U	37.2	U
	30.0	30.7	32.0	33.2	33.9	34.6	U	U	U
Avg	30.1	30.9	31.8	32.9	34.1	34.9	36.7	36.8	36.3
SD	0.14	0.13	0.25	0.30	0.60	0.44	0.92	0.76	0.81

Table 46: Results of sensitivity assessment for assay targeting M. cerebralis gene. Ten replicates for each dilution have been performed.

VIb22. Water samples

Primer optimization

DNA extracted from the filtered water with sterix and spiked with amplicon of *M. cerebralis*.

- Different concentrations of forward and reverse primers combination were analyzed

200 nM/200 nM, 200 nM/400 nM, 400 nM/200 nM, 400 nM/400 nM, 200 nM/600 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/400 nM, 400 nM/600 nM,.

- The probe was set at 200 nM as final concentration
- Ct values recorded using different combination of primer/probe concentrations (Table 47)

	200/200	200/400	400/200	400/400	200/600	600/200	600/600	600/400
	27.77	28.74	27.68	28.37	27.32	27.39	27.05	27.05
	27.45	29.05	27.74	28.47	27.12	27.27	26.91	27.02
Avg	27.61	28.90	27.71	28.42	27.22	27.33	26.98	27.04
SD	0.22	0.22	0.04	0.08	0.14	0.09	0.10	0.02

Table 47: Ct values recorded for different combination of primer concentrations of assay targeting M. cerebralis gene. Optimal concentrations of primer sets are highlighted

Data showed that the optimal primer set combination (lower Ct values) for forward and reverse were **600nM and 600nM** respectively.

Probe optimization

Optimisation of the probe concentration was performed using the same extracted DNA and the optimal primer set concentrations.

- Different concentrations of the probe were analyzed:
  100nM, 125nM, 150nM, 175nM, 200nM, 225nM, 250nM

- Recorded Ct values (Table 48)

	100nM	**125nM**	**150nM**	**175nM**	**200nM**	**225nM**	**250nM**
	U	28.4	33.0	U	U	27.1	27.6
	U	28.3	32.9	U	U	27.0	27.6
Avg	NA	28.38	32.92	NA	NA	27.05	27.58
STD	NA	0.09	0.09	NA	NA	0.12	0.00

Table 48: Ct values recorded for different probe concentrations of assay targeting M. cerebralis gene. Optimal concentration of probe is highlighted

Data showed that the optimal concentration of the probe was **225nM** which provided one of the lowest Ct value and better amplification curve (higher saturation).

Efficiency

- DNA extracted from the filtered water with sterivex and spiked with amplicon of *M. cerebralis* 10-fold serial diluted (n=6) and used to assess the efficiency.

- qPCR performed using the optimal conditions (600/600nM for both primers forward and reverse respectively and 225 nM for the probe)

The efficiency of the optimised assay was evaluated at **~94%** (slope is -3.47).

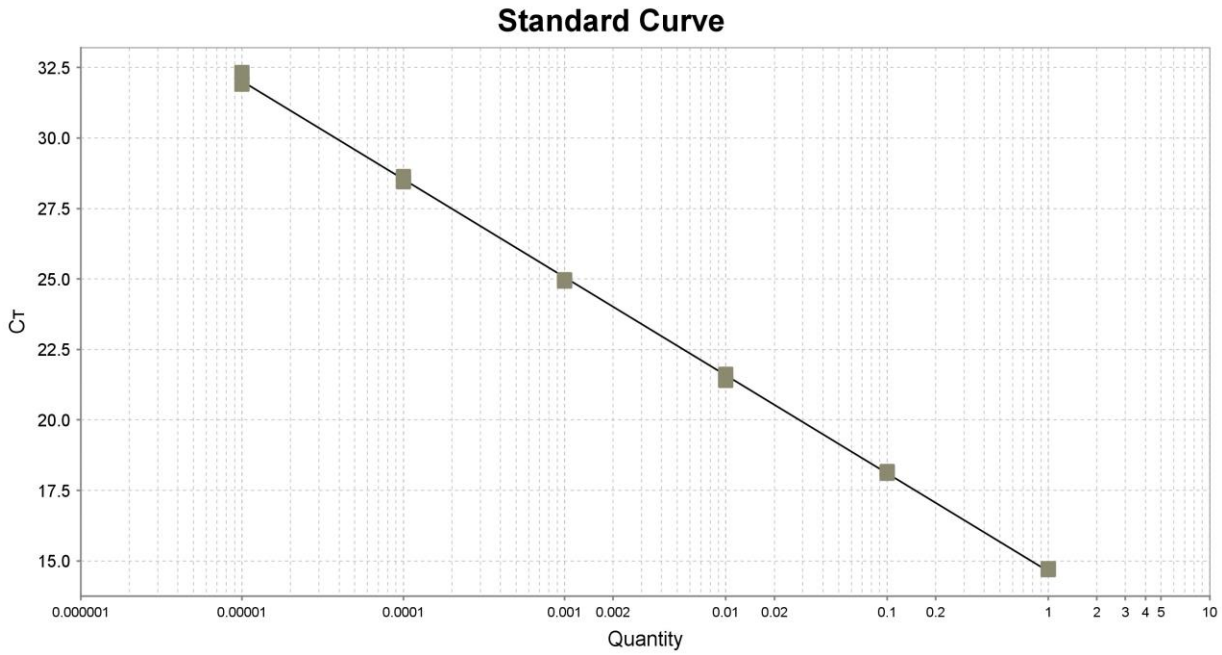

Figure 28: Standard curve of the assay targeting M. cerebralis DNA amplicon extracted from spiked and filtered water

Sensitivity

- Sample from 10E-6 diluted 1/10 from standard curve was used for the evaluation of the assay sensitivity
- Concentration of the probe was 225 nM and the concentration of the sets of primers, forward and reverse, was 600 nM and 600 nM respectively.
- 2 fold serial dilution was performed 4 times
- 10 replicates for each dilution were performed to define the sensitivity of the assay

Data showed that undetermined (U: no amplification) is recorded in 9 replicates out of 10 from the neat (10E-6 diluted 1/10 from standard curve) at an average Ct value of 36.5. Therefore, the threshold is set at Ct value of **35-36** (Table 49).

