Exploring freshwater challenges for conservation effort

Silje Halvorsen

UiA University of Agder

Doctoral Dissertations at the University of Agder 490

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Exploring freshwater challenges for conservation efforts: Insights into threatened and invasive fish species using environmental DNA

Dissertation for the degree philosophiae doctor (ph.d.)

University of Agder

Faculty of Engineering and Science

2024

Doctoral dissertations at the University of Agder, no: 490

ISSN: 1504-9272

ISBN: 978-82-8427-207-8

Printed by Make!Graphics

Kristiansand

Acknowledgements

Throughout the course of my doctoral studies, I encountered some milestones, both academic and personal. Among research and writing, I also welcomed the arrival of my two children, Ylva and Synne. Balancing the responsibilities of parenthood alongside the PhD presented its own set of challenges, but these experiences have taught me valuable lessons in time management and the importance of maintaining a sense of balance in life. Despite the potential obstacles one could expect to meet during a PhD-project, most things have fallen into place quite well. I am thankful for the opportunities and growth afforded to me during my years at the University of Agder.

In presenting this thesis, I am reminded of the effort and support I have had throughout this journey. First, I am grateful for the guidance of my two supervisors, Associate professor Audun Slettan and Associate professor Lars M. Korslund. Together, we form a good team characterized by open communication, not only about our projects but also about a wide range of topics. Audun has been my main supervisor for my bachelor's, master's, and PhD projects. He not only has a lot of knowledge, but also always maintains a calm and supportive character. It seems like he can never be stressed, no matter how big of a mistake his students (or himself) make in the lab, which is quite inspiring. Audun saw potential in me that I sometimes overlooked, and his support has been invaluable every step of the way. Furthermore, Lars has an incredible amount of energy and a huge capacity for doing almost anything within biology. Throughout the processes of planning projects, conducting fieldwork, and writing, he has consistently demonstrated an ability to recall every detail. His contributions have prevented drawbacks and been important for the success of our projects.

I am also deeply thankful for the support and assistance of friends, family, and colleagues throughout my time in the field. Special thanks to Kurt Johannesen and Per Øyvind Gustavsen for their assistance with electrofishing and capturing and handling eels. I am also grateful to Mikal Nilsen, Stian B. Stoveland, Fredrik Gustavsen, Tore Halvorsen, and Øystein S. Jørgensen for supporting me in the boat during sample collection along the coast. I would also like to express my gratitude to Eirik S. Jørgensen and Elise Rustad for assistance with water sample collection. Thanks also to Morten Mattingsdal for his contribution in handling DNA-sequencing data. Finally, thanks to Hanne Kloster, Stian B. Stoveland and

Henriette H. Hultmann for their consistently positive attitude, which have greeted me every day in the office. All these contributions were crucial for the completion of this PhD thesis.

Kristiansand, June 2024.

Silje Halvorsen

Sammendrag

Ferskvannsøkosystemer trues av habitatendringer, menneskeskapte barrierer, fremmede arter, forurensning, overfiske og klimaendringer. Selv om internasjonale lover og forskrifter støtter bevaring, kreves det fortsatt betydelig innsats for å gjenopprette og opprettholde en "god økologisk status" i ferskvannsmiljøer. Dette inkluderer økt forskning og overvåking.

Den europeiske ålen (*Anguilla anguilla*) er kritisk truet. En av utfordringene denne arten står overfor er vandringshindre, som begrenser dens habitat. Ved bruk av miljø-DNA analyser, har vi undersøkt hvorvidt ulike menneskeskapte barrierer hindrer oppvandring av ål i fire store elvesystemer. Våre analyser viser at vannkraftverk begrenser ålenes oppvandring. Dette understreker behovet for effektive forvaltningsstrategier som tar hensyn til artens behov for åpen tilgang til dens habitater.

I arbeidet med bevaring av truede fiskearter, er kunnskap om populasjons-størrelse essensielt. Vi ønsket å undersøke om det var mulig å bruke miljø-DNA fra vannprøver til å estimere antall individer av Europeisk ål. Vi undersøkte D-loop regionen i mitokondrie-DNA for å skille mellom ulike haplotyper. Vannprøvene ble hentet både i et kontrollert akvatisk miljø med kjente individtall, og fra bekker med ukjent individtall. Resultatene viste at det var mulig å telle antall åleindivider, både i kontrollmiljøet og i bekkene. Dette betyr at miljø-DNA i vannprøver kan gi genetisk informasjon om ål, men ytterligere forskning er nødvendig for å utvikle metoden til et kvantifiseringsverktøy.

