Monitoring Attempted Eradication of the Invasive Species Northern Pike (*Esox lucius*), and Re-Establishment of Three-Spined Stickleback (*Gasterosteus aculeatus*) in Gillsvann, using eDNA Analysis.

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Abstract

Anthropogenic activities are putting marine and freshwater ecosystems under increasing pressure and global climate change is causing transformations in ecosystems and food chains worldwide. Invasive species pose one of the greatest threats to natural diversity and the endemic species essential for ecosystem balance. In November 2021, Gillsvann in Kristiansand, Norway, was treated with rotenone with the aim of eradicating the invasive species Northern pike (*Esox lucius*) from the lake. The effectiveness of the treatment was monitored using environmental DNA (eDNA) analysis. The primary objective was to investigate the presence of Northern pike eDNA in the lake from November 2021 (after the treatment) to November 2022. Monthly water samples were collected from 14 different locations in Gillsvann and analyzed using real-time PCR with species-specific eDNA. The findings of the study revealed that the presence of Northern pike eDNA was observed consistently each month up until the month of April in the year 2022. However, from the month of May until November in the same year, there was no detection of Northern pike eDNA, suggesting that the species may have been successfully eliminated from the lake. The second objective was to analyze eDNA of three-spined stickleback (*Gasterosteus aculeatus*) to assess the re-establishment of endemic species. The results suggest that three-spined stickleback likely re-invaded the lake following the rotenone treatment, with eDNA levels returning to pre-treatment levels by June 2022. This indicates that the species has successfully re-established in Gillsvann. Overall, this study demonstrates the potential effectiveness of rotenone treatment for eradicating invasive species and the utility of eDNA analysis for monitoring the possible success of eradication efforts and re-establishment of native species.

Keywords: *Conservation, Invasive Species, Rotenone, eDNA, Real-Time PCR*

Abstrakt

Antropogene aktiviteter har en økende negativ effekt på marine og ferskvannsøkosystemer, samtidig som globale klimaendringer fører til transformasjoner i økosystemer og næringskjeder over hele verden. Fremmede arter utgjør en av de største truslene mot naturlig mangfold og de endemiske artene som er essensielle for balanse i økosystemet. I november 2021 ble Gillsvann i Kristiansand, Norge, behandlet med rotenon med mål om å utrydde den fremmede arten gjedde (*Esox lucius*) fra innsjøen. Effektiviteten av behandlingen ble overvåket ved hjelp av miljø-DNA (eDNA) -analyse. Det primære målet var å undersøke tilstedeværelsen av eDNA fra gjedde i innsjøen fra november 2021 (etter behandlingen) til november 2022. Månedlige vannprøver ble samlet inn fra 14 forskjellige lokasjoner i Gillsvann og analysert ved hjelp av real-time PCR med artsspesifikt eDNA. Resultatene av studien viste at tilstedeværelsen av eDNA fra gjedde ble observert jevnlig hver måned fram til april 2022. Imidlertid var det ingen påvisning av gjedde eDNA fra mai til november samme år, noe som tyder på at arten kan ha blitt utryddet fra innsjøen. Det andre målet var å analysere eDNA av trepigget stingsild (*Gasterosteus aculeatus*) for å vurdere reetablering av endemiske arter. Resultatene antyder at trepigget stingsild trolig re-invaderte innsjøen etter rotenonbehandlingen, med eDNA-nivåer som returnerte til pre-behandlingsnivå innen juni 2022. Dette indikerer at arten har lykkes med å reetablere seg i Gillsvann. Studien demonstrerer potensialet for effektiviteten av rotenonbehandling for å utrydde fremmede arter, samt nytten av eDNA-analyse for å overvåke mulig suksess i utryddelsesforsøk og reetablering av endemiske arter.

Nøkkelord: *Bevaring, Fremmede Arter, Rotenon, eDNA, Real-Time PCR*

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1. Introduction

1.1 Global Concern for Invasive Species

Marine and freshwater ecosystems worldwide are facing increased pressure from anthropogenic sources due to higher frequency of human interactions and exploitation of aquatic resources. At the same time, global climate change proposes concerning changes in ecosystems and food chains globally. Lakes and streams are particularly susceptible to human interferences and changing temperatures, causing diversity loss in many freshwater ecosystems over time (Havel et al., 2015). According to Havel et al. (2015), one of the greatest threats to natural diversity and endemic species who maintain ecosystem balance, are exotic invasive species. Humans have been notably good at breaking natural biogeographical barriers, intentionally spreading species, as well as bringing species along as hitchhikers (Havel et al., 2015). Although some introduced species fail to establish in a new ecosystem, many have become invasive and caused harm to the natural balance and diversity. Furthermore, the establishment of foreign organisms increases the concern for already endangered endemic species (Dextrase and Mandrak, 2006, Gurevitch and Padilla, 2004). The decline of native species frequently coincides with the invasion of non-native species in the same geographic location, which has led conservationists and researchers to believe that there is a strong correlation between invasions and extinctions (Gurevitch and Padilla, 2004). Invasive species may compete directly and/or indirectly for resources, or they may introduce harmful parasites and pathogens. Therefore, early evaluation of the potential impact of an introduced species, coupled with implementation of appropriate management measures and population monitoring, is particularly important in the context of effective conservation.

1.2 The Invasion of Pike in Gillsvann

In a lake named Gillsvann, located in Kristiansand, Norway, northern pike (*Esox lucius*, hereafter referred to as pike) was assumed introduced by people in 2018 for recreational fishing purposes (Løvdal and Omholt, 2020). The species has not been observed in Gillsvann prior to 2018, and it has never been observed in nearby lakes or streams (Løvdal and Omholt, 2020), suggesting that the pike has not naturally migrated from a nearby habitat. As illustrated in figure 1-1 below, pike has a limited natural distribution in eastern aquatic systems of Norway. Nevertheless, there are many cases of pike translocation is southern, western and areas further north in Norway, likely going back to the Middle Ages (Hesthagen

Figure 1-1: Areas in Norway with natural and introduced populations of pike per 2012 (Hesthagen and Sandlund, 2012)

et al., 2020). Although the release of foreign species to a new ecosystem is now illegal according to Norwegian legislation, the highest number of cases of pike translocation are from the past 30 to 40 years (Hesthagen et al., 2020). Pike has become a species of high attraction for sports fishing and other recreational uses, and this is likely a reason why people have released them into "suitable" aquatic systems they are not native to.
Pike is a highly predatory and opportunistic fish species that feeds on most fish they interact with. They undergo an ontogenetic shift, where they initially feed on zooplankton and move on to a piscivore diet at length of 3 to 10 cm (Jacobson et al., 2019). They are able to eat fish at an earlier stage compared to many other predatory fish, due to their large gape relative to their body size (Jacobson et al., 2019). A dietary study was performed through stomach content analysis by Jacobson et al. (2019) in the Baltic region. From the study, they found eight different species of fish in pike stomach. In early summer, the dominant species was three-spined stickleback (*Gasterosteus aculeatus*), while in late summer they found gobiids (*Gobiidae* fam.) to be the most dominant prey. Other prey found includes nine-spined stickleback (*Pungitius pungitius*), clupeids (*Clupeidae* fam.), eelpout (*Zoarcidae* fam.), roach (*Rutilus rutilus*), ruffe (*Gymnocephalus cernua*) and bleak (*Alburnus alburnus*) (Jacobson et al., 2019). Furthermore, studies from Alaska have found that their diets include a large portion of salmonid species, likely due to the high availability of these species in the respective area (Cathcart et al., 2019).

The ecology of Gillsvann is affected by high tide and low tide. This is because it lays 1 meter above sea level, and it is directly connected to the Topdalsfjord through two small water streams (see figure 1-2). Water flows from Gillsvann out to the fjord during low tide and flows from the fjord into the lake during high tide. The Topdalsfjord has an average salinity

of 12 ppt in the first meter of the surface layer (Schreck et al., 2021). According to Jacobsen et al. (2007), pike can survive in brackish waters with salinities up to12–15 ppt, and will not survive if exposed to salinities above 18 ppt. Oceanic water has an average salinity of 30 to 35 ppt, while fresh water is typically at 0 ppt. Therefore, considering the nearest locations pike has been observed until this day (see figure 1-3), there is no reason to believe the species has naturally migrated to Gillsvann. At certain times throughout the year, this brackish surface layer of the Topdalsfjord can get deeper, especially in early spring when rivers bring increased amount of fresh water to the fjord, because of ice and snow melting. This suggests that the newly established population of pike can potentially survive in the upper layer of the Topdalsfjord for a limited period. At 2 meters depth and below, the average salinity of the fjord is 23 ppt, which is not survivable for pike (Schreck et al., 2021).

Figure 1-2: Map of Gillsvann and its two river outlets to the Topdalsfjord – Justvikbukta (top right corner) and Eidsbukta (lower right corner). Map also shows a few small upstream rivers from nearby lakes (lower left corner) (Norgeskart.no)

Furthermore, the salinity tolerance of pike and the salinity variation of the Topdalsfjord throughout the year suggests that the species can potentially migrate from Gillsvann to other freshwater systems connected to the Topdalsfjord. This have raised concern for Tovdalselva and Otra, two large rivers providing important habitat for the vulnerable species Atlantic salmon (*Salmo salar*) and sea trout (*Salmo trutta*). Especially in early spring, the high

potential of pike to spread to these rivers, could have major impact on the natural salmonid stocks. According to a study performed by Sepulveda et al. (2013), the trophic adaptability of invasive pike can drive multiple species to low abundance, including salmonids.

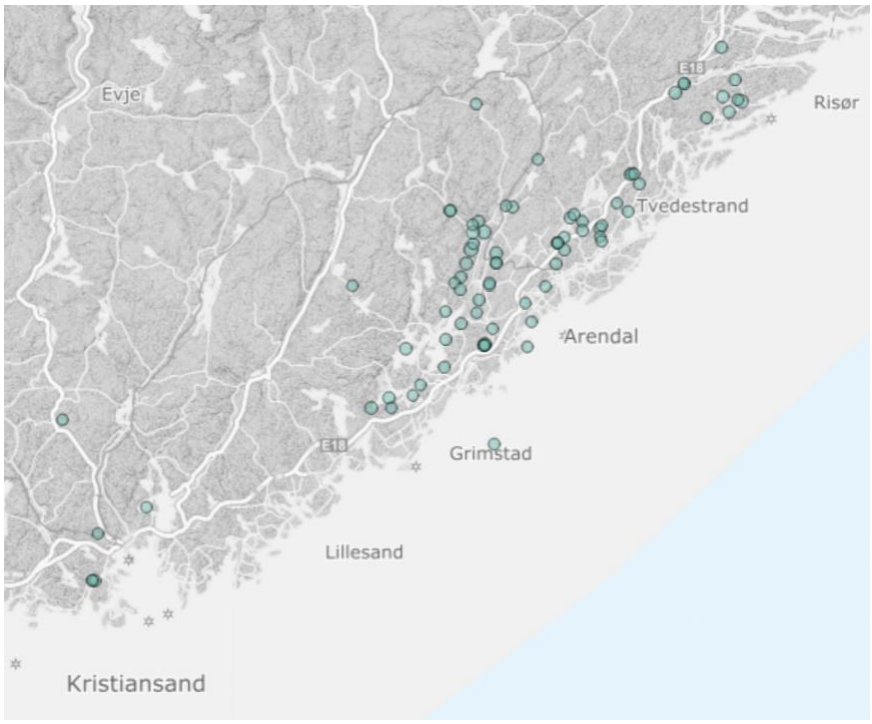

Figure 1-3: Known locations of pike in Agder county (Artsdatabanken.no). Green circles represent observations of pike.

A different study provides evidence that support the negative effect of introduction of pike on a native freshwater brown trout (*Salmo trutta*) population in a lake in Norway (Hesthagen et al., 2015). For this reason, it is not implausible that the low catch numbers of brown trout and perch that have been reported in recent years in Gillsvann (Løvdal and Omholt, 2020), can be related the introduction of- and increased abundance of pike. Additionally, this also suggest that pike may potentially pose a threat to the spawning and nursing habitats of salmon and sea trout in Tovdalselva and Otra. The commercial species of salmon and sea trout is of great interest to recreational fisheries and tourism. Hence, the public attention directed towards this specific event of invasion has been unusually intense. The engagement of the public is also based on certain false ideas that pike is a “dangerous” and aggressive species to humans. Locally, Gillsvann is a popular lake for swimming and other water activities during the summer months. Thus, locals have been concerned for themselves and their children due to the invasion of pike.

1.3 Rotenone: a Controversial but Effective Conservation Tool

Worldwide, rotenone is a controversial, yet widely used pesticide in conservation biology. Specifically in fish conservation, rotenone is called a piscicide due to its ability to eliminate fish. Amongst a range of cases in Norway, Trøndelag county is an example of an area where pike has been introduced in 94 lakes, and in 23 of those lakes, successful eradications of pike have been achieved by the use of rotenone treatment (Hesthagen et al., 2020). Additionally, rotenone treatment has been widely used in Norway to extirpate the harmful introduced fish parasite *Gyrodactylus salaris* (Hartvigsen, 1997*)*. Due to the ecological concerns for spread of pike, and its potential implications for already threatened species, Gillsvann was treated with rotenone early November 2021. This method was chosen due to its high likelihood of success, compared to traditional fishing methods. If possible, it would take a significantly long time to eradicate pike from the lake system using traditional methods, such as capturing fish in nets. However, if traditional methods were possible, the pike population would still have time to migrate and spread to nearby lakes or streams by the time it would take to remove the population from Gillsvann. On the contrary, the use of the pesticide rotenone is reliable, cost-effective, does not involve physical destruction of habitat and works immediately by blocking oxygen uptake on gills of fish and some invertebrates (Dalu et al., 2020). Nevertheless, rotenone treatment is regarded as a controversial management tool due to its impact on non-target species (Dalu et al., 2020), and the particular ecosystem must therefore be carefully examined before taking action.