	Neat	2E-1	2E-2	2E-3
	35.8	38.5	37.6	U
	38.8	U	39.0	U
	36.7	37.6	U	U
	36.2	38.4	U	38.5
	37.8	37.1	U	U
	36.0	36.4	U	U
	U	37.8	U	U
	35.4	U	37.3	U
	36.3	38.0	39.6	U
	35.8	U	U	U
Avg	36.5	37.7	38.3	38.5
SD	1.1	0.8	1.1	0.0

Table 49: Results of sensitivity assessment for assay targeting IHNv gene. Ten replicates for each dilution have been performed.

VII. Results & discussion

VII.a Pathogens screened from fish mucus and gill swabs

Viruses

IHNv, VHSv, IPNv, ISAv and EHNv reportable viruses have been tested in mucus and gills collected from fish of lakes 15 and 16. Amplification of the targeted gens of IHNv, VHSv, IPNv and ISAv were not detected in any collected fish swabs (Table 50).

Unconfirmed detections were observed for EHNv in few samples. Our results showed that 4 and 2 samples in gills and mucus from lake 15 respectively and 2 samples from mucus of fish from lake 16 had unconfirmed EHNv results (either peak of melting curve or a weak band on the gel of electrophoresis, Table 50 highlighted with bolded text). For instance, we have observed both a small band and peak of melt curve from the qPCR test in mucus swab# 11 (fish# 6) and gill swab# 64 (fish# 32) from lake 15. To confirm these results, further analysis is required such as sequencing DNA or improve the published qPCR method by designing a specific probe.

Lake #	Collection Date	Fish #	Gill Swab #	IHNv	VHSv	ISAv	IPNv	EHNv	Mucus swab #	IHNv	VHSv	ISAv	IPNv	EHNv
15	2021-09-08	1	2	U, U	U, U	U, U	U, U	N, N	1	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	2	4	U, U	U, U	U, U	U, U	N, N	3	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	3	6	U, U	U, U	U, U	U, U	N, N	5	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	4	8	U, U	U, U	U, U	U, U	N, N	7	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	5	10	U, U	U, U	U, U	U, U	N, N	9	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	6	12	U, U	U, U	U, U	U, U	N, N	11	U, U	U, U	U, U	U, U	**S, S**
15	2021-09-08	7	14	U, U	U, U	U, U	U, U	N, N	13	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	8	16	U, U	U, U	U, U	U, U	N, N	15	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	9	18	U, U	U, U	U, U	U, U	N, N	17	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	10	20	U, U	U, U	U, U	U, U	N, N	19	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	11	22	U, U	U, U	U, U	U, U	N, N	21	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	12	24	U, U	U, U	U, U	U, U	N, N	23	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	13	26	U, U	U, U	U, U	U, U	N, N	25	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	14	28	U, U	U, U	U, U	U, U	N, N	27	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	15	30	U, U	U, U	U, U	U, U	**S, N**	29	U, U	U, U	U, U	U, U	N,N
15	2021-09-08	16	32	U, U	U, U	U, U	U, U	N, N	31	U, U	U, U	U, U	U, U	N,N
15	2021-09-09	17	34	U, U	U, U	U, U	U, U	N, N	33	U, U	U, U	U, U	U, U	N,N
15	2021-09-09	18	36	U, U	U, U	U, U	U, U	N, N	35	U, U	U, U	U, U	U, U	N,N
15	2021-09-09	19	38	U, U	U, U	U, U	U, U	N, N	37	U, U	U, U	U, U	U, U	N,N
15	2021-09-09	20	40	U, U	U, U	U, U	U, U	N, N	39	U, U	U, U	U, U	U, U	N,N
15	2021-09-09	21	42	U, U	U, U	U, U	U, U	N, N	41	U, U	U, U	U, U	U, U	N,N
15	2021-09-09	22	44	U, U	U, U	U, U	U, U	N, N	43	U, U	U, U	U, U	U, U	N,N
15	2021-09-09	23	46	U, U	U, U	U, U	U, U	N, N	45	U, U	U, U	U, U	U, U	**N, S**
15	2021-09-10	24	48	U, U	U, U	U, U	U, U	N, N	47	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	25	50	U, U	U, U	U, U	U, U	N, N	49	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	26	52	U, U	U, U	U, U	U, U	N, N	51	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	27	54	U, U	U, U	U, U	U, U	N, N	53	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	28	56	U, U	U, U	U, U	U, U	N, N	55	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	29	58	U, U	U, U	U, U	U, U	N, N	57	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	30	60	U, U	U, U	U, U	U, U	N, N	59	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	31	62	U, U	U, U	U, U	U, U	N, N	61	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	32	64	U, U	U, U	U, U	U, U	**S, S**	63	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	33	66	U, U	U, U	U, U	U, U	N, N	65	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	34	68	U, U	U, U	U, U	U, U	N, N	67	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	35	70	U, U	U, U	U, U	U, U	N, N	69	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	36	72	U, U	U, U	U, U	U, U	**S, N**	71	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	37	74	U, U	U, U	U, U	U, U	N, N	73	U, U	U, U	U, U	U, U	N, N
15	2021-09-10	38	76	U, U	U, U	U, U	U, U	N, N	75	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	39	78	U, U	U, U	U, U	U, U	N, N	77	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	40	80	U, U	U, U	U, U	U, U	N, N	79	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	41	82	U, U	U, U	U, U	U, U	N, N	81	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	42	84	U, U	U, U	U, U	U, U	N, N	83	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	43	86	U, U	U, U	U, U	U, U	N, N	85	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	44	88	U, U	U, U	U, U	U, U	N, N	87	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	45	90	U, U	U, U	U, U	U, U	N, N	89	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	46	92	U, U	U, U	U, U	U, U	N, N	91	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	47	94	U, U	U, U	U, U	U, U	**S, N**	93	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	48	96	U, U	U, U	U, U	U, U	N, N	95	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	49	98	U, U	U, U	U, U	U, U	N, N	97	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	50	100	U, U	U, U	U, U	U, U	N, N	99	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	51	102	U, U	U, U	U, U	U, U	N, N	101	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	52	104	U, U	U, U	U, U	U, U	N, N	103	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	53	106	U, U	U, U	U, U	U, U	N, N	105	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	54	108	U, U	U, U	U, U	U, U	N, N	107	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	55	110	U, U	U, U	U, U	U, U	N, N	109	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	56	112	U, U	U, U	U, U	U, U	N, N	111	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	57	114	U, U	U, U	U, U	U, U	N, N	113	U, U	U, U	U, U	U, U	N, N
15	2021-09-11	58	116	U, U	U, U	U, U	U, U	N, N	115	U, U	U, U	U, U	U, U	N, N