Fremmede arter kan utgjøre en stor trussel for stedegne arter og hele økosystemer. Rotenonbehandling er en vanlig metode for å utrydde fremmede fiskearter i ferskvann. Likevel mangler det forskning som spesifikt adresserer effekten av en slik behandling på stedegne fiskearter, noe som er viktig for helhetlig økosystemforvaltning og bevaringsarbeid. I dette studiet undersøker vi de økologiske konsekvensene av en rotenonbehandling med formål om å utrydde den fremmede arten gjedde (*Esox lucius*) i en innsjø. Vi fokuserte på fire stedegne fiskearter med ulike livsstrategier: gjedde, europeisk ål, trepigget stingsild (*Gasterosteus aculeatus*) og ørret (*Salmo trutta*). Ved bruk av miljø-DNA (eDNA) fra vannprøver, overvåket vi disse artene over tid (før, under og etter behandling). Våre funn indikerer en vellykket, uassistert reetablering av de stedegne fiskeartene, samt utryddelse av gjedde.

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1. Introduction

1.1 Threatened freshwater ecosystems

The human impact on Earth presents a triple challenge of reducing climate change, improving the lives for a growing human population and halting biodiversity loss (Mace et al., 2018). Nowhere is the biodiversity crisis more acute than in freshwater ecosystems (Reid et al., 2019). Freshwater biodiversity faces a more severe decline compared to terrestrial ecosystems (Grooten & Almond, 2018; Reid et al., 2019) because of a multitude of threats, including habitat alteration, invasive species, pollution, overfishing, and climate change (Barrett et al., 2018).
The degradation and destruction of these systems may stem from a lack of understanding regarding the ecological functions of the services they offer and the tendency of managers to prioritize economic gains from a narrow range of services (Finlayson et al., 2018; Tickner et al., 2020). Despite the vital role freshwater ecosystems play, our current data on their status highlight the urgent need for more extensive research and monitoring efforts (Miranda, Hermoso, & Hassall, 2019). While international legislation has laid the groundwork for the protection and management of European inland waters, the journey towards achieving a “good ecological status” by 2027 is an ongoing challenge (Ferreira, Globevnik, & Schinegger, 2019).

1.2 Declines in freshwater fish species

Freshwater species are those who live all, or a critical part, of their life in freshwater or brackish estuaries (Arthington et al., 2016). By this definition, freshwater species make up 48% of all fishes and 25% of all vertebrates (Eschmeyer, 2016). Despite covering only 0.8% of the Earth’s surface, freshwater ecosystems support 10% of all species and approximately 15,000 fish species (Dudgeon et al., 2006; Strayer & Dudgeon, 2010). Freshwater fish are among the most threatened animal groups (Clavero et al., 2010) despite their significant social, cultural and economic importance (Cowx, 2015; Miqueleiz et al., 2020). Their population declines can be attributed to a range of factors,

including climate change, pollution, fishing, invasive species, and habitat loss (Barbarossa et al., 2020; Miqueleiz et al., 2020).

1.3 Invasive fish species

An invasive species is a non-native organism introduced to an ecosystem, typically causing environmental and economic damage, or posing a threat to human health (Beck et al., 2008). Freshwater ecosystems are particularly vulnerable to the introduction of invasive species (Moorhouse & Macdonald, 2015). Globally, 551 invasive freshwater fish species have been recorded as established (Bernery et al., 2022).

Invasive fish species can find their way into other aquatic habitats on their own, but human activities often facilitate their spread, directly or indirectly. For instance, if a fish species can tolerate higher temperatures than others, they may expand their habitat and outcompete native species as temperatures increase (Rahel & Olden, 2008; Zerebecki & Sorte, 2011). Directly human caused establishment of invasive fish species can result from for example escape from aquaculture (Consuegra et al., 2011), stocking for fisheries (Gozlan et al., 2010), or ballast transport (Wonham et al., 2000). The release of fish for sport fishing, game fishing, or as bait for angling is also a significant pathway for introducing fish species (Gozlan et al., 2010).

Invasive species can have detrimental impacts on native species, especially threatened species, such as the European eel (*Anguilla anguilla*). In addition to being a prey for fish predators, these impacts can for example include direct competition for food and habitat (Fletcher et al., 2019; Hata, Uemura, & Ouchi, 2021; Van Kessel et al., 2011) or introduction of diseases and parasites (Gilbert & Granath Jr, 2003; Skerratt et al., 2007).

In efforts to eradicate invasive fish species, rotenone treatments are commonly applied (Beaulieu et al., 2021; Recsetar & Bonar, 2015; Sandodden et al., 2022). In correct concentrations, fish will typically die within hours after treatment, affecting both target- and non-target species (Ling, 2003). Consequently, native fish will be adversely affected, leading to temporary or long-term declines in biodiversity and ecosystem function. However, the alternative of allowing invasive species to persist can have significant consequences, potentially causing long-term ecological damage and further

declines in native species. In such scenarios, the short-term negative impacts on native species may be better in order to restore the balance of the ecosystem (Woodford et al., 2013).