Rotenone is non-persistent in natural freshwater ecosystems and degradation time varies between a few days to a few months in temporal aquatic systems (Sousa, 1988). Lower temperatures correlate with longer degradation time, due to slow-down of biological and chemical processes (Sousa, 1988). According to Sousa (1988), the toxicity may last for about 30 days at a water temperature around 7°C. Furthermore, degradation time is also dependent on other variables such as UV-light, alkalinity and dissolved oxygen (Sousa, 1988). Rotenone does not directly harm organisms without gills, which means that it is strongly selective for fishes (Robertson and Smith-Vaniz, 2008). Nevertheless, there will be an indirect impact caused by removal of gill-breathing organisms in their habitat. Sufficient concentration of rotenone and area coverage is important to cover all pike habitats in the lake with an estimated water volume of 7 314 030 m^3 (Kjærstad et al., 2021), as well as nearby river outlets. Due to multiple biotic and abiotic factors, it is very likely that the ecosystem of Gillsvann will naturally recover over time. From fish populations in upstream lakes and

streams as well as the fjord, return and re-establishment of most native species are expected. Kjærstad et al. (2021) has reported that European perch (*Perca fluviatilis*), brown trout (*Salmo trutta*), European eel (*Anguilla anguilla*), crucian carp (*Carassius carassius*), tench (*Tinca tinca*), Atlantic mackerel (*Scomber scombrus*), Atlantic herring (*Clupea harengus*), Atlantic cod (*Gadus morhua*), European flounder (*Platichthys flesus*), nine-spined stickleback (*Pungitius pungitius*), three-spined stickleback (*Gasterosteus aculeatus*) and common goby (*Pomatoschistus microps*) are among the fish species that have been previously documented in Gillsvann. Nursing grounds of sea trout (anadromous adapted trout) and trout appear upstream from Gillsvann, and sea trout exploits the fjord for longer periods. Eels are catadromous species spending much of their lifetime in freshwater lakes and streams to grow and become sexually mature, and undertake long-distance migrations in the ocean to spawn (Durif et al., 2013). Considering the presumed utilization of upstream lakes and streams connected to Gillsvann, the species is anticipated to return to Gillsvann relatively soon after the treatment. Another native species that is assumed to return quickly is three-spined stickleback. Three-spined stickleback inhabits a wide range of aquatic environments from marine to freshwater systems in various latitudes in the Northern hemisphere (Sokolowska and Kulczykowska, 2006). Some populations are anadromous or catadromous and able to adapt to saltwater, brackish water as well as freshwater, while other populations live in freshwater or saltwater exclusively (Sokolowska and Kulczykowska, 2006). During spawning the males establish territory in shallow water with sand or small rocks, where they build nests out of algae (Sokolowska and Kulczykowska, 2006). Reproduction is stimulated by longer days and increased water temperatures in spring, and they may spawn in either freshwater, brackish water or saltwater depending on population adaptations. The life-cycle of three-spined stickleback is short and they have high offspring survival overall (Sokolowska and Kulczykowska, 2006). Altogether, there is good reason to believe that individuals of the three-spined stickleback population in the Topdalsfjord during treatment, will return to Gillsvann in a short time after rotenone degradation. Thus, this species will be monitored as an indicator of re-establishment of native species after rotenone treatment of Gillsvann. Crucian carp and tench are regarded as introduced species (Løvdal and Omholt, 2020). Their impact on the environment is not obvious and more difficult to determine, because they are not predatory fish like pike. Nonetheless, these two species are expected not to re-invade since they are not endemic to the area.

1.4 eDNA and Real-Time PCR

Environmental DNA (eDNA) methods are non-invasive, reliable, rapid to monitor and assess populations accurately in an environment (Cooper et al., 2021). eDNA is the sum of DNA shed from organisms in the environment, that provides a molecular signature of all organisms within that environment (Cooper et al., 2021). eDNA may be composed of DNA within cells or organelles from eukaryote cells derived from urine, excrements, skin, mucus and/or free eDNA, as well as extracellular DNA resulted by cell death (Lacoursière-Roussel et al., 2016). It may persist in the environment for a variated length of time depending on environmental conditions such as temperature, salinity, water movements and water exchange, as well as type of organism and development stage (Lacoursière-Roussel et al., 2016). Even though the organisms are eliminated, carcasses may continue to dispose DNA to the water and can therefore still be detected after elimination. Recent studies have shown that monitoring eDNA instead of using traditional sampling methods (e.g. capture fish, electro-fishing), may increase the power of detection and spatial coverage (Lacoursière-Roussel et al., 2016). Furthermore, eDNA based surveys provide an increased potential of successful detection of presence/absence of species in low abundance (Cooper et al., 2021), such as rare species or in this case, recently potentially eradicated and recovering populations. Nevertheless, it is important to note that increased detection sensitivity can lead to false positive results if contamination occurs between sites when collecting water, or in the laboratory when preparing and analyzing the samples (Balasingham et al., 2017). Therefore, it is essential to follow protocols for sampling, switch gloves frequently and use controls to minimize the risk of false positives.

This presence/absence survey on low abundance species requires optimization for maximum eDNA recovery and detection sensitivity (Cooper et al., 2021). Therefore, it is particularly important to optimize post-collected eDNA preservation and extraction workflows to avoid degradation or loss of eDNA, which can lead to false negative detections (Cooper et al., 2021). Filtration can be used to capture eDNA from the collected water. Pore size and material of the filters may affect the retention and sum of eDNA material captured (Lacoursière-Roussel et al., 2016). Overall, studies have shown that smaller pore size is increasing the retention as well as the amount of eDNA (Lacoursière-Roussel et al., 2016). Genetic material can be captured at pore sized between 0.02 μm and 180 μm (Lacoursière-Roussel et al., 2016). Extraction of eDNA from filters is commonly achieved using commercial kits, such as the DNeasy Blood and Tissue kit (QIAGEN) (Cooper et al., 2021).

The advantages commercial kits provide, are that their protocols are simple to follow, streamlined and yield comparable results as they provide a standardization across research groups (Cooper et al., 2021).

Furthermore, isolated eDNA can be analyzed using real-time polymerase chain reaction (real-time PCR) to look for presence of specific species. There are various methods available for detecting nucleic acid sequences from a sample. However, many of these methods have certain disadvantages including being time consuming, labor intensive, nonquantitative, too low sensitivity, require radioactive applications, or higher potential for cross contamination and human error (Valasek and Repa, 2005). A few examples of such methods are various types of hybridizations, HPCL (high performance liquid chromatography), PCR-ELISA (PCR- enzyme linked immunosorbent assay), scintillation proximity assay, and some gel electrophoresis PCR end-point methods (Valasek and Repa, 2005). For many years, PCR has been a power tool for quantitative nucleic acid analysis (McPherson and Møller, 2000). To measure presence of specific eDNA in a water sample, real-time PCR is advantageous over the earlier methods for several reasons. The method provides an extraordinarily wide dynamic range of quantifying with its high sensitivity, it is relatively quick and requires no post-PCR manipulation, which decreases chances of cross contamination (Valasek and Repa, 2005). PCR-tubes are never opened after a real-time PCR, which decreases the chance of contamination between previous PCR product and future samples. There have been many studies on detection of rare aquatic species using conventional PCR. In this method, primers are designed to target a highly conserved mitochondrial or nuclear region of the specific target species' DNA. Presence of PCR products are visualized as bands on agarose gel, and the product should also be sequenced to confirm that the target species is detected (Balasingham et al., 2017). However, traditional end-point PCR is not quantitative, it is time consuming and increases chances of contamination, which is why real-time PCR is more beneficial for this survey.

In simple terms, PCR involves combining a sample of DNA with oligonucleotide primers, deoxynucleotide triphosphates, and a thermostable DNA polymerase in a suitable buffer (McPherson and Møller, 2000). This mixture is repeatedly heated and cooled down for several hours depending on number of cycles, until the desired amount of amplification is achieved (McPherson and Møller, 2000). The principle is to copy and amplify specific fragments of DNA from a sample. DNA polymerases use the short, sequence specific

oligonucleotides that are added to the reaction to act as primers for the DNA synthesis (Valasek and Repa, 2005). The most common polymerase used in real-time PCR is the Taq DNA polymerase (from *Thermus aquaticus*) (Valasek and Repa, 2005). The enzyme Taq DNA polymerase can generate new strands of DNA using the DNA template and primers, and it is heat resistant which is crucial, because after each round of DNA copying, the double-stranded DNA (dsDNA) is melted by high temperatures (~95 °C) into single strands (Valasek and Repa, 2005). Next, the tubes are cooled down for the primers to direct the DNA polymerase, and initiate elongation on the now single stranded DNA (ssDNA), by adding complementary nucleotides to produce new dsDNA (Valasek and Repa, 2005). Subsequently, the new dsDNA must be melted before a new cycle of copying can begin (Valasek and Repa, 2005). Due to reactants being consumed after many cycles, the reaction will create twice as much specific dsDNA after each cycle (exponential phase of amplification plot), until it eventually reaches a plateau (Valasek and Repa, 2005). Self-annealing of increasing concentration of PCR product may also contribute to reaching the plateau (Valasek and Repa, 2005).

Real-time PCR is allowing quantification of DNA by taking advantage of the efficient DNA amplification occurring early in the reaction process, and measures PCR product (fluorescence) in the exponential phase (Valasek and Repa, 2005). This measurement correlates to the initial amount of the specific sequence of DNA in the sample, which enables quantification. Thus, real-time PCR is also known as “Quantitative PCR” or “qPCR”. “Real-time” refers to monitoring amplification of DNA in real time as the amplification is occurring (Valasek and Repa, 2005). The threshold cycle (Ct-value) is the point on the amplification curve where fluorescence exceed the background noise signals (Gunay et al., 2016) (see figure 1-4). The quantitative Ct-value intersects the signal curve in its exponential phase and is negatively associated with the (log) concentration of DNA detected in the sample (Gunay et al., 2016). In other

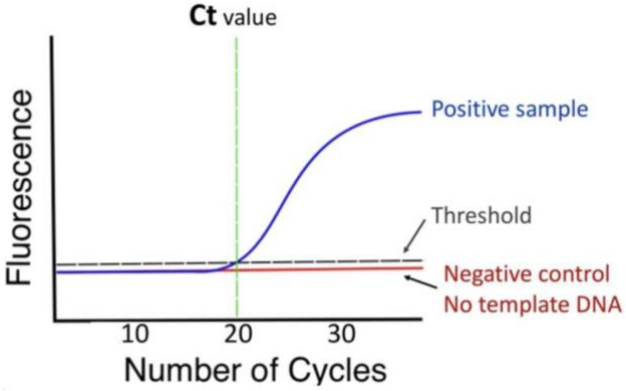

Figure 1-4: Linear amplification curve. Fluorescence intensity is plotted against number of reaction cycles. Threshold line is shown in a dashed gray line. The positive sample crosses the threshold line at Ct = 20. (Adam and Smith, 2022)

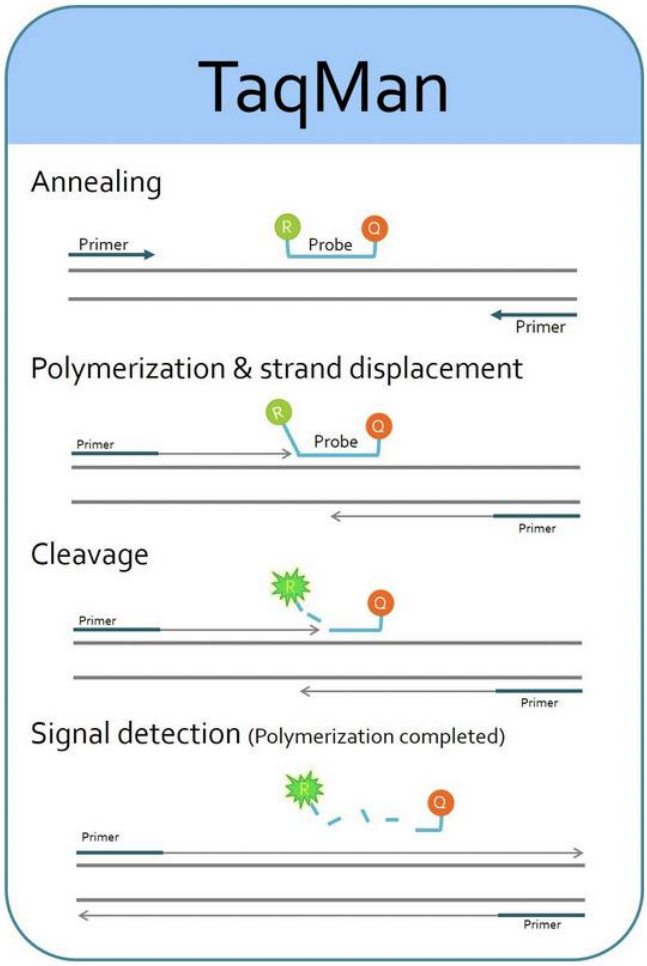

Figure 1-5: Principals of TaqMan real-time PCR. Grey lines represent template DNA (dsDNA), R = reporter, Q = quencher. DNA polymerase is not shown but lays at the arrow heads, on the synthesizing DNA strand (SMOBIO Technology).

terms, a lower Ct-value indicates a higher initial concentration of the target species' DNA in the sample as it corresponds to earlier fluorescence detection, and vice versa (Gunay et al., 2016).

To monitor the amount of DNA present in each cycle of the real-time PCR, the method generally involves fluorogenic probes (Valasek and Repa, 2005). Various probes exist including DNA-binding dyes, such as EtBr or SYBR green I, hydrolysis probes, hybridization probes, and more (Valasek and Repa, 2005). Overall, their task is to link fluorescence change to amplification of DNA. SYBR green I binds to the dsDNA in the minor groove, emitting higher fluorescence than when free in solution (Valasek and Repa, 2005). Thus, the higher the amount of specific DNA in the sample, the higher the fluorescence signal. TaqMan probe is a type of the hydrolysis probes (Valasek and Repa, 2005). They are also called “5’-nuclease probes” because the DNA polymerase exonuclease activity cleaves the probe starting at its 5’ end. The principals of the TaqMan real-time PCR are displayed in figure 1-5. The TaqMan probe is a specific DNA oligonucleotide with two fluorophores attached, one called “quencher” (Q) and one called “reporter” (R) (Valasek and Repa, 2005). When the quencher and the reporter are attached to the same oligonucleotide (are close together), the quencher (electron acceptor) detects signals from the reporter (electron donor) (Valasek and Repa, 2005). During the real-time PCR cycles, the DNA polymerase breaks the oligonucleotide apart (Valasek and Repa, 2005), shown in the cleavage step in figure 1-5. Consequently, the quencher and reporter separate which causes the reporter to release energy, and a fluorogenic signal can be detected. Thus, specific DNA fragments in the sample will be detectable when present, and strength of fluorogenic signal

(caused by hydrolysis) will correspond with amount of specific DNA in the sample. According to Valasek and Repa (2005), hydrolysis probes have similar precision as SYBR green I, but they have an advantage in measuring specific species since they only monitor sequence-specific amplification, which suggest greater insurance in species specific DNA analysis.

Real-time PCR is also dependent on proper instrumentation. Input of energy for excitation, as well as emission wavelength detection needs to be precise, specific and occur simultaneously for progressive detection of fluorescent signal (Valasek and Repa, 2005). Additionally, always maintaining a consistent temperature in all samples during PCR is important to avoid differences in amplification efficiency (Valasek and Repa, 2005). This is achieved by a heating block and heated air (Valasek and Repa, 2005). Finally, PCR needs computer hardware and software platforms that simplify results into graphical outputs such as the amplification (figure 1-4) and dissociation (melting point) curves. These provide information about the kinetics of amplification of target DNA and the characteristics of the amplified product, respectively (Valasek and Repa, 2005). Nevertheless, only the amplification curves are relevant to the present survey.