Lake #	Collection Date	Fish #	Gill Swab #	IHNv	VHSv	ISAv	IPNv	EHNv	Mucus swab #	IHNv	VHSv	ISAv	IPNv	EHNv
16	2021-09-12	1	1	U, U	U, U	U, U	U, U	N, N	1	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	2	2	U, U	U, U	U, U	U, U	N, N	2	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	3	3	U, U	U, U	U, U	U, U	N, N	3	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	4	4	U, U	U, U	U, U	U, U	N, N	4	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	5	5	U, U	U, U	U, U	U, U	N, N	5	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	6	6	U, U	U, U	U, U	U, U	N, N	6	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	7	7	U, U	U, U	U, U	U, U	N, N	7	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	8	8	U, U	U, U	U, U	U, U	N, N	8	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	9	9	U, U	U, U	U, U	U, U	N, N	9	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	10	10	U, U	U, U	U, U	U, U	N, N	10	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	11	11	U, U	U, U	U, U	U, U	N, N	11	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	12	12	U, U	U, U	U, U	U, U	N, N	12	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	13	13	U, U	U, U	U, U	U, U	N, N	13	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	14	14	U, U	U, U	U, U	U, U	N, N	14	U, U	U, U	U, U	U, U	**S, N**
16	2021-09-12	15	15	U, U	U, U	U, U	U, U	N, N	15	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	16	16	U, U	U, U	U, U	U, U	N, N	16	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	17	17	U, U	U, U	U, U	U, U	N, N	17	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	18	18	U, U	U, U	U, U	U, U	N, N	18	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	19	19	U, U	U, U	U, U	U, U	N, N	19	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	20	20	U, U	U, U	U, U	U, U	N, N	20	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	21	21	U, U	U, U	U, U	U, U	N, N	21	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	22	22	U, U	U, U	U, U	U, U	N, N	22	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	23	23	U, U	U, U	U, U	U, U	N, N	23	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	24	24	U, U	U, U	U, U	U, U	N, N	24	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	25	25	U, U	U, U	U, U	U, U	N, N	25	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	26	26	U, U	U, U	U, U	U, U	N, N	26	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	27	27	U, U	U, U	U, U	U, U	N, N	27	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	28	28	U, U	U, U	U, U	U, U	N, N	28	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	29	29	U, U	U, U	U, U	U, U	N, N	29	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	30	30	U, U	U, U	U, U	U, U	N, N	30	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	31	31	U, U	U, U	U, U	U, U	N, N	31	U, U	U, U	U, U	U, U	N, N
16	2021-09-12	32	32	U, U	U, U	U, U	U, U	N, N	32	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	33	33	U, U	U, U	U, U	U, U	N, N	33	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	34	34	U, U	U, U	U, U	U, U	N, N	34	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	35	35	U, U	U, U	U, U	U, U	N, N	35	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	36	36	U, U	U, U	U, U	U, U	N, N	36	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	37	37	U, U	U, U	U, U	U, U	N, N	37	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	38	38	U, U	U, U	U, U	U, U	N, N	38	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	39	39	U, U	U, U	U, U	U, U	N, N	39	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	40	40	U, U	U, U	U, U	U, U	N, N	40	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	41	41	U, U	U, U	U, U	U, U	N, N	41	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	42	42	U, U	U, U	U, U	U, U	N, N	42	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	43	43	U, U	U, U	U, U	U, U	N, N	43	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	44	44	U, U	U, U	U, U	U, U	N, N	44	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	45	45	U, U	U, U	U, U	U, U	N, N	45	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	46	46	U, U	U, U	U, U	U, U	N, N	46	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	47	47	U, U	U, U	U, U	U, U	N, N	47	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	48	48	U, U	U, U	U, U	U, U	N, N	48	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	49	49	U, U	U, U	U, U	U, U	N, N	49	U, U	U, U	U, U	U, U	N, N
16	2021-09-13	50	50	U, U	U, U	U, U	U, U	N, N	50	U, U	U, U	U, U	U, U	**S, N**
16	2021-09-13	51	51	U, U	U, U	U, U	U, U	N, N	51	U, U	U, U	U, U	U, U	N, N
16	2021-09-14	52	52	U, U	U, U	U, U	U, U	N, N	52	U, U	U, U	U, U	U, U	N, N
16	2021-09-14	53	53	U, U	U, U	U, U	U, U	N, N	53	U, U	U, U	U, U	U, U	N, N
16	2021-09-14	54	54	U, U	U, U	U, U	U, U	N, N	54	U, U	U, U	U, U	U, U	N, N
16	2021-09-14	55	55	U, U	U, U	U, U	U, U	N, N	55	U, U	U, U	U, U	U, U	N, N
16	2021-09-14	56	56	U, U	U, U	U, U	U, U	N, N	56	U, U	U, U	U, U	U, U	N, N
16	2021-09-14	57	57	U, U	U, U	U, U	U, U	N, N	57	U, U	U, U	U, U	U, U	N, N
16	2021-09-14	58	58	U, U	U, U	U, U	U, U	N, N	58	U, U	U, U	U, U	U, U	N, N
16	2021-09-14	59	59	U, U	U, U	U, U	U, U	N, N	59	U, U	U, U	U, U	U, U	N, N
16	2021-09-14	60	60	U, U	U, U	U, U	U, U	N, N	60	U, U	U, U	U, U	U, U	N, N

Table 50: Viruses screening results from mucus and gill swabs collected from fish of Lake 15 and 16.