1.4 Migration barriers

Migration barriers, whether natural or man-made, represent physical or environmental obstacles that disrupt the natural movements of fish within waterways. Globally, the proliferation of riverine barriers, such as hydroelectric power stations and dams, is on the rise, transforming once continuous riverscapes into fragmented habitats (Silva et al., 2018). When fish encounter these barriers during their migratory journeys, they face significant challenges in accessing crucial habitats for spawning, feeding, and completing their life cycles (Nyqvist et al., 2017; Piper et al., 2017). These barriers can also lead to fragmented fish populations, reduced gene flow, and ultimately contributing to declines in population abundance and genetic diversity (Silva et al., 2018; Van Leeuwen et al., 2018).

1.5 The endangered European eel

The European eel has intrigued scholars since Greek antiquity, with its mysterious nature and life cycle posing questions even to Aristotle, as evidenced in his works "Animal History" and "Animal Generation" (Dufour et al., 2003). Even today, there are gaps of knowledge about their migration routes and behaviour (Wysujack et al., 2014). The *Anguilla* genus originated over 50 million years ago during the Eocene period (Tsukamoto & Aoyama, 1998). The European eel emerged as a distinct species around 3 million years ago, alongside 15 other species within the *Anguilla* genus (Tsukamoto & Arai, 2001). Throughout their existence, the *Anguilla* genus has withstood significant environmental changes on Earth, including the ice age around 22,000 years ago and continental drift, which likely altered the distance between their growth habitat and

spawning areas (Knights, 2003). Despite its remarkable adaptability and robustness, the fact that the stock has nearly depleted in recent years raises questions.

The European eel is a facultative catadromous fish species that primarily spawns in the sea and undertakes feeding migrations in freshwater. However, some eels skip the freshwater phase entirely or shift between fresh, brackish, and saltwater throughout their growth phase (Capoccioni et al., 2014; Durif et al., 2023). Found in Europe and North Africa, from Norway to Morocco, including the Mediterranean and Baltic Seas (Bornarel et al., 2018), these eels have a unique life cycle. Eels spawn in the Sargasso Sea, 5000 km from Europe and North Africa (Righton et al., 2016). Migration starts between August and December, with spawning in early December (Righton et al., 2016). After spawning, the parents die, and the larvae return to the coastlines. During migrations, both adults and larvae are guided by Earth's magnetic fields (Durif et al., 2022; Moore & Riley, 2009). The leptocephalus larvae metamorphose into glass eels before entering continental waters, and later into yellow eels (Vøllestad, 1992). In freshwater, they're secretive, foraging at night (Baras et al., 1998). After years, yellow eels metamorphose into silver eels for the return journey to the Sargasso Sea (Wysujack et al., 2014).

Since the 1980s, the European eel stock is reduced by approximately 90%, leading to a critically endangered status on the IUCN Red List (Jacoby & Gollock, 2014). This decline is attributed to a combination of factors, including overfishing and aquaculture (Castonguay et al., 1994), parasitic infestations (Feunteun, 2002), contamination (Belpaire et al., 2009), changes in sea conditions and global warming (Drouineau et al., 2018; Friedland, Miller, & Knights, 2007), as well as the destruction of their habitats and the construction of dams and barriers (Halvorsen et al., 2020; Kettle, Vollestad, & Wibig, 2011). The surge in freshwater construction during the 1950s and 1960s have resulted in the loss of approximately 50-90% of eel habitats across Europe (Feunteun, 2002; Tesch, 1977). Migration obstacles that are taller than 50-60% of an eel’s body length, or that create high flow rates or velocities, can hinder their upstream movement (Porcher, 2002; Thorstad et al., 2010). Examples of these barriers include fish ladders, pipelines, basins, and hydroelectric power stations. The modification of freshwater environments by humans presents a significant threat to the European eel, yet it is a challenge that can be addressed through effective management strategies.

Understanding the population genetics of fish species is crucial for effective conservation management. The European eel comprises a single population (Palm et al., 2009), distributed across various habitats, each with unique environmental pressures and threats. Accurate estimates of the number of individuals in different areas are vital for informed conservation efforts, enabling targeted interventions and habitat protection measures. Additionally, such estimates can help monitor the effectiveness of conservation strategies and adapt them as needed to ensure the survival of this critically endangered species.

1.6 Environmental DNA: the fastest growing biomonitoring tool

Biological monitoring is important to assess the status of ecosystems, detecting presence of invasive species, characterize biological diversity and investigating environmental contamination. Rapid technological advancements have resulted in a range of alternative techniques for monitoring biodiversity, for example acoustic sensors (Føre et al., 2017), underwater video systems (Cappo, Speare, & De'ath, 2004) and robotic samplers (Schwarzbach et al., 2014). However, one approach has experienced significant growth in recent times: environmental DNA (eDNA). eDNA is genetic material obtained directly from environmental samples such as soil, water, or air, rather than from an individual organism (Ogden, 2022). It is used to detect and monitor species presence and biodiversity without needing to observe or capture the organisms directly. Because of its time efficiency and sensitivity, eDNA analysis is now the fastest growing biomonitoring tool (Takahashi et al., 2023).