Combining the knowledge provided regarding characteristics and uses of real-time PCR and eDNA, it is possible to quantify eDNA of the target species (pike and three-spined stickleback) when the specific real-time PCR tests are designed for the two species. When designing the tests, it is important to consider the choice of DNA marker for primer and probe sequences. This marker affects both specificity and sensitivity of the PCR (Brežná and Piknová, 2013). A target sequence that is very abundant in the cell will ensure better sensitivity due to lower limit of detection (Brežná and Piknová, 2013). Examples of abundant markers are ribosomal, mitochondrial or plastid DNA, and they are frequently used by scientists, thus more likely available in genetic databases (Brežná and Piknová, 2013). For detection of fish DNA, mitochondrial DNA markers are frequently used, due to its high number in animal tissue (Brežná and Piknová, 2013). The gene for cytochrome c oxidase subunit I (CO1) is a mitochondrial marker, and it is proposed as the standard gene for identification of animal species by zoologists (Brežná and Piknová, 2013). Cytochrome b (cytb) is another frequently sequenced target gene found in the mitochondria (Brežná and Piknová, 2013). The selected area for the PCR analysis of the mitochondrial markers mentioned above are short (around 100 base pairs) (Brežná and Piknová, 2013), which is

advantageous for detecting eDNA in the case of this lake system. The fragments of DNA in the water are degrading over time, thus having a shorter sequence will contribute to higher probability of detection, as longer sequences may break apart more easily in the environment.

When running real-time PCR tests on isolated eDNA, it may be beneficial to also run quality control checks of the samples. The purity and total concentration of DNA in the samples are not known unless they are measured. Nanodrop spectrometers allow scientists to quickly quality check their extracted nucleic acid and protein samples, using as little as 1-2 µl of the sample (Matlock, 2015). Nucleic acids and proteins absorb ultraviolet light (UV-light) at wavelength 260 nm and 280 nm, respectively (Wilfinger et al., 1997). The ratio of the absorbance of a sample at these two wavelengths has been widely used as a measure of purity of nucleic acid and protein extractions from a sample (Matlock, 2015). The optimal A_{260}/A_{280} ratio for what is considered "pure" DNA is ~1.8 (Wilfinger et al., 1997). There may be various substances in the sample that absorb UV-light at a wavelength of 230 nm, that can be potential inhibitors of PCR. Hence, the A_{260}/A_{230} ratio is usually also given. The value given for the A_{260}/A_{230} ratio, is often higher than the value given for the A_{260}/A_{280} ratio, and the value should be in the range 2.0-2.2 when the sample consists of "pure" nucleic acids (Matlock, 2015). These ratios can be helpful to examine potential sources of error in the methods. Typically, a low A_{260}/A_{230} ratio (<2.0) suggests that there can be residual substances from the extraction or from the water (Matlock, 2015). On the contrary, a high A_{260}/A_{230} ratio (>2.2) can be caused by measuring a blank sample on a dirty pedestal, or using a solution for the blank sample that is not suitable (should be the same pH as the sample) (Matlock, 2015). Likewise, abnormal values for the A_{260}/A_{280} ratio may also raise concerns about the liability of the results. Low A_{260}/A_{280} ratio (<1.8) either indicates that the sample is contaminated by proteins or reagents associated with the extraction protocol (Matlock, 2015). Another reason can be that the concentration of nucleic acids in the sample is very low (<10 ng/µL) (Matlock, 2015). Additionally, it is important to note that even though ratios are within the optimal range, it does not mean that the sample is perfectly pure and carry no issues. On the other hand, with appropriate measures, the buffer components present in the master mix can counteract inhibitors in the sample, thereby allowing PCR to effectively amplify samples with low purity.

1.5 Aims of Study

This study's purpose is to monitor the effect of rotenone treatment in eradicating the invasive species Northern pike, using eDNA analysis. Additionally, the study will present the investigation of re-establishment of endemic species in Gillsvann after the treatment, using eDNA analysis of three-spined stickleback as an indicator of return of endemic species. A presence/absence recording of eDNA of these species will be used to determine the potential eradication and re-establishment by analyzing samples collected from the lake every month for one year after rotenone treatment. Furthermore, this provides an ideal case study to demonstrate the potential of success for rotenone use in management of invasive species, as well as the long-term effects of using rotenone on endemic species in a lake system.

2. Methods

2.1 Water Collection

Water samples from 14 different locations at Gillsvann were collected once every month from September 2021 to November 2022. Water samples from September 2021 until June 2022 were collected by PhD student Silje Halvorsen and Master student Eirik Jørgensen. Eirik Jørgensen also did an additional collection of water in November 2022. Sampling locations can be seen in figure 2-1. Sampling location 1, 5, 6, 8, 10 and 11 were in the lake itself. Sampling location 7.1, 7.2, 7.3, 9.1, 9.2 and 9.3 were taken in the fjord from the harbor next to the two river outlets (represented as 7 or 9), and the sampling was done from the inner to the outer part of the harbor (1 to 3 respectively). The last two locations, 2 and 3, were in two smaller rivers upstream from Gillsvann originating from Grovann and Øygardstjønn, respectively. The locations for the study were selected to represent Gillsvann and its nearby potential pike inhabited areas the best way possible for this method. Surface water was collected in 1-liter containers with sealed lids from shore in all 14 locations. Gloves were always used and disposed after every sample to avoid any contamination between locations.

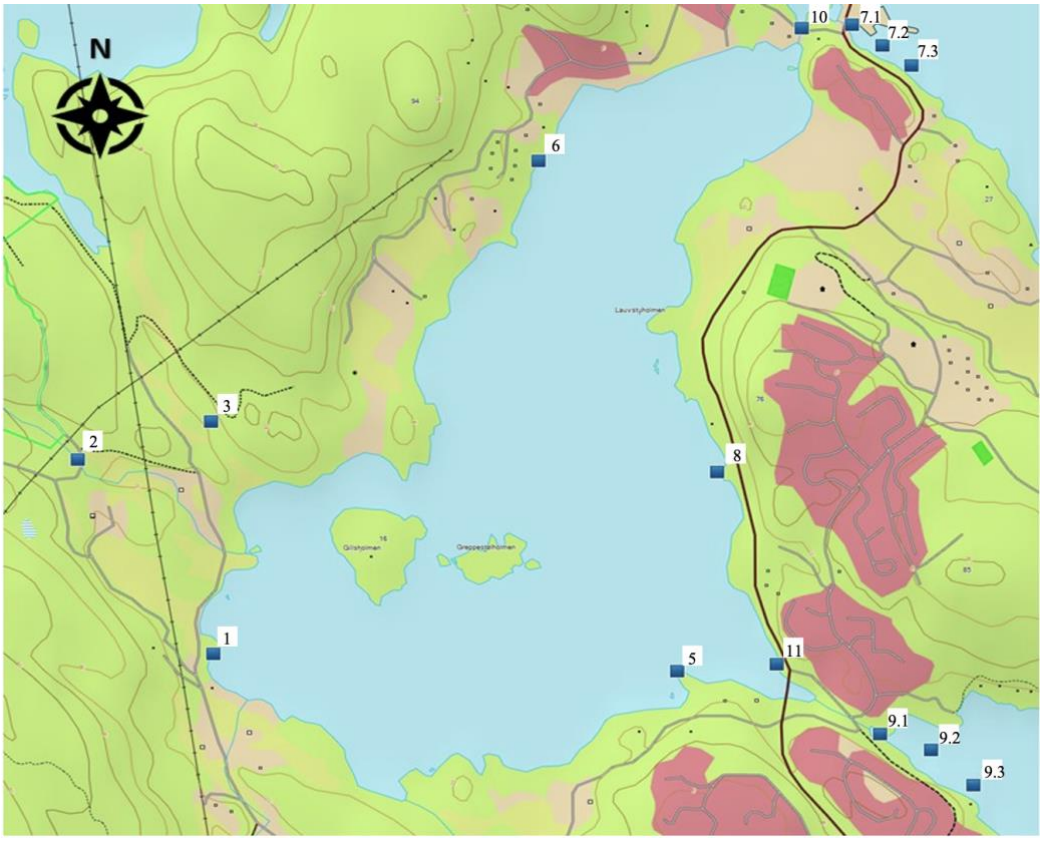

Figure 2-1: Map of sample locations at Gillsvann. Location 2 and 3 represent samples in upstream rivers. Location 7.1, 7.2, 7.3 and 9.1, 9.2, 9.3 represent downstream samples in the respective bays/coves in Topdalsfjord close to outlets from Gillsvann. Sample 10 is also taken downstream. (Map illustration: Made by Lars Korslund and modified by Elise Rustad)

Well-marked sample containers were kept in coolers with cooling elements until filtration. Location, date, time, outside temperature and weather were noted for every location. For each water collection, high tide was avoided due to inflow of fjord water to Gillsvann. Collecting water during high tide would give DNA material representative to the ocean water, rather than Gillsvann in the areas close to where the ocean water flows into the lake. At some of the sampling dates, a container of distilled water was brought out in the field to provide a negative control. Containers were cleaned with 10% chlorine between each sampling.

2.2 Filtration of Water Samples

From 1-liter containers filled with water, about 400 to 1000ml was filtered using a ILMVAC vacuum pump (GmbH) and cellulose nitrate filters with pore size 0.45 µm (Thermo Scientific Nalgene). The presence of floating organic matter in the water samples caused the filters to become blocked after a certain amount of time, resulting in a filtration termination. Thus, filtration volume varied from 400-1000 ml. The filters were folded 3 to 4 times and placed in a 1.5 ml Eppendorf tube using a sterile tweezer. A total of 14 Eppendorf tubes, each containing one filter representing the location, was marked well, and placed in a freezer at -20°C. Negative controls were filtered and treated similarly. Filtration was done by the respective students who collected the water immediately after collection.

2.3 eDNA Isolation from Filters

DNeasy Blood and Tissue Kit (QIAGEN) was used in the isolation procedure. However, some adjustments were made to the procedure making it suitable for the collected eDNA on the filters. Filters were thawed and cut into approximately 1 mm pieces using a scalpel. The pieces were placed into a marked 1.5 ml microcentrifuge tube containing 0.5 mm Zirconia/Silica beads (BioSpec Products) using a sterile tweezer. 720 µl of Buffer ATL was added to the tube. The microcentrifuge tube was sealed with a twist cap and shaked for 45 sec at 2800 opm (oscillations per minute) using a bead beating machine (MagNa Lyser, Roche). The microcentrifuge tube was placed in a water bath at 56°C for 30 minutes and shaked again using the same settings. 80 µl proteinase K were added to the tube and tube was incubated at 56°C for 2 hours. After the incubation, the microcentrifuge tube was vortexed and centrifuged at 8000 rpm for 1 min, and the supernatant (approximately 500 µl) was pipetted over to another marked 1.5 ml Eppendorf tube. Equal volume of Buffer AL and 96% Ethanol were also added to the same Eppendorf tube, and the supernatant was vortexed between each

addition. The solution was then transferred to a DNeasy Blood and Tissue spin column containing filter for DNA, 500 µl at a time. Spin column was centrifuged at 8000 rpm for 1 min after each time solution was added, and the collection tube was emptied after each run. For a total volume around 1.5 ml, this step was done three times to assemble all the DNA in the supernatant in the spin column filter. 500 µl of washing solution AW1 (with added ethanol) were then added to the spin column, and it was centrifuged at 8000 rpm for 1 min. Collection tube was eliminated and substituted. Likewise, 500 µl of AW2 (with added ethanol) were added and spin column was centrifuged, but this time at 13400 rpm (max speed) for 3 min. Collection tube was eliminated and substituted with a 1.5 ml Eppendorf tube without lid. 100 µl of AE were added to the center of the spin column. The spin column was incubated at room temperature for 1 min before being centrifuged at 8000 rpm for 1 min. Depending on amount of DNA solution required for real-time PCR analysis, volume of AE was decided. For this experiment, a lower volume of Buffer AE (100 µl) was chosen to increase the concentration of DNA in the solution. eDNA concentrations of the species were expected to be low initially, so reducing dilution with lower volume of buffer would increase chances of real-time PCR to detect eDNA in the sample. On the other hand, this reduces volume of DNA yield and will therefore provide lower volume of DNA yield to work with in total. Final DNA yield was transferred to a marked Eppendorf tube and put in the freezer at -20°C. This procedure was done for every sample from every location and therefore provided 14 tubes of DNA yield from each month. Negative controls were isolated in an identical way. PhD student Silje Halvorsen isolated eDNA from filters collected from September 2021 until April 2022.

2.4 gDNA Isolation from Muscle Tissue

Tissue DNA was also isolated from an individual of three-spined stickleback and an individual of pike to be used as positive controls during analysis of the collected eDNA. In this procedure the DNeasy Blood and Tissue kit was used again, and the protocol was followed stepwise for tissue. In general terms, about 0.3 cm^3 of muscle tissue was cut directly out of the fish, chopped into smaller pieces, and placed into a 1.5 ml microcentrifuge tube. 180 µl of Buffer ATL and 20 µl of proteinase K were added to the tube and mixed by vortexing. The tube was incubated for 1-2 hours at 56°C, until the mixture was completely lysed. During the incubation time, the tube was vortexed occasionally to make sure the enzymes penetrated and dissolved all the muscle cells. When everything was completely

lysed the mixture was vortexed for 15 sec, before proceeding to step two in the protocol (QIAGEN, 2020) that was explained in the previous section. The DNA was diluted in 200 µl AE buffer.

2.5 Quantity and Purity Measurement of Isolated DNA

DNA samples were tested in an absorption spectrometer (NanoDrop One, Thermo scientific) to estimate concentration of DNA isolated in the sample, as well as to estimate the purity of the sample. Desired DNA samples were taken out of the freezer, thawed, mixed slightly and centrifuged. The absorption instrument was cleaned with distilled water. 1.5 µl of Buffer AE were pipetted to the sampling area and instrument arm was lowered to measure the blank sample. Sampling area was cleaned again and 1.5 µl of DNA sample were added to the sampling area. Arm was lowered and DNA sample was measured. The DNA concentration (ng/µl) and absorption rates (A260/A280, A260/A230) were noted. Gloves were used to avoid potential contamination.

2.6 Real-Time PCR

Real Time PCR tests were established, tested and performed on DNA samples for both species (*Esox lucius* and *Gasterosteus aculeatus*). DNA samples from all 14 locations from September 2021 until November 2022 were analyzed. Negative controls and positive controls were also analyzed. A well-functioning real-time PCR test for *E. lucius*, retrieved from scientific literature (Olsen et al., 2015, Olsen et al., 2016), was already run by PhD student Silje Halvorsen for a number of samples from September 2021 until April 2022. Hence, the same test was used on the remaining samples until April 2022, and all samples collected after April 2022. The primers and probe selected for pike recognizes the CO1 gene. Three-spined stickleback real-time PCR test was also established based on primers and probes discovered in scientific literature (Thomsen et al., 2012). Length of three-spined stickleback probe and primers were modified (shortened and extended, respectively), for optimal Tm (melting temperature) by using the software *Primer express* (Thermofisher). They recognize and anneal to an area of the cytb gene. Samples were analyzed by a qPCR with species-specific TaqMan®-Probe, with a reporter 6-FAM fluorescence in the 5'-end and a quencher in the 3'-end. Primers and probes are given in table 2-1.