IHNv: Infectious Hematopoietic necrosis virus, VHSv: Viral hemorrhagic septicemia virus, ISAv: Infectious salmon anemia virus, IPNv: Infectious pancreatic necrosis, EHNv: Epizootic Haematopoietic Necrosis Virus.

U: Undetermined (no amplification recorded); N: Negative (no band present), S: Suspicious (faint band or peak at ~85 Tm), P: Positive (band present and peak at ~85 Tm).

Bacteria

Overall, no amplification under the cut off has been recorded related to *R. salmoninarum*, *Aeromonas salmonicida* or *Yersinia ruckeri* in all mucus and gill samples. Amplification in few samples have been recorded with Ct values higher than the cut off and only one well amplified out of the duplicates (Table 51, highlighted with bolded text).

In one analysis, the negative control had amplification. These results have not passed the quality control. Because of the lack of remaining samples, we were not able to repeat the analysis using new samples. Therefore, these data were not taken into account in our analysis (Table 51, noted as N/A).

Taken together, no confirmed bacteria genes were detected on fish mucus and gill swabs according to the quality control criteria of the testing.

Lake #	Collection Date	Fish #	Mucus swab #	R sal	A sal	Y ruckeri	Gill Swab	R sal	A sal	Y ruckeri
15	2021-09-08	1	1	U,U	U,U	U,U	2	U,U	U,U	U,U
15	2021-09-08	2	3	U,U	U,U	U,U	4	U,U	U,U	U,U
15	2021-09-08	3	5	U,U	U,U	U,U	6	U,U	U,U	U,U
15	2021-09-08	4	7	U,U	U,U	U,U	8	U,U	U,U	U,U
15	2021-09-08	5	9	U,U	U,U	U,U	10	U,U	U,U	U,U
15	2021-09-08	6	11	U,U	U,U	U,U	12	U,U	U,U	U,U
15	2021-09-08	7	13	U,U	U,U	U,U	14	U,U	U,U	U,U
15	2021-09-08	8	15	U,U	U,U	U,U	16	U,U	U,U	U,U
15	2021-09-08	9	17	U,U	U,U	U,U	18	U,U	U,U	U,U
15	2021-09-08	10	19	U,U	U,U	U,U	20	U,U	U,U	U,U
15	2021-09-08	11	21	U,U	U,U	U,U	22	U,U	U,U	U,U
15	2021-09-08	12	23	U,U	U,U	U,U	24	U,U	U,U	U,U
15	2021-09-08	13	25	U,U	U,U	U,U	26	U,U	U,U	U,U
15	2021-09-08	14	27	U,U	U,U	U,U	28	U,U	U,U	U,U
15	2021-09-08	15	29	U,U	U,U	U,U	30	U,U	U,U	U,U
15	2021-09-08	16	31	U,U	U,U	U,U	32	U,U	U,U	U,U
15	2021-09-09	17	33	U,U	U,U	U,U	34	**39.2,U**	U,U	U,U
15	2021-09-09	18	35	U,U	U,U	U,U	36	U,U	U,U	U,U
15	2021-09-09	19	37	**39.3,U**	U,U	U,U	38	U,U	U,U	U,U
15	2021-09-09	20	39	U,U	U,U	U,U	40	U,U	U,U	U,U
15	2021-09-09	21	41	U,U	U,U	U,U	42	U,U	U,U	U,U
15	2021-09-09	22	43	U,U	U,U	U,U	44	U,U	U,U	U,U
15	2021-09-09	23	45	U,U	U,U	U,U	46	U,U	U,U	U,U
15	2021-09-10	24	47	U,U	U,U	U,U	48	U,U	U,U	U,U
15	2021-09-10	25	49	U,U	U,U	U,U	50	U,U	U,U	U,U
15	2021-09-10	26	51	U,U	U,U	U,U	52	U,U	U,U	U,U
15	2021-09-10	27	53	U,U	U,U	U,U	54	U,U	U,U	U,U
15	2021-09-10	28	55	U,U	U,U	U,U	56	U,U	U,U	U,U
15	2021-09-10	29	57	U,U	U,U	U,U	58	U,U	U,U	U,U
15	2021-09-10	30	59	U,U	U,U	U,U	60	U,U	U,U	U,U
15	2021-09-10	31	61	U,U	U,U	U,U	62	U,U	U,U	U,U
15	2021-09-10	32	63	U,U	U,U	U,U	64	U,U	U,U	U,U
15	2021-09-10	33	65	U,U	U,U	U,U	66	U,U	U,U	U,U
15	2021-09-10	34	67	U,U	U,U	U,U	68	U,U	U,U	U,U
15	2021-09-10	35	69	U,U	U,U	U,U	70	U,U	U,U	U,U
15	2021-09-10	36	71	U,U	U,U	U,U	72	U,U	U,U	U,U
15	2021-09-10	37	73	N/A	U,U	U,U	74	N/A	U,U	U,U
15	2021-09-10	38	75	N/A	U,U	U,U	76	N/A	U,U	U,U
15	2021-09-11	39	77	N/A	U,U	U,U	78	N/A	U,U	U,U
15	2021-09-11	40	79	N/A	U,U	U,U	80	N/A	U,U	U,U
15	2021-09-11	41	81	N/A	U,U	U,U	82	N/A	U,U	U,U
15	2021-09-11	42	83	N/A	U,U	U,U	84	N/A	U,U	U,U
15	2021-09-11	43	85	N/A	U,U	U,U	86	N/A	U,U	U,U
15	2021-09-11	44	87	N/A	U,U	U,U	88	N/A	U,U	U,U
15	2021-09-11	45	89	N/A	U,U	U,U	90	N/A	U,U	U,U
15	2021-09-11	46	91	N/A	U,U	U,U	92	N/A	U,U	U,U
15	2021-09-11	47	93	N/A	U,U	U,U	94	N/A	U,U	U,U
15	2021-09-11	48	95	N/A	U,U	U,U	96	N/A	U,U	U,U
15	2021-09-11	49	97	N/A	**35.9, U**	U,U	98	N/A	U,U	U,U
15	2021-09-11	50	99	N/A	U,U	U,U	100	N/A	U,U	U,U
15	2021-09-11	51	101	N/A	U,U	U,U	102	N/A	U,U	U,U
15	2021-09-11	52	103	N/A	U,U	U,U	104	N/A	U,U	U,U
15	2021-09-11	53	105	N/A	U,U	U,U	106	N/A	**38.5, U**	U,U
15	2021-09-11	54	107	N/A	U,U	**38.2, U**	108	N/A	U,U	U,U
15	2021-09-11	55	109	N/A	U,U	U,U	110	N/A	U,U	U,U
15	2021-09-11	56	111	N/A	U,U	U,U	112	N/A	**39.2, U**	U,U
15	2021-09-11	57	113	N/A	U,U	U,U	114	N/A	U,U	U,U
15	2021-09-11	58	115	N/A	U,U	U,U	116	N/A	U,U	U,U

Lake #	Collection Date	Fish #	Gill Swab	R sal	A sal	Y ruckeri	Mucus swab #	R sal	A sal	Y ruckeri
16	2021-09-12	1	1	U,U	U,U	U,U	1	U,U	U,U	U,U
16	2021-09-12	2	2	U,U	U,U	U,U	2	U,U	U,U	U,U
16	2021-09-12	3	3	U,U	U,U	U,U	3	U,U	U,U	U,U
16	2021-09-12	4	4	U,U	U,U	U,U	4	U,U	U,U	U,U
16	2021-09-12	5	5	U,U	U,U	U,U	5	U,U	U,U	U,U
16	2021-09-12	6	6	U,U	U,U	U,U	6	U,U	U,U	U,U
16	2021-09-12	7	7	U,U	U,U	U,U	7	U,U	U,U	U,U
16	2021-09-12	8	8	U,U	U,U	U,U	8	U,U	U,U	U,U
16	2021-09-12	9	9	U,U	U,U	U,U	9	U,U	U,U	U,U
16	2021-09-12	10	10	U,U	U,U	U,U	10	U,U	U,U	U,U
16	2021-09-12	11	11	U,U	U,U	U,U	11	U,U	U,U	U,U
16	2021-09-12	12	12	U,U	U,U	U,U	12	U,U	U,U	U,U
16	2021-09-12	13	13	U,U	U,U	U,U	13	U,U	U,U	U,U
16	2021-09-12	14	14	U,U	U,U	U,U	14	U,U	U,U	U,U
16	2021-09-12	15	15	U,U	U,U	U,U	15	U,U	U,U	U,U
16	2021-09-12	16	16	U,U	U,U	U,U	16	U,U	U,U	U,U
16	2021-09-12	17	17	U,U	U,U	U,U	17	U,U	U,U	U,U
16	2021-09-12	18	18	U,U	U,U	U,U	18	U,U	U,U	U,U
16	2021-09-12	19	19	U,U	U,U	U,U	19	U,U	U,U	U,U
16	2021-09-12	20	20	U,U	U,U	U,U	20	U,U	U,U	U,U
16	2021-09-12	21	21	U,U	U,U	U,U	21	U,U	U,U	U,U
16	2021-09-12	22	22	U,U	U,U	U,U	22	U,U	U,U	U,U
16	2021-09-12	23	23	U,U	U,U	U,U	23	U,U	U,U	U,U
16	2021-09-12	24	24	U,U	U,U	U,U	24	U,U	U,U	U,U
16	2021-09-12	25	25	U,U	U,U	U,U	25	U,U	U,U	U,U
16	2021-09-12	26	26	U,U	U,U	U,U	26	U,U	U,U	U,U
16	2021-09-12	27	27	U,U	U,U	U,U	27	U,U	U,U	U,U
16	2021-09-12	28	28	U,U	U,U	U,U	28	U,U	U,U	U,U
16	2021-09-12	29	29	U,U	U,U	U,U	29	U,U	U,U	U,U
16	2021-09-12	30	30	U,U	U,U	U,U	30	U,U	U,U	U,U
16	2021-09-12	31	31	U,U	U,U	U,U	31	U,U	U,U	U,U
16	2021-09-12	32	32	U,U	U,U	U,U	32	U,U	U,U	U,U
16	2021-09-13	33	33	U,U	U,U	U,U	33	U,U	U,U	U,U
16	2021-09-13	34	34	U,U	U,U	U,U	34	U,U	U,U	U,U
16	2021-09-13	35	35	U,U	U,U	U,U	35	U,U	U,U	U,U
16	2021-09-13	36	36	U,U	U,U	U,U	36	U,U	U,U	U,U
16	2021-09-13	37	37	U,U	U,U	U,U	37	U,U	U,U	U,U
16	2021-09-13	38	38	U,U	U,U	U,U	38	U,U	**35.4,U**	U,U
16	2021-09-13	39	39	U,U	U,U	U,U	39	U,U	U,U	U,U
16	2021-09-13	40	40	U,U	U,U	U,U	40	U,U	U,U	U,U
16	2021-09-13	41	41	U,U	U,U	U,U	41	U,U	U,U	U,U
16	2021-09-13	42	42	U,U	U,U	U,U	42	U,U	U,U	U,U
16	2021-09-13	43	43	U,U	U,U	U,U	43	U,U	U,U	U,U
16	2021-09-13	44	44	U,U	U,U	U,U	44	U,U	U,U	U,U
16	2021-09-13	45	45	U,U	U,U	U,U	45	U,U	U,U	U,U
16	2021-09-13	46	46	U,U	U,U	U,U	46	U,U	U,U	U,U
16	2021-09-13	47	47	U,U	U,U	U,U	47	U,U	U,U	U,U
16	2021-09-13	48	48	U,U	U,U	U,U	48	U,U	U,U	U,U
16	2021-09-13	49	49	U,U	U,U	U,U	49	U,U	U,U	U,U
16	2021-09-13	50	50	U,U	U,U	U,U	50	U,U	U,U	U,U
16	2021-09-13	51	51	U,U	U,U	U,U	51	U,U	U,U	U,U
16	2021-09-14	52	52	U,U	U,U	U,U	52	U,U	U,U	U,U
16	2021-09-14	53	53	U,U	U,U	U,U	53	U,U	U,U	U,U
16	2021-09-14	54	54	U,U	U,U	U,U	54	U,U	**35.4,U**	U,U
16	2021-09-14	55	55	U,U	U,U	U,U	55	U,U	U,U	U,U
16	2021-09-14	56	56	U,U	U,U	U,U	56	U,U	U,U	U,U
16	2021-09-14	57	57	U,U	U,U	U,U	57	U,U	U,U	U,U
16	2021-09-14	58	58	U,U	U,U	U,U	58	U,U	U,U	U,U
16	2021-09-14	59	59	U,U	U,U	U,U	59	U,U	U,U	U,U
16	2021-09-14	60	60	U,U	U,U	U,U	60	U,U	U,U	U,U