Table 2-1: Primers and probes for *E. lucius* and *G. aculeatus.* DNA fragment sizes including primers and probe are 94 and 101 base pairs (bp), respectively.

	Esox lucius	***Gasterosteus aculeatus***
F-primer	5'-CCTTCCCCCGCATAAATAATATAA-3'	5'-ACGCCACCTTAACACGTTTCTT-3'
R-primer	5'-GTACCAGCACCAGCTTCAACAC-3'	5'-AGAGCCTGTCTGGTGAAGGAAA-3'
Probe	-6FAM-5'-CTTCTGACTTCTCCCC-3'-MBG-NFQ	-6FAM-5'-GGTGCCACACTTGTT-3'-MBG-NFQ

The qPCR was performed by a StepOne Plus real-time PCR machine (Applied Biosystem), with the ability to analyze 96 samples in one run. The PCR mix for one 0,1 ml Micro Fast Tube Strips (Thermo Fisher) contained the following ingredients: 12.5 µl TaqMan Environmental Master Mix (Applied Biosystems), 4.3 µl nuclease free H_2O, 2 µl forward primer (10 µM), 2 µl reverse primer (10 µM), 1.2 µl probe (10 µM) and 3 µl DNA sample (or H_2O for blank). Total volume in each tube was 25 µl. After preparing tubes, lids were placed on the tubes and the tubes were centrifuged before being placed into the PCR instrument. All samples were run through qPCR in triplets (three replicate analyses of the same location) and there were also one blank (H_2O + PCR-mix) and one positive (genomic DNA + PCR-mix) control present in every run.

Two different temperature profiles for the qPCR cycles were used. Initially, the following temperature profile was used for detecting *E. lucius*: 1 incubation at 50 °C for 2 min and 95°C for 10 min, followed by 60 cycles at 95 °C for 15 sec, 57°C for 1 min, and 60°C for 30 sec. Fluorescence was measured during the last step for every cycle. However, this profile was switched with the following profile that was used for both *E. lucius (*all samples, excluding some samples analyzed by Silje Halvorsen until April 2022) and *G. aculeatus* (all samples): 1 incubation at 50 °C for 2 min and 95°C for 10 min, followed by 60 cycles at 95 °C for 15 sec and 60°C for 1 min. Similarly, fluorescence was measured during the last step for every cycle. Ct-values for positive samples of both species were recorded, and mean Ct-values for the three replicate analyses of each location (triplets) were calculated.

2.7 Measure of Real-Time PCR Sensitivity by Dilution Series of gDNA and eDNA

To find an approximate concentration for the lower limit of detection by the real-time PCR, dilution series were tested for both species. 5 µl of desired sample were diluted with 45 µl of nuclease free water in a 1.5 ml microtube. Microtube containing one tenth of the concentration from the original sample was vortexed for 1 min, and thereafter centrifuged for

15 sec at max speed. Next, 5 µl of the diluted sample were added to another microtube, together with 45 µl of nuclease free water, which was also vortexed and centrifuged. This dilution series was prepared for 7 dilutions. After running the PCR for 7 dilutions, the final positive sample was diluted in a 50/50 ratio. This was done once more if necessary. Positive controls (gDNA) and one positive sample from Gillsvann for both species were investigated for sensitivity. Lower limit of detection of positive control DNA samples were calculated using Nanodrop One absorption measurements of the original sample. The DNA concentration retrieved from this measurement was used to find the concentrations in the diluted sample using this approach for a 10^x diluted sample: concentration in original sample (ng/µl) / 10^x. Furthermore, the estimated species-specific DNA concentration in the PCR reaction tube from the final positive sample of the dilution series was calculated using the concentration at the lower limit of detection and the volume of the PCR reaction tube. This method assumes that lower limit of detection for specific eDNA and gDNA are approximately equal. For all real-time PCR runs on dilution series, the settings, preparations, and ingredients were the same as described in the previous section.

2.8 Specificity Evaluation using Traditional PCR and Multiple Sequence Alignments

To test real-time PCR specificity for the two target species, traditional PCR followed by gel-electrophoresis methods and multiple sequence alignments were done. Primers given in table 2-1, were used for the traditional PCR. Initially, the plan was to isolate PCR products and send them to a sequencing service center, to verify that the sequences belong to the respective target species. One gDNA sample and one positive eDNA sample from each species were selected. 5 µl nuclease free H_2O, 10 µl Phusion Green Master Mix, 1µl (10 µM) forward primer and 1µl (10 µM) reverse primer were combined in one PCR reaction tube. 3 µl of gDNA sample, eDNA sample or nuclease free water (for blank control) was pipetted into the PCR tube. A sealing lid strip was placed on the tube strip before placing the strip into an Applied Biosystems Verti^{TM} 96-Well Thermal Cycler PCR machine. The following temperature profile was used for both species: first stage at 98°C for 30 sec, followed by second stage with 40 cycles at 98°C for 10 sec, 60°C for 10 sec and 72°C for 30 sec, and lastly a third stage at 72°C for 5 min followed by an infinite incubation at 4°C until samples were taken out for further use. When agarose gel-electrophoresis gave no visible product, 3 µl of the reaction was utilized as a template for another PCR run to increase chances of visible product. The remaining 18 µl of the reaction were stored in the freezer or fridge while the second PCR was running.

Next, agarose gel for gel-electrophoresis was prepared. 1.8 g of agarose powder and 100 ml of TAE-buffer (Tris-acetate-EDTA, diluted to 1X conc.) were mixed in a small Erlenmeyer flask. The mixture was heated to boiling point in the microwave and mixed gently multiple times, until everything was melted. Thereafter, the flask was cooled down to 60°C. When desired temperature was reached, 10 µl of gel stain (SYBR^{TM} Safe DNA Gel Stain, Thermo Fisher Scientific) were added and mixed by gently twirl the flask. Suitable sized gel mold with a comb of desired number of sample wells was placed on a flat surface, and gel-mixture was gently poured into the container. After about 60 min, when the agarose gel had hardened, the comb was removed and the gel was placed into the electrophoresis tank filled with TAE-buffer, connected to a power supply (Biorad PowerPac 300). Around 18 µl of PCR product samples were pipetted into each well and 10 µl of GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific) were loaded into the first well. A loading dye buffer was added to the samples prior to electrophoresis (when master mixes besides Phusion Green was used), to increase viscosity and observe migration of sample on the gel. Electrophoresis was turned on at 85V. After 60 min, gel was taken out of the tank and moved to a Syngene NuGenius documentation system for DNA analysis.

Unfortunately, PCR product of good enough quality was not obtained after multiple attempts, using three different master mixes (TaqMan, Phusion Green and 2X Phusion HSII High Fidelity). Therefore, it was decided to use a different approach to evaluate specificity. Multiple sequence alignment tool Clustal Omega (EMBL-EBI, 2023) was used to check whether the primers and probes of the two target species would work on other ecologically relevant species. This was done by aligning the sequences of the primers and probes together with the respective DNA sequences (COI or cytb) of species that could potentially be present in the eDNA samples. 10 different sequences of each desired species, retrieved from the NCBI GenBank, were initially aligned in FASTA format in Clustal Omega to study the genes for mutations that could be significant. Thereafter, one COI sequence and one cytb sequence of each desired species were aligned with each of the primers and probes for pike and three-spined stickleback, respectively. The following species (besides the target species) were studied in the multiple sequence alignment, reasoned by the fact that they were present in the lake at the time of rotenone treatment: *S. trutta*, *P. fluviatilis*, *C. carassius*, *A. anguilla* and *T. tinca*. Three-spined stickleback primers and probe were additionally aligned with *P. pungitius*, due to their morphological and ecological similarities.

3. Results

3.1 eDNA Results

eDNA samples from 14 locations at Gillsvann, over a time of 14 months were analyzed using real-time PCR. The first two months of water collection occurred prior to the rotenone treatment, while the remaining monthly collections were done after the treatment. eDNA samples were analyzed for presence of pike DNA, as well as three-spined stickleback DNA to monitor the expected eradication of pike and to discover a potential reestablishment of three-spined stickleback. Furthermore, the methods were assessed for sensitivity and specificity using dilution series and multiple sequence alignment, respectively.

According to the real-time PCR results in table 3-1 and 3-2, pike DNA was not found in Gillsvann after April 2022, suggesting that pike was likely successfully eradicated from the lake. Prior to the rotenone treatment, fragments of pike DNA were detected in samples from most locations in Gillsvann, as well as in some of the samples collected in the river outlets (sample 7.1, 7.2 and 9.2). After the treatment, eDNA detections of pike increased to all locations, despite the two smaller upstream rivers (sample 2 and 3). In February, March and April 2022, there was a decline of locations with positive results. For most of the runs in these months, all three replicate analyses of each location (triplets) were negative. April 2022 is the time of the final two positive detections of pike (sample 9.1 and 11). Only one of the three replicate analyses of the two locations were positive. Overall, Ct-means for each triplet of replicate analyses of samples lays between 29 at the lowest, and 41 at the highest. Standard deviation calculations of the Ct-means from table 3-1 and 3-2 can be found in the appendix (table B-1). The variation of the triplets of Ct-values is generally low with standard deviations between ± 0.04 and ± 3.64, with most of the numbers laying in the lower end of that scale.

Table 3-1: Real-time PCR results from September 2021 to April 2022. Target species: *E. lucius.* Sample numbers are shown in the first column. + is given for positive samples, while – is given for negative samples. Mean Ct-values for each of the corresponding replicate analysis of each location (triplets) are also given. Sample 1 from December 2021 was missing from the sample collection and not tested.

Sample #	**30-sep-21**		**28-oct-21**		**24-nov-21**		**22-dec-21**		**2-feb-22**		**4-mar-22**		**6-apr-22**	
	PCR	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*
1	+++	33	+++	33	+++	36			---	0	---	0	---	0
2	---	0	---	0	---	0	---	0	---	0	---	0	---	0
3	---	0	---	0	---	0	---	0	---	0	---	0	---	0
5	+++	35	+++	34	+++	34	+--	35	---	0	+--	34	---	0
6	+++	33	+++	29	+++	32	+++	34	---	0	+++	34	---	0
7.1	---	0	+--	36	+++	32	+++	33	---	0	++-	30	---	0
7.2	---	0	++-	36	+++	24	++-	34	---	0	++-	34	---	0
7.3	---	0	---	0	+++	33	+++	32	---	0	+--	34	---	0
8	+++	34	+++	33	+++	35	+--	36	++-	35	---	0	---	0
9.1	---	0	---	0	+++	35	+++	33	+--	41	+--	30	+--	36
9.2	++-	34	---	0	+++	37	++-	35	++-	33	---	0	---	0
9.3	---	0	---	0	++	39	+++	34	+--	33	---	0	---	0
10	+++	32	+--	35	+++	33	+++	34	+--	33	++-	32	---	0
11	+++	34	---	0	+++	34	+++	39	---	0	++-	34	+--	34

Table 3-2: Real-time PCR results from May 2022 to November 2022. Target species: *E. lucius*. Sample numbers are shown in the first column. + is given for positive samples, while – is given for negative samples. Mean Ct-values for each of the corresponding triplets are also given.

Sample #	**9-may-22**		**16-jun-22**		**8-jul-22**		**12-aug-22**		**19-sep-22**		**11-oct-22**		**9-nov-22**	
	PCR	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*
1	---	0	---	0	---	0	---	0	---	0	---	0	---	0
2	---	0	---	0	---	0	---	0	---	0	---	0	---	0
3	---	0	---	0	---	0	---	0	---	0	---	0	---	0
5	---	0	---	0	---	0	---	0	---	0	---	0	---	0
6	---	0	---	0	---	0	---	0	---	0	---	0	---	0
7.1	---	0	---	0	---	0	---	0	---	0	---	0	---	0
7.2	---	0	---	0	---	0	---	0	---	0	---	0	---	0
7.3	---	0	---	0	---	0	---	0	---	0	---	0	---	0
8	---	0	---	0	---	0	---	0	---	0	---	0	---	0
9.1	---	0	---	0	---	0	---	0	---	0	---	0	---	0
9.2	---	0	---	0	---	0	---	0	---	0	---	0	---	0
9.3	---	0	---	0	---	0	---	0	---	0	---	0	---	0
10	---	0	---	0	---	0	---	0	---	0	---	0	---	0
11	---	0	---	0	---	0	---	0	---	0	---	0	---	0

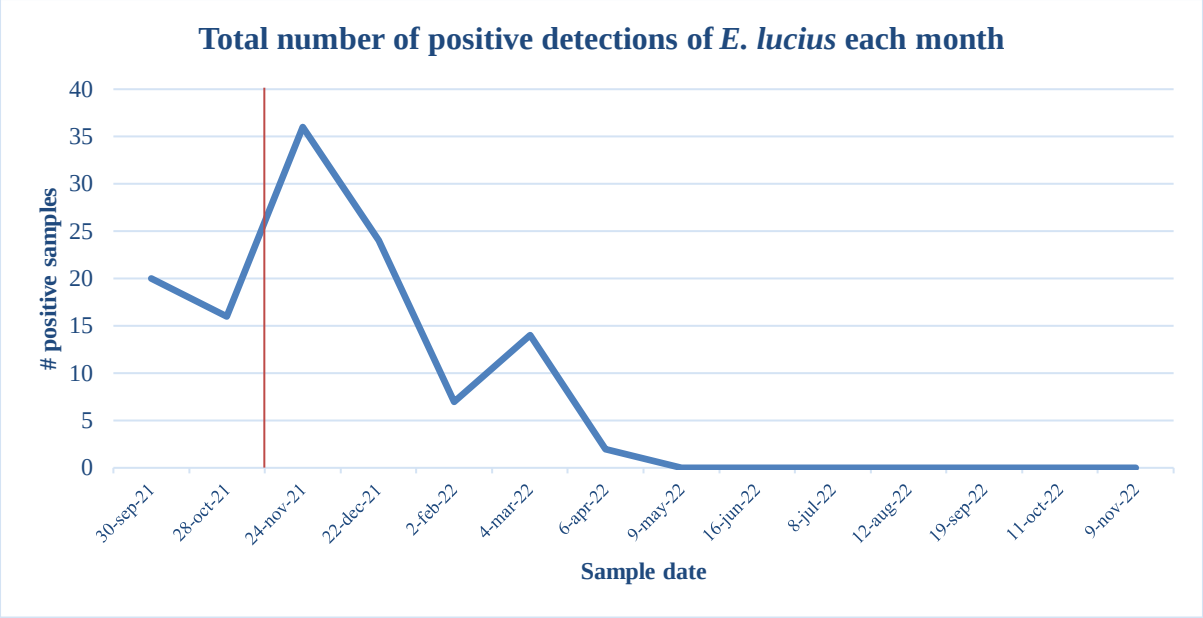

Figure 3-1: Total number of positive samples of *E. lucius* for each sample date displayed in a continuous graph. The three replicate samples of each location (triplets) are used as individual samples. Hence, possible maximum total is 42 samples. Number of positive samples are shown at the y-axis. Red line represents time of rotenone treatment (beginning of November). Note: 22-dec-21 is missing three reactions.

The results from table 3-1 and 3-2 are visually displayed in the graph above (fig. 3-1). The total number of positive detections from each sampling date are shown in a time series from September 2021 to November 2022, with a red line indicating when rotenone release was put into action. Each sampling date has a maximum of 42 positive reactions, as there are 14 sampling locations, and each location is analyzed three times (in triplets). For December 2021, one location is missing, so this run could potentially have three more positive detections. The graph shows the clear increase of positive detections immediately after the treatment, and the gradual decrease of positive detections until May 2022 where it stabilizes at 0 for the remaining runs until November 2022.

Table 3-3 and 3-4 represents the real-time PCR data from eDNA sample runs targeting three-spined stickleback in Gillsvann. In contrast to the results of pike shown above, three-spined stickleback eDNA is never completely absent from the water samples. Nevertheless, there is decline in number of positive detections of three-spined stickleback DNA in the samples after the rotenone treatment. In one of the two upstream rivers from Gillsvann (sample 2), the species was never detected. On the other hand, three-spined stickleback was frequently detected in the river outlets in the Topdalsfjord (sample 7.1, 7.2, 7.3, 9.1, 9.2 and 9.3) both prior to and after the treatment. A few more positive locations were found in October 2021 compared to September 2021, prior to the treatment. All locations were positive in November 2021 (besides sample 2), immediately after rotenone release. The next couple of months,

most of the positive detections were found in the river outlets (7.1, 7.2, 7.3, 9.1, 9.2 and 9.3) as well as in the upstream river (sample 3). In May 2022, there is an increase of locations of positive detections in Gillsvann (sample 5, 6 and 11), while in June 2022 some of these locations show up as negative, while sample 8 was found to be positive. Furthermore, from July 2022 and the remaining months, most locations were detected positive for three-spined stickleback. The three replicate analyses' Ct-means obtained from the real-time PCR runs on three-spined stickleback in Gillsvann are between 34 and 45, somewhat higher than the pike runs. The variation in the three-spined stickleback Ct-values for each triplet were found to be relatively low, similarly to that of pike. The standard deviation of the triplet means can be found in the appendix, table B-2. The standard deviations lay between ± 0.01 and ± 4.61, with most of the numbers laying in the lower end of that scale.

Table 3-3: Real-time PCR results from September 2021 to April 2022, targeting *G. aculeatus*. Sample numbers are shown in the first column. + is given for positive samples, while – is given for negative samples. Mean Ct-values for each of the three replicate analyses for each location (triplets) are also given. Sample 1 and 11 from December 2021 were missing from the sample collection and not tested.

Sample #	**30-sep-21**		**28-oct-21**		**24-nov-21**		**22-Dec-21**		**2-feb-22**		**4-mar-22**		**6-apr-22**	
	PCR	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*
1	---	0	+--	40	+--	40			---	0	---	0	---	0
2	---	0	---	0	---	0	---	0	---	0	---	0	---	0
3	---	0	+--	40	++-	40	+--	40	+--	42	+--	40	+++	39
5	---	0	++-	40	++-	39	+--	38	---	0	---	0	---	0
6	---	0	+++	37	+++	37	---	0	---	0	---	0	---	0
7.1	+++	39	+++	39	+++	38	+--	42	+++	40	---	0	+--	40
7.2	+++	41	+++	40	+++	39	+--	40	++-	40	++-	40	+++	40
7.3	+++	39	++-	42	+++	39	++-	41	+++	40	+++	37	+--	40
8	---	0	+--	40	+++	40	---	0	---	0	---	0	---	0
9.1	+++	36	+++	35	++-	40	+--	41	++-	40	+--	41	++-	40
9.2	+++	40	+++	39	+++	40	---	0	++-	40	++-	40	+--	42
9.3	+++	40	+++	39	+++	40	---	0	---	0	+++	41	+--	41
10	+++	34	+++	36	+++	39	---	0	---	0	---	0	---	0
11	+++	39	+++	37	+++	39			---	0	---	0	---	0

Table 3-4: Real-time PCR results from May 2022 to November 2022, targeting *G. aculeatus*. Sample numbers are shown in the first column. + is given for positive samples, while – is given for negative samples. Mean Ct-values for each of the corresponding triplets are also given.

Sample #	9-may-22		16-jun-22		8-jul-22		12-aug-22		19-sep-22		11-oct-22		9-nov-22	
	PCR	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*	*PCR*	*Ct*
1	---	0	---	0	++-	41	+++	39	+++	38	+++	37	+++	36
2	---	0	---	0	---	0	---	0	---	0	---	0	---	0
3	---	0	---	0	---	0	---	0	+--	41	++-	39	---	0
5	++-	40	---	0	+++	39	+++	39	++-	40	+++	38	+++	37
6	+--	42	---	0	+--	39	++-	41	+++	37	+++	56	+++	34
7.1	+++	35	+++	38	+++	35	+++	36	+++	37	+++	34	---	0
7.2	+++	39	++-	40	+++	35	+++	36	+++	37	+++	36	+--	40
7.3	+++	40	++-	39	+++	37	+++	36	+++	37	+++	37	+--	40
8	---	0	+--	41	+--	41	+++	45	+++	38	+++	37	+++	38
9.1	+++	37	+++	38	+++	36	+++	37	+++	37	+++	35	+++	34
9.2	+++	38	---	0	+++	39	+++	38	+++	38	++-	39	+++	35
9.3	+++	39	---	0	++-	40	+++	38	+++	39	+++	39	+++	36
10	+++	39	---	0	+++	38	+++	39	+++	38	+++	36	+++	35
11	+++	39	++-	38	+++	36	+++	38	+++	39	+++	35	+++	38

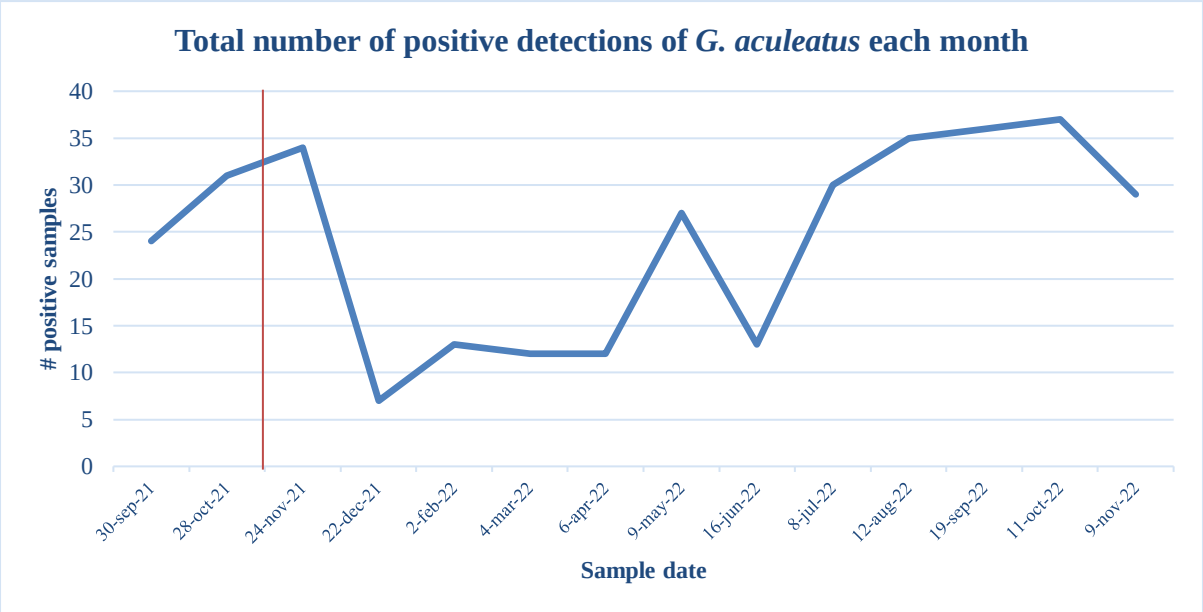

Figure 3-2: Total number of positive samples of *G. aculeatus* for each sample date displayed in a continuous graph. The three replicate samples of each location (triplets) are used as individual samples. Hence, possible maximum total is 42 samples. Number of positive samples are shown at the y-axis. Red line represents time of rotenone treatment (beginning of November). Note: 22-dec-21 is missing six reactions.

The trend of positive detections of three-spined stickleback over time is displayed in the graph above (fig. 3-2). There was a slight increase of total number of positive reactions immediately after the time of rotenone treatment (red line). Thereafter, there was a drastic decline of positive detections in December 2021. Positive reactions remained low in numbers each month until May 2022 when numbers increased to being higher than September 2021,

before the rotenone treatment. In June 2022 numbers dropped once again but recovered to nearly all reactions being positive throughout the autumn of 2022. A slight drop of reactions with positive detections occurred in November 2022. Overall, the graph shows that the number of positively detected locations for three-spined stickleback is somewhat the same prior to treatment compared to half a year after the treatment, with a clear drop in the months in between.

3.2 Real-Time PCR Sensitivity: gDNA and eDNA Dilution Series

Dilution series of genomic DNA for both target species were done to test the sensitivity of the real-time PCR assay. DNA concentrations of isolated genomic DNA (control samples) of pike and three-spined stickleback were 88.3 ng/µl and 76.5 ng/µl, respectively. These numbers are listed in table 3-5 below. Table 3-5 is also showing an estimate of the purity of the samples, given by absorption wavelength ratios. Most of them were relatively close to the optimal ratios of 1.8 for A260/A280 and 2.2 for A260/A230, described in the introduction. The A260/A230 ratio for three-spined stickleback was a little lower than optimal. Both target species' tissue DNA sample, as well as one positive eDNA sample were diluted. The pike and three-spined stickleback DNA concentration in the last positive eDNA sample were estimated from the respective genomic DNA dilution series with known DNA concentration. The results are displayed in table 3-6, 3-7, 3-8 and 3-9 below.

Table 3-5: Nanodrop one measurements on isolated tissue DNA from the two target species. DNA concentrations are given in ng/µl.

Sample	***DNA conc. (ng/µl)***	***A260/A280***	***A260/A230***
E. lucius	88.3	2.12	2.17
G. acetulatus	76.5	1.74	0.91

Table 3-6: *E. lucius* genomic DNA dilution series. Concentration of original sample (gDNA) was retrieved from Nanodrop one measurement (table 3-5). DNA samples were diluted with nuclease free water. DNA concentrations are given as ng/µl.

Soultion	***Dilution***	***PCR***	***Ct (mean)***	***Calculated concentration (ng/µl)***
gDNA	0	+++	17	88.30
1	10	+++	20	8.83
2	10^2	+++	24	8.83×10^{-1}
3	10^3	+++	27	8.83×10^{-2}
4	10^4	+++	31	8.83×10^{-3}
5	10^5	+++	34	8.83×10^{-4}
6	10^6	+++	38	8.83×10^{-5}
7	10^7	+--	39	8.83×10^{-6}
8	20^7	+--	39	4.42×10^{-6}
9	40^7	+--	40	2.21×10^{-6}
10	80^7	---	0	0

Table 3-7: eDNA dilution series from positive sample (Sample 6. 28-oct-21). Target species: *E. lucius.* DNA samples were diluted with nuclease free water.

Soultion	***Dilution***	***PCR***	***Ct (mean)***
eDNA sample	0	+++	34
1	10	+++	37
2	20	++-	40
3	40	++-	40
4	80	++-	40
5	160	+++	40
6	320	---	0

Using the nanodrop measurement of DNA concentration in table 3-5, the concentration of pike DNA in the dilution series of the gDNA sample was calculated. The real-time PCR was able to detect DNA down to a 40^7 dilution, and Ct-value given for this run was 40, which is much higher than the original sample. The calculated DNA concentration of the last positive sample was 2.21×10^{-6} ng/µl. With a 3 µl DNA sample volume and a total PCR reaction volume of 25 µl, concentration of pike DNA in the reaction tube was calculated to be 2.7×10^{-7} ng/µl. The dilution series of the positive eDNA sample did also have a mean Ct-value of 40 at the last positive detection of pike. This sample was only diluted 160 times in nuclease free water.

Table 3-8: *G. aculeatus* genomic DNA dilution series. Concentration of original sample (gDNA) was retrieved from Nanodrop one measurement (table 3-5). DNA samples were diluted with nuclease free water. DNA concentrations are given as ng/µl.

Soultion	***Dilution***	***PCR***	***Ct (mean)***	***Calculated concentration (ng/µl)***
gDNA	0	+++	21	76.5
1	10	+++	27	7.65
2	10^2	+++	30	7.65×10^{-1}
3	10^3	+++	34	7.65×10^{-2}
4	10^4	+++	37	7.65×10^{-3}
5	10^5	+++	41	7.65×10^{-4}
6	20^5	---	0	0

Table 3-9: eDNA dilution series from positive sample (Sample 6. 28-oct-21). Target species: *G. aculeatus.* DNA samples were diluted with nuclease free water.

Soultion	***Dilution***	***PCR***	***Ct (mean)***
eDNA sample	0	+++	33
1	10	+++	37
2	20	++-	40
3	40	+--	41
4	80	---	0

The final positive detection in the dilution series of three-spined stickleback genomic DNA, was diluted 10^5 times (sample 5, table 3-8). The mean Ct-value of the triplet of replicate samples was 41. Using the concentration given for three-spined stickleback in table 3-5, an estimated concentration of DNA in solution 5 was calculated to be 7.65×10^{-4} ng/µl. The lower limit of detection in the real-time PCR reaction tube with 3 µl DNA sample and total reaction volume of 25 µl is 9.18×10^{-5} ng/µl. Hence, the real-time PCR was likely able to detect DNA of pike at a lower concentration (2.7×10^{-7} ng/µl) compared to results of three-spined stickleback. Furthermore, table 3-9 shows the dilution series of a positive eDNA sample for three-spined stickleback. This sample was diluted 80 times, and the last positive detection of DNA was seen in the 40 times diluted sample (sample 3, table 3-9). The Ct-value of the lest positive reaction was 41.

3.3 Real-Time PCR Specificity: Traditional PCR and Multiple Sequence Alignments

Traditional PCR was attempted multiple times to make product that would be sequenced, to ensure that the sequences belong to the respective species. However, the results obtained from the gel-electrophoresis were not sufficient to send the product to a sequencing service

center. In the figures below (fig. 3-3, 3-4 and 3-5) some examples are given of the unsuccessful attempts. All runs were done using eDNA samples that were already detected positive by real-time PCR. Due to lack of time and resources, multiple sequence alignments were used as a different approach to evaluate the specificity, comparing primers and probes with respective sequences of various ecologically relevant species. The results are given in table 3-10 and 3-11.