Table 51: Bacteria screening results in gills and mucus swabs from fish collected in lake 15 and 16.

R sal: Renibacterium salmoninarum; A sal: Aeromonas salmonicida; Y ruckeri: Yersinia ruckeri

U: Undetermined (no amplification recorded); N/A Not applicable

Parasites

No amplification in all mucus and gill samples have been recorded in our analysis related to both *C. shasta* and *M. cerebralis* parasites (Table 52). In one analysis, the negative control had amplification with high Ct values (38.1, 36.9) but all the samples had no amplification. This could be due to aerosol contamination from positive to the negative control during either extraction steps or plate loading. Therefore, no tested parasites (*M. cerebralis* and *C. shasta*) targeted genes were detected on fish mucus and gill swabs collected in lake 15 and 16.

Lake #	Collection Date	Fish #	Mucus swab #	C. shasta	M. cerebralis	Gill Swab #	C. shasta	M. cerebralis
15	2021-09-08	1	1	U,U	U,U	2	U,U	U,U
15	2021-09-08	2	3	U,U	U,U	4	U,U	U,U
15	2021-09-08	3	5	U,U	U,U	6	U,U	U,U
15	2021-09-08	4	7	U,U	U,U	8	U,U	U,U
15	2021-09-08	5	9	U,U	U,U	10	U,U	U,U
15	2021-09-08	6	11	U,U	U,U	12	U,U	U,U
15	2021-09-08	7	13	U,U	U,U	14	U,U	U,U
15	2021-09-08	8	15	U,U	U,U	16	U,U	U,U
15	2021-09-08	9	17	U,U	U,U	18	U,U	U,U
15	2021-09-08	10	19	U,U	U,U	20	U,U	U,U
15	2021-09-08	11	21	U,U	U,U	22	U,U	U,U
15	2021-09-08	12	23	U,U	U,U	24	U,U	U,U
15	2021-09-08	13	25	U,U	U,U	26	U,U	U,U
15	2021-09-08	14	27	U,U	U,U	28	U,U	U,U
15	2021-09-08	15	29	U,U	U,U	30	U,U	U,U
15	2021-09-08	16	31	U,U	U,U	32	U,U	U,U
15	2021-09-09	17	33	U,U	U,U	34	U,U	U,U
15	2021-09-09	18	35	U,U	U,U	36	U,U	U,U
15	2021-09-09	19	37	U,U	U,U	38	U,U	U,U
15	2021-09-09	20	39	U,U	U,U	40	U,U	U,U
15	2021-09-09	21	41	U,U	U,U	42	U,U	U,U
15	2021-09-09	22	43	U,U	U,U	44	U,U	U,U
15	2021-09-09	23	45	U,U	U,U	46	U,U	U,U
15	2021-09-10	24	47	U,U	U,U	48	U,U	U,U
15	2021-09-10	25	49	U,U	U,U	50	U,U	U,U
15	2021-09-10	26	51	U,U	U,U	52	U,U	U,U
15	2021-09-10	27	53	U,U	U,U	54	U,U	U,U
15	2021-09-10	28	55	U,U	U,U	56	U,U	U,U
15	2021-09-10	29	57	U,U	U,U	58	U,U	U,U
15	2021-09-10	30	59	U,U	U,U	60	U,U	U,U
15	2021-09-10	31	61	U,U	U,U	62	U,U	U,U
15	2021-09-10	32	63	U,U	U,U	64	U,U	U,U
15	2021-09-10	33	65	U,U	U,U	66	U,U	U,U
15	2021-09-10	34	67	U,U	U,U	68	U,U	U,U
15	2021-09-10	35	69	U,U	U,U	70	U,U	U,U
15	2021-09-10	36	71	U,U	U,U	72	U,U	U,U
15	2021-09-10	37	73	U,U	U,U	74	U,U	U,U
15	2021-09-10	38	75	U,U	U,U	76	U,U	U,U
15	2021-09-11	39	77	U,U	U,U	78	U,U	U,U
15	2021-09-11	40	79	U,U	U,U	80	U,U	U,U
15	2021-09-11	41	81	U,U	U,U	82	U,U	U,U
15	2021-09-11	42	83	U,U	U,U	84	U,U	U,U
15	2021-09-11	43	85	U,U	U,U	86	U,U	U,U
15	2021-09-11	44	87	U,U	U,U	88	U,U	U,U
15	2021-09-11	45	89	U,U	U,U	90	U,U	U,U
15	2021-09-11	46	91	U,U	U,U	92	U,U	U,U
15	2021-09-11	47	93	U,U	U,U	94	U,U	U,U
15	2021-09-11	48	95	U,U	U,U	96	U,U	U,U
15	2021-09-11	49	97	U,U	U,U	98	U,U	U,U
15	2021-09-11	50	99	U,U	U,U	100	U,U	U,U
15	2021-09-11	51	101	U,U	U,U	102	U,U	U,U
15	2021-09-11	52	103	U,U	U,U	104	U,U	U,U
15	2021-09-11	53	105	U,U	U,U	106	U,U	U,U
15	2021-09-11	54	107	U,U	U,U	108	U,U	U,U
15	2021-09-11	55	109	U,U	U,U	110	U,U	U,U
15	2021-09-11	56	111	U,U	U,U	112	U,U	U,U
15	2021-09-11	57	113	U,U	U,U	114	U,U	U,U
15	2021-09-11	58	115	U,U	U,U	116	U,U	U,U