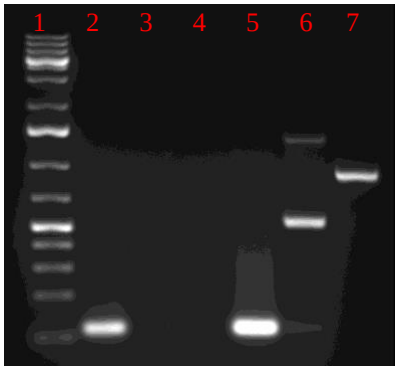

Figure 3-3: PCR results displayed by Syngene NuGenius with UV-light showing fluorescence. From left: DNA ladder, 1st run of control DNA, 1st run of eDNA sample, 1st run of blank sample, 2nd run of control DNA, 2nd run of eDNA sample, 2nd run of blank sample. TaqMan master mix used in PCR. Target: *E. lucius.*

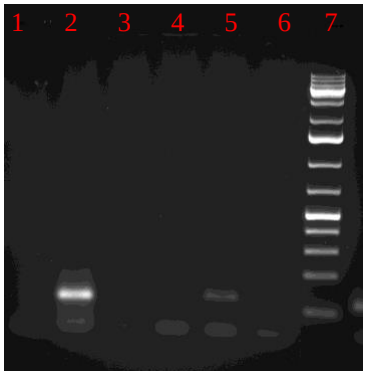

Figure 3-4: PCR results displayed by Syngene NuGenius with UV-light showing fluorescence. From left: 1st run of control DNA, 1st run of eDNA sample, 1st run of blank sample, 2nd run of control DNA, 2nd run of eDNA sample, 2nd run of blank sample, DNA ladder. Phusion Green master mix used in PCR. Target: *G. aculeatus*

Figure 3-5: PCR results displayed by Syngene NuGenius with UV-light showing fluorescence. From left: 1st run of control DNA, 1st run of eDNA sample, 1st run of blank sample, 2nd run of control DNA, 2nd run of eDNA sample, 2nd run of blank sample. 2X Phusion HSII High Fidelity master mix used in PCR. Dye added to wells in gel. Target: *E.lucius*

Figure 3-3 shows the DNA bands obtained after the gel electrophoresis using primers targeting pike in the PCR runs. Well number 2, 3 and 4 from the left are from the initial run and well number 5, 6 and 7 are bands formed using PCR product from the first run as template for another PCR run. There was more product after the second PCR run, but unfortunately there were unclear results, and the blank control sample (7) was contaminated. Additionally, the bands in the positive control columns (2 and 5), seems to be too short to be the correct DNA-fragments of 94 bp.

Figure 3-4 shows an example of results obtained when testing for three-spined stickleback DNA. Here, only the eDNA reaction (2 and 5) formed bands, while the positive controls (1 and 4) were negative. Furthermore, the second run using PCR product as DNA template (5), unexpectedly seemed to be less clear than the initial run (2). This should in theory give much higher fluorogenic signal, due to higher amount of amplified product.

Finally, the results presented in figure 3-5 were hidden by the dye used to stain the samples in the wells. Nevertheless, the positive control (1) did show through the dark shadow, but after

the second run of PCR, the positive control band was less visible (4). Additionally, disturbance of fluorogenic signals in the first part of the gel can be seen relatively clearly in the first three samples (1, 2 and 3), indicating contamination.

Overall, results of good quality were not obtained, and sequencing of PCR products were therefore not done. Thus, the results presented above were not considered when evaluating the specificity of the real-time PCR tests for pike and three-spined stickleback. Nevertheless, specificity could still be evaluated from the multiple sequence alignment presented below.

Table 3-10: Multiple sequence alignment of cytochrome c oxidase subunit 1 (CO1) sequences of 6 ecologically relevant species, compared with the primers and probe of *E.lucius*. The first alignment (*E.lucius*) is included for reference. * represent similarity between bases. R-primer is transformed to reverse complementary form to match DNA sequences in the alignments. Clustal Omega (EMBL-EBI) was used for multiple sequence alignments.

Species aligned	F-primer	Probe	R-primer
E. lucius	--CCTTCCCCCGCATAAATAATATAA-- GCCTTCCCCCGCATAAATAATATAAGC ************************	--CTTCTGACTTCTCCCC--- AGCTTCTGACTTCTCCCCCC ****************	--GTGTTGAAGCTGGTGCTGGTAC--- AGGTGTTGAAGCTGGTGCTGGTACTG ************************
G. aculeatus	--CCTTCCCCCGCATAAATAATATAA--- AGCATTTCCACGAATAAACAACATGAGC * ** ** ** ***** ** ** *	--CTTCTGACTTCTCCCC-- AGCTTCTGATTACTCCCACC ******* * *****	--GTGTTGAAGCTGGTGCTGGTAC-- GGGGTTGAAGCTGGTGCAGGGACAC * *************** ** **
S. trutta	--CCTTCCCCCGCATAAATAATATAA---- GCATTCCCCCGAATGAATAACATAAGC * ******** ** ***** ****	--CTTCTGACTTCTCCCC-- AGCTTCTGACTCCTCCCTCC ********* *****	--GTGTTGAAGCTGGTGCTGGTAC-- GGAGTTGAAGCCGGCGCTGGCACAG * ******** ** ***** **
P. fluviatilis	--CCTTCCCCCGCATAAATAATATAA-- AGCTTTCCCTCGAATAAATAACATGAGC * ***** ** ******** ** *	--CTTCTGACTTCTCCCC--- AGCTTTTGACTCCTACCCCC *** ***** ** ***	--GTGTTGAAGCTGGTGCTGGTAC--- AGGAGTCGAAGCTGGGGCTGGTACCG * ** ******** ********
C. carassius	--CCTTCCCCCGCATAAATAATATAA-- GCATTCCCACGAATAAATAATATAAGC * ***** ** **********	--CTTCTGACTTCTCCCC-- GCTTCTGATTACTTCCCCC ******* * ** ***	--GTGTTGAAGCTGGTGCTGGTAC-- GGTGTTGAAGCCGGGGGCTGGAACCG ********** ** ***** **
A. anguilla	CCTTCCCCCGCATAAATAATATAA-- CATTCCCCCGAATAAATAATATAAGC * ******** **********	--CTTCTGACTTCTCCCC-- GCTTCTGACTTTTACCCCC ********** * ***	--GTGTTGAAGCTGGTGCTGGTAC-- GGAGTAGAGGCCGGAGCTGGTACA * ** ** ** ** ********
T. tinca	--CCTTCCCCCGCATAAATAATATAA-- GCATTCCCACGAATAAACAATATAAGT * ***** ** ***** *******	--CTTCTGACTTCTCCCC-- GTTTTTGACTCCTACCCCC ** ***** ** ***	--GTGTTGAAGCTGGTGCTGGTAC-- GGTGTTGAGGCTGGAGCCGGGACAC ******* ***** ** ** **

The primers and probe used in the real-time PCR runs for pike were aligned with 6 different species, where all alignments show one or more mismatches between CO1 sequences and primers/probe. The results are presented in table 3-10. Additionally, 10 CO1 sequences of each of the species mentioned in table 3-10, retrieved from the GenBank, were aligned beforehand to look for relevant mutations. There were found a few mutations in relevant areas of *G. aculeatus*, *S. trutta* and *T. tinca*, where the base mutations changed to a base that did not affect the number of similarities between the primers or probe. Furthermore, there were found two base mutations for *S. trutta* in probe area, one mutation in F-primer area and one mutation in R-primer area that would contribute to reduce the number of similarities given in table 3-10. For *P. fluviatilis*, one base mutation in R-primer area, and one base

mutation in probe area were found, which would reduce and increase the number of similarities by one, respectively. Only one base mutation in one of the *T. tinca* sequences had a base mutation that would increase the number of similarities in the F-primer area by one. Nevertheless, all the mutations mentioned above did not increase the number of similarities between the primers/probe and the respective species enough for there to be a full match in any of the cases.

Table 3-11: Multiple sequence alignment of cytochrome b (cytb) sequences of 7 ecologically relevant species, compared with the primers and probe of *G. aculeatus.* The first alignment (*G. aculeatus*) is included for reference. * represent similarity between bases. R-primer is transformed to reverse complementary form to match DNA sequences in the alignments. Clustal Omega (EMBL-EBI) was used for multiple sequence alignments. *E.lucius* and *S.trutta* alignments are missing the final two bases of the F-primer and R-primer.

Species aligned	F-primer	Probe	R-primer
G. aculeatus	--ACGCCACCTTAACACGTTTCTT--- :AACGCCACCTTAACACGTTTCTTTG **********	--GGTGCCACACTTGTT-- :TGGTGCCACACTTGTTCA **********	--TTTCCTTCACCAGACAGGCTCT-- :TTTTCCTTCACCAGACAGGCTCTAA **********
E. lucius	---ACGCCACCTTAACACGTTTC--- \TAACGCAACCCTTACACGATTCTTC **** *** * ***** ***	---GGTGCCACACTTGTT--- [GAGGCGCAACAGTAATTACT ** ** *** * **	---TCCTTCACCAGACAGGCTCT---- 'ATTCCTTCATGAAACTGGCTCAAAC ******* * ** *****
S. trutta	--ACGCCACCTTAACACGTTTC--- CAACGCCACCCTAACACGATTTTT(******** ******* **	--GGTGCCACACTTGTT--- TTGGAGGCGCCCTTGTACA ** * * * *****	--TCCTTCACCAGACAGGCTCT--- ATTTTTACATGAAACCGGCTCTAA * * ** * ** ******
P. fluviatilis	--ACGCCACCTTAACACGTTTCTT--- CAATGCCACCCTCACTCGATTCTTG * ****** * ** ** *****	--GGTGCCACACTTGTT--- :AGGTGCCACCCTCATCCA ******** ** *	--TTTCCTTCACCAGACAGGCTCT-- CTTTTCCTTCATGAAACAGGCTCGAA ********* * ********
C. carassius	--ACGCCACCTTAACACGTTTCTT--- :AACGCAACATTAACACGATTCTTCG **** ** ******** *****	-GGTGCCACACTTGTT-- AGGCGCTACAGTAATTAC ** ** *** * **	--TTTCCTTCACCAGACAGGCTCT :TATTTCTTCACGAAACAGGGTCA ** ****** * ***** **
A. anguilla	--ACGCCACCTTAACACGTTTCTT-- :AACGCCACATTAACCCGATTCTTC ******* ***** ** *****	--GGTGCCACACTTGTT-- ;AGGTGCTACAGTAATTA ***** *** * **	-TTTCCTTCACCAGACAGGCTCT-- TATTCCTCCATGAAACAGGATCAAA ***** ** * ***** **
T. tinca	--ACGCCACCTTAACACGTTTCTT-- :AATGCAACACTTACACGATTCTTCG * ** ** * ***** *****	--GGTGCCACACTTGTT-- ;AGGCGCAACAGTAATTA ** ** *** * **	--TTTCCTTCACCAGACAGGCTCT- \GTTCCCTACATAGGAGATGCTTTA ** *** ** ** * *** *
P. pungitius	--ACGCCACCTTAACACGTTTCTT-- :AACGCTACCCTAACCCGATTCTTTG **** *** **** ** *****	--GGTGCCACACTTGTT--- :TGGAGCCACATTAGTTCA ** ****** * ***	--TTTCCTTCACCAGACAGGCTCT--- CTGTTCCTCCACCAAACCGGCTCAAAC ***** ***** ** *****

Table 3-11 presents the results of the multiple sequence alignments of the primers and probe of three-spined stickleback. Furthermore, 10 cytb sequences of each of the species selected for alignment, were also aligned to check for mutations in relevant areas for primers and probe of three-spined stickleback. Overall, there were only found a few mutations in relevant areas. These include one mutation in one sequence of *P. fluviatilis* in the F-primer area, causing one more similarity in the respective alignment shown in table 3-11. There were also found several mutations for some of the other species on the list in the F-primer and R-primer area, but they would not affect the results as the base changes were still not similar to the complimentary base on the primers.

4. Discussion

4.1 Temporal and Spatial Trends of Northern Pike

The application of the real-time PCR assay detected presence of pike DNA in Gillsvann almost five months after rotenone treatment, which was longer than expected. Nevertheless, pike was last detected in April 2022, suggesting that pike was eradicated from the lake system. There are many proposed reasons to why the DNA of pike persisted in the water for such a long time after the application of rotenone. Rotenone treatment was done in the late fall, prior to freezing, to maximize the rotenone toxicity persistence. At that time, the water temperature is colder, and it influences the efficiency of the biochemical processes that contributes to degradation of genetic material in the water. Since DNA persistence depends on endogenous nucleases, water conditions, UV radiation and decomposers (bacteria and fungi), the rate of decomposition is slower when temperature is cooler and light penetration is limited by season and ice coverage. Furthermore, Gillsvann is closely connected to the tidal currents. Saltwater from the Topdalsfjord enters Gillsvann during high tide and sinks to the bottom of Gillsvann because of the higher density. This likely contributes to a buildup of high-density saline water near the bottom of Gillsvann. A typical freshwater lake has an oxygen depleted zone near the bottom of the lake, due to uptake of dissolved oxygen by decomposers. However, in Gillsvann this zone is also influenced by the differences in density of the water. The high-density water may not mix well with the fresh water above, which can create a physical barrier that limits the exchange of nutrients and dissolved oxygen between the layers. Therefore, when dead fish and organic material sunk to the bottom of Gillsvann after the rotenone treatment, this density stratification may also have been a reason to why degradation of DNA took longer than expected, as conditions may not be favorable for survival of decomposing organisms.