Lake #	Collection Date	Fish #	Mucus swab #	C. shasta	M. cerebralis	Gill Swab	C. shasta	M. cerebralis
16	2021-09-12	1	1	U,U	U,U	1	U,U	U,U
16	2021-09-12	2	2	U,U	U,U	2	U,U	U,U
16	2021-09-12	3	3	U,U	U,U	3	U,U	U,U
16	2021-09-12	4	4	U,U	U,U	4	U,U	U,U
16	2021-09-12	5	5	U,U	U,U	5	U,U	U,U
16	2021-09-12	6	6	U,U	U,U	6	U,U	U,U
16	2021-09-12	7	7	U,U	U,U	7	U,U	U,U
16	2021-09-12	8	8	U,U	U,U	8	U,U	U,U
16	2021-09-12	9	9	U,U	U,U	9	U,U	U,U
16	2021-09-12	10	10	U,U	U,U	10	U,U	U,U
16	2021-09-12	11	11	U,U	U,U	11	U,U	U,U
16	2021-09-12	12	12	U,U	U,U	12	U,U	U,U
16	2021-09-12	13	13	U,U	U,U	13	U,U	U,U
16	2021-09-12	14	14	U,U	U,U	14	U,U	U,U
16	2021-09-12	15	15	U,U	U,U	15	U,U	U,U
16	2021-09-12	16	16	U,U	U,U	16	U,U	U,U
16	2021-09-12	17	17	U,U	U,U	17	U,U	U,U
16	2021-09-12	18	18	U,U	U,U	18	U,U	U,U
16	2021-09-12	19	19	U,U	U,U	19	U,U	U,U
16	2021-09-12	20	20	U,U	U,U	20	U,U	U,U
16	2021-09-12	21	21	U,U	U,U	21	U,U	U,U
16	2021-09-12	22	22	U,U	U,U	22	U,U	U,U
16	2021-09-12	23	23	U,U	U,U	23	U,U	U,U
16	2021-09-12	24	24	U,U	U,U	24	U,U	U,U
16	2021-09-12	25	25	U,U	U,U	25	U,U	U,U
16	2021-09-12	26	26	U,U	U,U	26	U,U	U,U
16	2021-09-12	27	27	U,U	U,U	27	U,U	U,U
16	2021-09-12	28	28	U,U	U,U	28	U,U	U,U
16	2021-09-12	29	29	U,U	U,U	29	U,U	U,U
16	2021-09-12	30	30	U,U	U,U	30	U,U	U,U
16	2021-09-12	31	31	U,U	U,U	31	U,U	U,U
16	2021-09-12	32	32	U,U	U,U	32	U,U	U,U
16	2021-09-13	33	33	U,U	U,U	33	U,U	U,U
16	2021-09-13	34	34	U,U	U,U	34	U,U	U,U
16	2021-09-13	35	35	U,U	U,U	35	U,U	U,U
16	2021-09-13	36	36	U,U	U,U	36	U,U	U,U
16	2021-09-13	37	37	U,U	U,U	37	U,U	U,U
16	2021-09-13	38	38	U,U	U,U	38	U,U	U,U
16	2021-09-13	39	39	U,U	U,U	39	U,U	U,U
16	2021-09-13	40	40	U,U	U,U	40	U,U	U,U
16	2021-09-13	41	41	U,U	U,U	41	U,U	U,U
16	2021-09-13	42	42	U,U	U,U	42	U,U	U,U
16	2021-09-13	43	43	U,U	U,U	43	U,U	U,U
16	2021-09-13	44	44	U,U	U,U	44	U,U	U,U
16	2021-09-13	45	45	U,U	U,U	45	U,U	U,U
16	2021-09-13	46	46	U,U	U,U	46	U,U	U,U
16	2021-09-13	47	47	U,U	U,U	47	U,U	U,U
16	2021-09-13	48	48	U,U	U,U	48	U,U	U,U
16	2021-09-13	49	49	U,U	U,U	49	U,U	U,U
16	2021-09-13	50	50	U,U	U,U	50	U,U	U,U
16	2021-09-13	51	51	U,U	U,U	51	U,U	U,U
16	2021-09-14	52	52	U,U	U,U	52	U,U	U,U
16	2021-09-14	53	53	U,U	U,U	53	U,U	U,U
16	2021-09-14	54	54	U,U	U,U	54	U,U	U,U
16	2021-09-14	55	55	U,U	U,U	55	U,U	U,U
16	2021-09-14	56	56	U,U	U,U	56	U,U	U,U
16	2021-09-14	57	57	U,U	U,U	57	U,U	U,U
16	2021-09-14	58	58	U,U	U,U	58	U,U	U,U
16	2021-09-14	59	59	U,U	U,U	59	U,U	U,U
16	2021-09-14	60	60	U,U	U,U	60	U,U	U,U

Table 52: Parasite screening results in gills and mucus swabs from fish collected in lake 15 and 16.

C. shasta: Ceratomyxa shasta, M. cerebralis: Myxobolus cerebralis

U: Undetermined (no amplification recorded)

VII.a Pathogens screened from lake 15 and 16 waters

Water was collected from lake 15 (n=21) and 16 (n=20) and filtered through a 0.22 sterivex filter. Assays targeting bacteria (*A. salmonicida*, *R. salmoninarum* and *Y. ruckeri*) and parasites (*C. shasta* and *M. cerebralis*) have been optimised and tested using qPCR. Results showed no amplification for all the tested samples (Table 53). Therefore, no bacteria (*A. salmonicida*, *R. salmoninarum* and *Y. ruckeri*) and parasites (*C. shasta* and *M. cerebralis*) targeted genes were detected in the water of either lake.