Due to the long persistence time of pike DNA in Gillsvann, a question raised is to what extent the fish in Gillsvann was able to escape the toxicity of the rotenone after the treatment, and how long they were able to survive in the lake. Like explained in the introduction, rotenone toxicity may persist for a month in temperate, cold water systems. Therefore, the final surviving individuals of pike could potentially have managed to survive and escape the toxicity for a few weeks. However, any longer survival would suggest an incomplete eradication. If the eradication efforts were incomplete, and individuals of pike managed to escape to an area that was untreated, it is anticipated that pike DNA detection would persist

throughout the entire monitoring period. Pike can adapt to brackish water and could potentially have used this adaptability to escape the pesticide by swimming out to the fjord through the river outlet in Justvikbukta (Eidsbukta outlet was supposedly blocked by a net). However, as there were no detections of pike DNA after April 2023, this indicates that pike was successfully eradicated and did not recover after the treatment. Although the results from the analysis may indicate extermination, there is still a possibility of surviving individuals in Gillsvann. Surprisingly, a recreational fisherman caught an individual of pike in April 2023, five months after the final eDNA analysis performed in the current survey. This raises questions whether the rotenone treatment was not sufficient to eradicate all individuals, or if the species has migrated to Gillvann recently or been re-introduced by humans after the treatment. Regardless of the background, the species is present in the lake one and a half year after the treatment, which is a misfortune for the ecosystem. Due to lack of positive signals after April 2022 until November 2022, it is important to evaluate the sensitivity of the real-time PCR survey in relation to the water volume and size of Gillsvann. According to Kjærstad et al. (2021), the lake has an area of 1.11 km^2, an estimated water volume of 7 314 030 m^3 and an average depth of 6.6 m. Multiple studies have revealed that the detection probability of the real-time PCR decreases with density of the target species (Wilcox et al., 2016, Moyer et al., 2014, Robson et al., 2016). For instance, Wilcox et al. (2016) found that probability of detecting a stream-dwelling char (*Salvelinus fontinalis*) was 0.18 at densities of one fish per stream kilometer, while the detection probability was > 0.99 at densities of ≥ 3 fish per 100 m). Presuming that there are very few surviving individuals of pike (< 3 per 100 m) after the rotenone treatment, there is a chance that the current survey failed to detect the presence of the species, resulting in false negative results. Additionally, the study performed by Robson et al. (2016) determined a minimum detection of limit of invasive Mozambique tilapia (*Oreochromis mossambicus*) as 1 fish per 400 m^3 after 4 days, which emphasizes the potential of the qPCR to fail to detect fish at lower densities. Furthermore, to distinguish between presence of live fish versus remains of carcasses, it is possible to study presence of RNA fragments in water samples. Environmental RNA (eRNA) degrades more quickly than eDNA, allowing for detection of metabolically active fish in the lake (Yates et al., 2021). eRNA monitoring was not done in the present survey as it was beyond the initial plan. However, an eRNA based survey has the potential to be a valuable method for identifying a more accurate timeframe for eradication and subsequent re-colonization by endemic species and could be of help to evaluate presence of live fish after degradation of rotenone toxicity.

Successful eDNA detection of organisms in freshwater and marine ecosystems also depend on proper design of primers and probes, as well as suitable temperature profiles for PCR analysis. In the present survey, primers and probes were retrieved from papers with already established and approved PCR tests. Although the likelihood of this experimental design to fail to target the desired species was low, testing for specificity by sequencing PCR product from Gillsvann would validate and increase confidence of the results from the analysis. Due to time constraint and lack of resources and experience with traditional PCR, this method did not give adequate results to sequence PCR product. Therefore, an alternative method for evaluating specificity in the relevant ecosystem was proposed. The multiple sequence alignment results (table 3-10) suggest that neither of the primers or probe, who fully match the pike CO1 sequence, would attach to any of the CO1 sequences for the aligned ecologically relevant species. Although this is theoretical, it elevates the confidence that the primers and probe would not produce signal in the PCR runs when no pike eDNA is present. Mutations of the relevant genes were also considered, and the results showed that the chances of mutations of significance were low. The present species-specific primer and probe establishment thus enforces low chances of false positive detections reasoned by poor specificity. Alternatively, to assess specificity, species-specific primers and probes for pike and three-spined stickleback could be used to conduct real-time PCR on ecologically relevant genomic DNA samples. These samples should be appropriately diluted to approximate concentrations similar to the eDNA of the species present in Gillsvann. Negative PCR results would thus indicate absence of false positive reactions resulted by unspecific bindings of the primers and probes. The current survey did not utilize this method because of the unavailability of genomic DNA obtained from the ecologically relevant species.

The spatial distribution of positive detections of pike during the monitoring period was also noteworthy. The highest number of positive detections of pike were observed in November and December 2021. This is likely due to the high concentration of DNA fragments in the water disposed due to decomposition of dead fish. The upstream river samples, 2 and 3, remained negative throughout the whole period, enforcing the confidence of results as these rivers were selected to represent locations with expected negative results. The life-history of pike does not suggest that the species would migrate upstream from Gillsvann. Sample 1, 5, 6 and 8, were all positive prior to treatment like expected since they were taken in areas of Gillsvann known to be preferred habitats for pike. The locations remained positive for the two months after rotenone treatment, but in February 2022 only sample 8 was found to

contain DNA of pike. Surprisingly, in the next month (March 2022), positive detections were observed again in sample 5 and 6, while sample 8 turned negative. A possible reason why some locations appear positive after being detected negative in earlier samples, could be explained by water movements and water exchange within the lake and between the lake and the fjord. In addition, bioturbation by benthic organisms, as well as transportation of water from one location to another by human interactions (e.g., boats, swimming) or other animals (e.g., birds) could influence the spatial detections of genetic material in the surface water.

The water samples connected to the downstream river outlets of Gillsvann were collected during, or close to low tide. This was done to prevent collecting samples representing the content of water from the fjord, rather than the lake. One interesting observation was found in the data from February and March 2022 (table 3-1). Sample location 10 was taken in the beginning of the stream going down do Justvikbukta (7.1, 7.2 and 7.3), while location 11 was taken in the beginning of the stream going down do Eidsbukta (9.1, 9.2 and 9.3). In February 2022, only negative detections were observed in Justvikbukta, but sample 10, closely associated to those samples was positive. Furthermore, Eidsbukta had fewer positive detections in March 2022, while Justvikbukta turned positive. Sample 11, associated to Eidsbukta was negative in February, but positive in March. These results were not as expected, as it would be anticipated that Justvikbukta would appear positive if sample 10 was positive, and similarly Eidsbukta was positive if sample 11 was positive, due to water running from Gillsvann to the respective river outlets during the time of water collection. However, the real-time PCR has a high detection ability, which enables it to detect eDNA even at very low concentrations. The dilution series of the positive eDNA sample and the gDNA sample (section 3.2) suggest that the real-time PCR can detect pike DNA down to a concentration of ~ 2.7×10^{-7} ng/µl in the reaction tube. Therefore, it is possible that the corresponding locations may not simultaneously test positive due to the high rate of water exchange near the streams. Over time, a single pike individual (or degrading carcass) release DNA fragments into the water in low concentrations. The distribution of these fragments may be relatively dispersed and depend on multiple biotic and abiotic factors discussed earlier.

Furthermore, to lower the chances of false negative detections (failure to detect target species when they are present), increasing the volume of water filtered and the pore size of the filters could be considered. Table A-1 (appendix) shows the volume of water that was filtered for each sample taken. At each location about 250 – 1000ml of water was filtered, which means

that some samples were derived from 4x the volume of water than others, due to clogging by organic material. When eDNA is dispersed in such low concentrations in the water body, it could have been advantageous to increase the volume of water filtered by collecting more water at each location. Although this is an added complication, the unexpected results for sample 10 and 11 in February/March described above, did not seem to correlate with lower filtration volumes compared to the other sample locations for the respective months (table A-1, appendix). However, filtered water volume may be of significance when considering the overall eDNA detection probability of the real-time PCR for species of low abundance. One possible solution is to incorporate an extra initial filtration step utilizing a larger pore size filter. This would eliminate larger organic particles present in the water before passing through the small pore size filter utilized in this assay to capture smaller biological material and cells derived from fish. Finally, all negative field controls, as well as blank PCR controls performed during the experiment turned out negative for the pike runs, leaving no indication of contamination.

4.2 Temporal and Spatial Trends of Three-Spined Stickleback

During the entire monitoring period, positive samples of three-spined stickleback were detected. There was an initial increase after the rotenone treatment in early November 2022, followed by a decline a few months later, and a subsequent recovery after approximately six months. The results suggest that the three-spined stickleback population was able to re-establish in Gillsvann half a year after the potential eradication. An complete eradication of the population is subject to debate, because no complete absence of DNA fragments were found in the monthly water samples after the rotenone treatment and before the potential invasion of new individuals. Nevertheless, based on the highly diverse biological and ecological characteristics of three-spined stickleback populations, it is likely that they are anadromous and/or catadromous individuals. This implies that they could have returned to Gillsvann from the fjord in short time after the toxicity of rotenone degraded. Similar to the results of pike, three-spined stickleback DNA detections peaked in November 2021, likely due to the high number of carcasses decomposing and releasing DNA into the environment. Overall, the samples that remained positive in December, February and March are the samples located in Justvikbukta and Eidsbukta, as well as one of the upstream rivers (sample 3), suggesting that these locations are likely to consist of DNA disposed by living individuals. Due to their smaller size relative to pike, three-spined stickleback are likely to

decompose faster, requiring less time for complete decomposition. At present, it is unknown whether a population of three-spined stickleback exists in the upstream lake, Grovann. Yet, the results indicate that three-spined stickleback were present in the associated river (sample 3) between October 2021 and April 2022, as well as in October and November 2022. This suggests that they were either eradicated by the pesticide (carcasses disposed DNA for a few months post-treatment) and subsequently re-established, or that DNA fragments from an upstream lake population were continually dispersing downstream (in addition to the possible carcasses). Furthermore, it is probable that the three-spined stickleback population returned shortly after the rotenone toxicity subsided in the two river outlets downstream of Gillsvann. This is because three-spined stickleback are endemic to the area and presumably already utilized a range of suitable habitats connected to the Topdalsfjord prior to the treatment. It is also possible that some individuals managed to respond to the lack of oxygen in time and escaped to nearby habitats in the fjord, as they can adapt well to the changes in salinity. In contrast, pike can only survive in a higher salinity for a short period of time implying that they would not benefit from such response.

Furthermore, the results from May 2022 indicates a recovery of the population as most sample locations were found to contain three-spined stickleback eDNA. On the contrary, in June 2022, there is a decline in number of locations with positive detections. Only sample locations 8 and 11 were positive, besides the samples collected in the two river outlets. The unexpected change from positive to negative detections may be explained by the biotic and abiotic factors that were discussed for the random distribution of pike DNA detections from February to April 2022. On the contrary, since three-spined stickleback are expected to re-colonize relatively fast, the unpredicted spatial trend in positive detections may also be due to disposal of eDNA fragments by movement of living individuals (e.g., feces) present in Gillsvann. After June 2022, the findings show full recovery in all locations in and around Gillsvann, indicating a successful re-establishment of the population of three-spined stickleback. Overall, the observed variation in eDNA of three-spined stickleback can be readily attributed to seasonal and yearly fluctuations, as well as the species' phenology. This reflects how important it is to fully understand of the biological and ecological characteristics of the specific populations of the species, which is currently a significant gap of knowledge for this diverse species. The lower limit of detection by the real-time PCR, which was estimated and calculated to be ~ 9.18×10^{-5} ng/µl (see section 3.2) allows for catching the variated distribution and patchiness of the three-spined stickleback eDNA fragments in the

lake. The lower detection limit of three-spined stickleback was higher than that of pike (~ 2.7 x 10^{-7} ng/µl). Yet, three-spined stickleback was presumed to be more abundant post-treatment due to rapid re-invasion, suggesting higher probability of detection even with a slightly reduced real-time PCR sensitivity compared to the test for pike.

In the same way as for pike, multiple sequence alignments of three-spined stickleback primers and probe were performed to evaluate the specificity of the established PCR test. The results show that neither of the primers or the probe are fully complimentary to the aligned sequences, other than the three-spined stickleback sequence itself (table 3-11). Therefore, the primers and probes used in this real-time PCR test, would in theory not attach to any of the ecological relevant species selected in this alignment. Likewise, 10 cytb sequences of each ecologically relevant species were studied for possible mutations, but no mutations of significant importance for the PCR test specificity were found. Hence, chances of false positive detections due to failure of primers and probe specificity are low. Additionally, the probe for three-spined stickleback was modified from the original probe in the respective literature. This was because shortening the probe a few bases would result in an optimal melting temperature (Tm) for the probe in the TaqMan PCR, due to the minor groove binder (MGB). If the probe remained the same length (+5 bases) with MGB, there would be a higher Tm. This is not optimal for other molecules in the PCR reaction, and there would be a higher chance for a non-specific hybridization of other unspecific PCR products, which could result in false positive detections. Similar to the pike study, none of the negative field controls or blank PCR controls conducted during the three-spined stickleback survey yielded any positive results, leaving no indication of contamination.

4.3 Future Perspectives

eDNA and real-time PCR provide a powerful tool for monitoring fish populations in real time. The methods offer several advantages over traditional methods of fish monitoring, including non-invasiveness, cost-effectiveness, increased sensitivity, and time efficiency. Like explained previously, eDNA does not require capturing or handling of fish, which reduce the risk of stress, damage and fish mortality. Additionally, collecting water is a much cheaper and time-effective way to collect data compared to methods such as electrofishing or netting. Real-time PCR may increase the power of detection, spatial coverage and enable a higher frequency of aquatic wildlife data sampling and can monitor populations at very low densities. Although eDNA detection has been successful in freshwater systems in recent

studies, it is surprising how well the approach performs on marine water samples. This is considering the larger water-volume to biomass ratio of marine ecosystems compared to freshwater, the effects of sea-currents and wave action, and the impact of salinity on the preservation and extraction of eDNA (Thomsen et al., 2012). These factors suggest that eDNA in marine water is likely less concentrated, more rapidly dispersed, and may be extracted less efficiently from the water column (Thomsen et al., 2012). Put together, this has resulted in a growing interest in the incorporation of eDNA and real-time PCR in strategies for fisheries management and aquatic conservation and will undoubtedly be of great importance in future research.

The use of eDNA for species detection is leading to the accumulation of high amounts of genetic data linked to both temporal and spatial variables, such as seasonal and regional fluctuations. Handling such large volumes of data can pose a significant computational challenge for researchers. To address this issue, it may be an idea to expand database capacities, enhance data management, and establish archives for eDNA data (Díaz-Ferguson and Moyer, 2014). One practical example of relevance would be to keep the DNA extracted from the water samples in Gillsvann, which can serve as a reference for evaluating future population dynamics and structure, as well as for monitoring biodiversity and community composition. Additionally, continuing to monitor the community in Gillsvann is essential to be able to study the long-term effects of the rotenone treatment and how the community structure changes over time. The current study is only able to evaluate re-establishment of one endemic species for one year after the treatment, and new findings indicate either unsuccessful eradication and/or re-introduction of pike in Gillsvann. Therefore, implementing eDNA analysis of additional endemic species, as well as continuing to monitor the lake for pike eDNA, would provide extended knowledge about rotenone as a tool for conservation and the potential of endemic species to recover from a major extermination event. In particular, it would be reasonable to investigate selected samples from before and after rotenone treatment by amplifying specific genomic regions using universal PCR primers, followed by DNA sequencing using next-generation sequencing (NGS) methods. This approach would identify all the species that match these universal primers. The method is known as metabarcoding. Although it may be more costly and time-consuming than real-time PCR (Bylemans et al., 2019), if the number of reactions to be analyzed is not excessively large, this would be an essential step to undertake to study the ecosystem’s response to the treatment. Furthermore, it is well established that invasive species are of emerging concern,

and the current situation suggests that further mitigation strategies must be done as the pike population is still exploiting the habitat of lake. The primary concern and reason why the initial mitigation measure was implemented, was due to the vulnerable local populations of Atlantic salmon and sea trout in Tovdalselva. Hence, it would also be of great interest to expand the area of pike monitoring to this site to confirm that the species is absent from the river.