Sample #	Lake	Depth	Location	Date sampled	Date filtered	Amount Filtered (mL)	R sal	A sal	Y ruckeri	C.shasta	M. cerebralis
1	15	Surface	East shore near crib net	08-Sep	08-Sep	500	U,U	U,U	U,U	U,U	U,U
2	15	3m		08-Sep	09-Sep	500	U,U	U,U	U,U	U,U	U,U
3	15	bottom (~4m)		08-Sep	09-Sep	450	U,U	U,U	U,U	U,U	U,U
4	15	Surface	East side of island, halfway between shore and island	08-Sep	09-Sep	1000	U,U	U,U	U,U	U,U	U,U
5	15	3m		08-Sep	11-Sep	250	U,U	U,U	U,U	U,U	U,U
6	15	5m (bottom)		08-Sep	11-Sep	250	U,U	U,U	U,U	U,U	U,U
7	15	Surface	South shore	08-Sep	09-Sep	1000	U,U	U,U	U,U	U,U	U,U
8	15	3m		08-Sep	09-Sep	450	U,U	U,U	U,U	U,U	U,U
9	15	5m		08-Sep	11-Sep	250	U,U	U,U	U,U	U,U	U,U
10	15	Surface	South shore between shore and island	08-Sep	11-Sep	700	U,U	U,U	U,U	U,U	U,U
11	15	3m		08-Sep	11-Sep	250	U,U	U,U	U,U	U,U	U,U
12	15	5m		08-Sep	11-Sep	250	U,U	U,U	U,U	U,U	U,U
13	15	Surface	West end	08-Sep	11-Sep	1000	U,U	U,U	U,U	U,U	U,U
14	15	3m		08-Sep	11-Sep	850	U,U	U,U	U,U	U,U	U,U
15	15	5m		08-Sep	11-Sep	650	U,U	U,U	U,U	U,U	U,U
16	15	5m	North shore halfway between island and shore	08-Sep	11-Sep	300	U,U	U,U	U,U	U,U	U,U
17	15	Surface		08-Sep	11-Sep	1000	U,U	U,U	U,U	U,U	U,U
18	15	3m		08-Sep	11-Sep	650	U,U	U,U	U,U	U,U	U,U
19	15	Surface	between both islands	08-Sep	11-Sep	1000	U,U	U,U	U,U	U,U	U,U
20	15	3m		08-Sep	11-Sep	250	U,U	U,U	U,U	U,U	U,U
21	15	5m		08-Sep	11-Sep	350	U,U	U,U	U,U	U,U	U,U
1601	16	Surface	off 'shellf' south shore	13-Sep	14-Sep	900	U,U	U,U	U,U	U,U	U,U
1602	16	3m		13-Sep	14-Sep	1000	U,U	U,U	U,U	U,U	U,U
1603	16	5m (~bottom)		13-Sep	14-Sep	950	U,U	U,U	U,U	U,U	U,U
1604	16	Surface	small bay south east shore	13-Sep	14-Sep	1000	U,U	U,U	U,U	U,U	U,U
1605	16	3m		13-Sep	14-Sep	1000	U,U	U,U	U,U	U,U	U,U
1606	16	5m		13-Sep	14-Sep	1000	U,U	U,U	U,U	U,U	U,U
1607	16	Surface	outlet bay	13-Sep	14-Sep	1000	U,U	U,U	U,U	U,U	U,U
1608	16	3m		13-Sep	14-Sep	1000	U,U	U,U	U,U	U,U	U,U
1609	16	5m		13-Sep	14-Sep	550	U,U	U,U	U,U	U,U	U,U
1610	16	Surface	off good fishing point (north east)	13-Sep	14-Sep	1000	U,U	U,U	U,U	U,U	U,U
1611	16	3m		13-Sep	14-Sep	1000	U,U	U,U	U,U	U,U	U,U
1612	16	5m		13-Sep	14-Sep	1000	U,U	U,U	U,U	U,U	U,U
1613	16	Surface	middle of lake	13-Sep	14-Sep	1000	U,U	U,U	U,U	U,U	U,U
1614	16	3m		13-Sep	14-Sep	1000	U,U	U,U	U,U	U,U	U,U
1615	16	5m		13-Sep	14-Sep	850	U,U	U,U	U,U	U,U	U,U
1616	16	Surface	entrance to launch	13-Sep	15-Sep	750	U,U	U,U	U,U	U,U	U,U
1617	16	1m		13-Sep	15-Sep	1000	U,U	U,U	U,U	U,U	U,U
1618	16	2m		13-Sep	15-Sep	1000	U,U	U,U	U,U	U,U	U,U
1619	16	Surface	near island west shore	13-Sep	15-Sep	1000	U,U	U,U	U,U	U,U	U,U
1621	16	5m		13-Sep	15-Sep	850	U,U	U,U	U,U	U,U	U,U

Table 53: Bacteria and Parasites screening results in water collected in lakes 15 and 16.

R sal: Renibacterium salmoninarum; A sal: Aeromonas salmonicida; Y ruckeri: Yersinia ruckeri, C. shasta: Ceratomyxa shasta, M. cerebralis: Myxobolus cerebralis

U: Undetermined (no amplification recorded)

VIII. Conclusion

In this study, we have optimised the molecular assay to screen for viruses IHNv, VHSv, IPNv and ISAv, EHNv, the bacteria of concern, *Aeromonas salminicida*, *Yersinia ruckeri* and *Renibacterium salmoninarum* as well as parasites, *Ceratomyxa shasta* and *Myxobolus cerebralis*. Collection of the samples was performed non lethally by swabbing fish mucus and gills.

Screening of the viruses, bacteria and parasites using the optimised conditions reported in this study. Last September 2021, 58 mucus and 58 gills swabs have been collected for screening viruses and bacteria of concern from lake 15 fish and 60 mucus and 60 gills swabs have been sampled from fish lake 16. Water samples were also collected at different locations and depths of lakes 15 and 16.

In summary, the results showed that the collected samples from both lakes had no confirmed amplification. Some amplifications with high Ct values (higher than cut off or one out of two amplified) have been recorded in few samples. This cannot be considered as positive samples as aerosol contamination can happen during the process which was observed in our negative controls.

As for EHNv, we have observed both a small band and peak from the qPCR test in mucus swab (fish# 6) and gill swab (fish# 32) from lake 15. Further analysis is needed to confirm these results.