Improving supporting eDNA technologies such as databases, data management and establishing eDNA archives could provide desirable baselines for researchers in the future. Another emerging area of eDNA interest for researchers is to use this method as a tool for assessing biological events such as spawning, recruitment and settlement by analyzing fluctuations in target eDNA concentrations at the time and place of the particular life-history event (Díaz-Ferguson and Moyer, 2014). However, this requires substantial knowledge about eDNA persistence and PCR sensitivity. Especially for marine systems, this remains a challenge as the volume of water in relation to biomass is very high and contributes to higher rate of dispersion and eDNA dilution. It is crucial to gain a better understanding of the dispersal of eDNA in aquatic population monitoring, particularly with regards to the influence of abiotic factors such as temperature and salinity on the accuracy of results. Although this way of using eDNA surveys remains a challenge, it has great potential for elevated understanding of aquatic community dynamics and structure, which is essential for optimal conservation and management.

5. Conclusion

The eDNA analysis carried out in the present survey revealed that the presence of pike was detected prior to the rotenone treatment of Gillsvann, but the positive signals ceased a few months post-treatment and remained negative for the rest of the monitoring period. Additionally, presence of three-spined stickleback in Gillsvann throughout the entire monitoring period was revealed. In conclusion, the current study demonstrates the potential use of eDNA surveys for evaluating the success of eradication efforts in aquatic ecosystems, further reinforcing previous findings that suggest eDNA analysis are more sensitive than traditional methods in detecting species at low abundance. Despite the possibility of false negative and false positive detections caused by a variety of factors, the implementation of rigorous and carefully followed protocols can effectively minimize their occurrence. Surveys based on eDNA analysis are therefore an important tool to assist in management of non-native species and native species of low abundance, both for early detection and rapid response, as well as for assessing success of eradication and re-establishment. Furthermore, new findings emphasize the challenge of eradicating an invasive pike population in Gillsvann and highlights the necessity for continued monitoring of the lake, as well as the consideration of additional eradication measures.

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Appendices

A. Filtration volumes

Table A-1: Volume of water filtered from the flasks, containing water from Gillsvann for each location every month. Volumes from December 2021 were not recorded. A total of 1 liter of water was collected at each location. All volumes are in milliliters (ml).

Sample #:	30.09.21	28.10.21	24.11.21	22.12.21	02.02.22	04.03.22	06.04.22	09.05.22	16.06.22	08.07.22	12.08.22	19.09.22	11.10.22	09.11.22
1	450	650	250		500	400	700	700	800	900	650	600	600	500
2	350	550	800		700	1000	700	700	800	800	750	650	700	800
3	450	900	750		800	1000	700	800	400	700	800	650	700	700
5	550	900	750		700	700	800	600	800	700	700	700	700	700
6	500	800	1000		700	900	800	700	700	650	650	700	750	600
7.1	350	750	800		700	900	900	600	700	700	750	700	700	500
7.2	350	500	800		600	800	900	800	700	800	700	650	600	500
7.3	400	500	700		600	800	700	700	700	800	700	700	600	700
8	550	900	700		700	800	900	800	600	600	700	650	800	800
9.1	400	600	750		600	700	900	700	700	700	650	750	700	600
9.2	550	600	750		700	800	700	800	700	800	700	650	550	700
9.3	450	750	750		700	800	600	800	700	750	750	750	600	700
10	900	700	650		700	800	900	600	700	850	500	650	700	700
11	500	800	800		700	700	900	700	700	750	600	650	700	800

B. Standard Deviation

Table B-1: Standard deviations (SD) of mean Ct-values for each triplet tested for the target species *E. lucius*. SD was only calculated for Ct-means with two or three positive results in each triplet.

Sample number:	30-sep-21	28-Oct-21	24-nov-21	22-dec-21	2-feb-22	4-mar-22	6-apr-22	9-may-22	16-jun-22	8-jul-22	12-aug-22	19-sep-22	11-oct-22	9-nov-22
1	0.74	1.01	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.88	0.59	0.04	0.82	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	0.93	0.50	0.07	1.81	0.00	0.43	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7.1	0.00	0.00	0.23	1.54	0.00	3.64	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7.2	0.00	0.20	0.14	0.14	0.00	0.62	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7.3	0.00	0.00	0.44	0.87	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	0.35	0.46	0.08	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
9.1	0.00	0.00	0.19	0.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
9.2	0.28	0.00	0.39	0.69	0.32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
9.3	0.00	0.00	2.16	1.65	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	0.15	0.00	0.10	0.90	0.00	0.97	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
11	1.34	0.00	0.11	0.76	0.00	0.70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table B-2: Standard deviations (SD) of mean Ct-values for each triplet tested for the target species *G. aculeatus.* SD was only calculated for Ct-means with two or three positive results in each triplet.

Sample number:	*30-sep-21*	*28-Oct-21*	*24-nov-21*	*22-dec-21*	*2-feb-22*	*4-mar-22*	*6-apr-22*	*9-may-22*	*16-jun-22*	*8-jul-22*	*12-aug-22*	*19-sep-22*	*11-oct-22*	*9-nov-22*
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.58	0.65	0.40	0.37	0.63
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.51	0.00	0.00	0.00	0.44	0.00	0.00	0.00	0.00	0.00	0.43	0.00
5	0.00	1.23	0.14	0.00	0.00	0.00	0.00	0.29	0.00	0.33	0.92	0.81	0.88	0.53
6	0.00	0.07	0.55	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.39	0.15	0.25	0.08
7.1	0.45	0.85	0.39	0.00	0.52	0.00	0.00	0.19	0.22	0.07	0.34	0.41	0.05	0.00
7.2	2.04	1.53	1.12	0.00	0.18	0.02	0.29	0.42	0.04	0.63	0.20	0.49	0.38	0.00
7.3	0.55	4.61	0.35	0.38	0.85	0.35	0.00	0.41	1.30	0.58	0.16	0.34	0.55	0.00
8	0.00	0.00	0.55	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.50	0.54	0.47	0.18
9.1	0.65	0.11	0.01	0.00	0.18	0.00	0.18	0.27	0.28	0.58	0.13	0.07	0.30	0.21
9.2	1.08	0.30	0.36	0.00	1.78	0.40	0.00	0.80	0.00	1.41	0.23	0.48	0.14	0.13
9.3	0.43	0.89	1.26	0.00	0.00	0.50	0.00	0.14	0.00	0.39	1.06	0.11	0.90	0.49
10	0.40	0.35	0.58	0.00	0.00	0.00	0.00	1.46	0.00	1.33	0.67	0.28	0.51	0.38
11	1.54	0.52	1.03	0.00	0.00	0.00	0.00	0.79	0.20	0.47	0.87	0.67	0.29	0.28

C. DNA purity

Table C-1: DNA concentration (ng/µl) and ratios of wavelength absorption form Nanodrop one measurements. Sample 1-11 from September 2021 to February 2022. Sample 1 and 11 from December 2021 were missing from the sample collection and not measured.

	30-sep-21			*28-oct-21*			*24-nov-21*			*22-dec-21*			*2-feb-22*		
Sample	**ng/µl**	**A260/ A280**	**A260/ A230**	**ng/µl**	**A260/ A280**	**A260/ A230**	**ng/µl**	**A260/ A280**	**A260/ A230**	**ng/µl**	**A260/ A280**	**A260/ A230**	**ng/µl**	**A260/ A280**	**A260/ A230**
1	57.4	2.04	1.06	23.4	2.15	1.45	22.9	2.06	1.04				26.5	1.95	0.71
2	15.5	1.84	0.53	11.1	2.25	0.56	11.8	2.23	0.71	6.6	2.95	0.28	9	2.09	0.24
3	26.6	2.03	1.04	12.5	2.44	0.99	26.4	1.78	0.85	6.9	3.15	0.61	8.7	2.26	0.25
5	18.1	2.05	1.93	17.7	2.32	0.73	32.5	2.13	1.38	39.4	2.18	1.34	32.4	1.94	0.71
6	10.8	2.05	209.7	47.6	2	1.52	51.2	2.06	1.71	67.2	2.07	1.07	20.5	1.89	1.43
7.1	14.3	1.77	1.44	28.9	1.85	1.29	37.3	2.15	1.13	20.8	2.08	0.75	21.4	1.89	0.46
7.2	12.7	1.79	0.53	33	1.85	0.75	20.8	2.18	1.13	17.6	2.13	1.34	23	1.75	0.55
7.3	21.1	1.73	0.97	13.1	2.13	0.68	64.9	1.91	1.27	26	1.94	0.52	20.7	1.59	0.59
8	13.3	2.09	0.82	22.4	2.06	0.84	18.7	2.35	1.34	41.6	2.26	1.95	22.3	1.8	1.27
9.1	57.3	1.91	1.34	31.8	1.94	0.83	38	2.12	1.55	27.7	2.1	1.2	294.4	1.35	0.77
9.2	27.4	1.94	0.97	27.3	1.94	0.91	24.9	1.84	0.72	24.4	1.97	0.95	31.71	1.71	1.23
9.3	23.1	1.83	1.11	27	2	0.64	18.2	1.94	0.99	25.3	2.03	1.19	26.6	1.72	1.06
10	100.2	1.9	1.17	39.2	1.82	0.92	127	1.96	1.53	24.7	2.11	1.89	32.9	1.86	1.25
11	16.2	1.79	0.62	25.4	1.96	0.96	30.5	2.18	1.67				39.3	1.85	1.41

Table C-2: DNA concentration (ng/µl) and ratios of wavelength absorption form Nanodrop one measurement. Sample 1-11 from March 2022 to July 2022

	4-mar-22			*6-apr-22*			*9-may-22*			*16-jun-22*			*8-jul-22*		
Sample	**ng/µl**	**A260/ A280**	**A260/ A230**	**ng/µl**	**A260/ A280**	**A260/ A230**	**ng/µl**	**A260/ A280**	**A260/ A230**	**ng/µl**	**A260/ A280**	**A260/ A230**	**ng/µl**	**A260/ A280**	**A260/ A230**
1	5.7	1.96	0.41	33.2	1.74	1.04	17.7	1.76	0.91	27.5	1.89	1.53	35.6	1.86	1.4
2	18.5	1.79	0.85	19.2	1.66	1.08	9.9	1.64	0.94	14.6	1.68	0.77	12	1.69	0.75
3	17	1.75	0.61	10.2	1.58	1.86	7.5	1.7	-4.53	6.3	1.97	0.28	11.1	2.08	0.53
5	35.2	1.86	1.84	94.7	1.82	1.36	32.5	1.71	0.95	17.9	2.03	0.6	31.1	1.9	0.99
6	52.7	1.83	1.34	60.3	1.83	1.45	21.9	1.66	0.96	24.1	2.08	1.51	27.2	2.07	1.33
7.1	39.5	1.82	1.05	58.7	1.73	1.09	65.5	1.62	0.85	35.7	2.07	1.03	34.6	2.03	1.09
7.2	25.3	1.72	1.11	36.4	1.66	0.87	62.1	1.69	0.87	29	2.24	1.35	61.8	2.05	1.76
7.3	16.8	1.57	1.05	25.8	1.54	0.72	55.1	1.63	0.93	18.4	2.43	1.6	54.4	2	0.95
8	26.3	1.62	0.81	46.8	1.81	1.14	24.3	1.76	1.02	34.3	2.12	1.41	38.8	1.99	1.26
9.1	19.2	1.67	0.45	90.2	1.82	1.46	45	1.65	0.89	31.5	2.05	1.12	26.6	2.07	1.33
9.2	20.1	1.7	0.41	33.9	1.58	0.69	44.5	1.59	0.84	19.9	2.26	0.99	48.6	2.08	2.01
9.3	29.9	1.65	0.63	67.8	1.59	1.02	35.2	1.63	0.94	31.6	2.2	1.15	50.3	2.06	1.51
10	26.6	1.69	1.01	72.3	1.77	1.09	21.4	1.87	1.46	17.6	2.3	1.05	40.2	1.99	0.98
11	33.2	1.74	0.93	47.3	1.68	0.86	28.4	1.76	1.08	20.2	2.24	1.07	40	1.98	1.18

Table C-3: DNA concentration (ng/µl) and ratios of wavelength absorption form Nanodrop one measurement. Sample 1-11 from August 2022 to November 2022.

	12-aug-22			*19-sep-22*			*11-oct-22*			*9-nov-22*		
Sample	**ng/µl**	**A260/ A280**	**A260/ A230**	**ng/µl**	**A260/ A280**	**A260/ A230**	**ng/µl**	**A260/ A280**	**A260/ A230**	**ng/µl**	**A260/ A280**	**A260/ A230**
1	18.1	1.9	0.6	19.4	2.02	1.16	27.9	1.88	1.13	37.8	1.81	1.2
2	9	1.76	0.25	22.7	1.8	0.84	24.8	1.74	0.72	14.8	1.71	0.67
3	14.7	1.75	0.31	24	1.88	1.21	14.4	1.81	0.8	11.9	1.71	0.79
5	12.3	1.87	0.33	30.1	1.91	1.3	34	1.95	1.1	41.9	1.88	1.14
6	14.7	1.84	0.59	21.7	1.98	1.5	30.8	1.95	1.16	32.5	1.83	1.21
7.1	19.3	2.07	1.12	35.3	1.87	1.25	32.9	1.75	0.88	27.1	1.58	0.68
7.2	22.5	1.91	0.94	25.8	1.88	1.23	61.6	1.56	0.73	31	1.6	0.73
7.3	19	2.08	0.58	17.8	1.91	1.02	69.8	1.52	0.73	26.8	1.63	0.65
8	8.5	2.1	0.75	24.3	1.89	1.33	31.2	2	1.21	43.6	1.87	1.02
9.1	14.3	2	0.8	18.5	1.92	1.03	24.4	1.96	0.97	58.8	1.88	1.25
9.2	37.2	1.97	1.34	25.2	1.9	0.65	38.3	1.59	0.68	52.8	1.59	0.82
9.3	22.7	1.9	0.63	30.8	1.78	0.95	51.7	1.56	0.66	26.5	1.68	0.71
10	17.9	2.03	1.27	31.8	1.93	0.8	29.7	1.96	1.04	44.4	1.95	1.17
11	15.5	2.08	1.28	32.6	1.84	0.75	24	2.02	0.71	49	1.94	1.42
N.C..				2	1.91	0.21	1.5	1.48	0.15			

D. Composition of Buffers

Table D-1: Composition of buffer solutions prepared in the lab

Buffer	**Composition**
50X TAE Electrophoresis Buffer (1X Buffer)	40 mM Tris 20 mM acetic acid 1 mM EDTA
Loading dye buffer	50% Glycerol 50% 10mM TrisHCl pH 8