The methodology originally developed in the 1980s for detection of bacteria in marine sediments (Ogram, Sayler, & Barkay, 1987), while applications in water started to develop in the 2000s, studying eukaryotes and procaryotes in fecally contaminated surface water (Layton et al., 2006; Martellini, Payment, & Villemur, 2005). With the development of high throughput sequencing, eDNA methods detecting invasive species in freshwater (Ficetola, Taberlet, & Coissac, 2016) and monitoring of marine mammals (Thomsen et al., 2012) emerged. Because of the enormous benefits for biomonitoring, the development of eDNA methods has been described as a “quiet revolution transforming conservation” (Takahashi et al., 2023).

While traditional methods such as fishing or traps have historically been chosen for species detection in aquatic environments, the advent of eDNA approaches presents a non-invasive alternative. These methods allow for the sampling of genetic material without causing significant damage to the species or their habitats (Antognazza et al., 2019). Moreover, eDNA techniques are often more sensitive and economically advantageous compared to traditional methods (Itakura et al., 2019; Thomsen & Willerslev, 2015), while also being capable of detecting rare and elusive species (Takahara et al., 2020).

While eDNA analysis offers several advantages, it is crucial to understand the potential limitations of this methodology. eDNA longevity is influenced by various factors, including environmental conditions, time of year, and biotic factors (Lacoursière-Roussel et al., 2018). Therefore, eDNA can persist in the environment from a few hours to several months after being released (Pilliod et al., 2014). Because eDNA may be in the environment even after the target organism is no longer present, presence/absence data can be misleading (Song, Small, & Casman, 2017). Our current understanding of how eDNA persist under different environmental conditions for different species is limited, but this information is critical for deciding on the most appropriate time window to conduct eDNA surveys (Beng & Corlett, 2020). Imperfect sampling and false detections can occur and give false positives (eDNA is detected in the absence of target organisms) (Ficetola, Taberlet, & Coissac, 2016) or false negatives (eDNA is not detected but the target organism is present) (Morin et al., 2001).

Another limitation with eDNA surveys is obtaining accurate information about species abundance or biomass, which is sometimes possible but often difficult (Doi et al., 2015; Halvorsen et al., 2023; Pilliod et al., 2013; Takahara et al., 2012). Additionally, eDNA does not provide detailed information about a species' ecology, such as life history, sex ratio or breeding status (Evans et al., 2017). However, ongoing research on eDNA methodologies suggests that it may become possible to obtain more detailed genetic information through eDNA analysis in the future.

1.7 Variations in the eDNA methodology

Along with the expansion of eDNA approaches, multiple sampling and laboratory techniques are developed, and eDNA is becoming more method-diverse (Takahashi et al., 2023). While this diversity might seem risky due to the lack of common methodological standards, it also provides the flexibility to tailor methodologies to specific projects, which can be advantageous (Goldberg et al., 2016). Implementing a careful sampling plan that selects the most efficient eDNA sampling protocol is crucial for optimizing the efficiency of eDNA capture techniques (Muha et al., 2019). For example, Jeunen et al. (2019) identified an optimal protocol for seawater samples: filtering seawater through cellulose-nitrate membranes followed by extraction with Qiagen's DNeasy Blood & Tissue Kit. This method outperformed the other combinations of capture and extraction techniques tested. Further, the different steps in the methodology, based on water samples, will be discussed.

Water volume

Sampling a larger volume of water usually finds more biodiversity, but the volume sampled must be manageable. Most researchers collect samples ranging from 500 to 1000 mL, with many preferring 1000 mL (Takahashi et al., 2023). This volume is efficient and effective for detecting various species (Andruszkiewicz et al., 2017). Increasing the water volume may be needed when expecting low DNA concentrations, but this can clog filters, requiring pre-filtration with larger pore-sized filters (Takasaki et al., 2021), which again usually reduces DNA yield (Majaneva et al., 2018).
Having more replicates in the field generally improves species detection. By 2021, over 60% of studies used 1-3 replicates (West et al., 2020). Combining multiple subsamples into one can better represent the area (Mena et al., 2021), balancing logistical concerns with study goals.

Water sample preservation

Filtering water samples on-site has become more common since 2012, favoured for space efficiency and protection of eDNA fragments (Takahashi et al., 2023). Alternatively, water samples can be chilled, frozen, or preserved with chemicals during transport to minimize eDNA degradation sample (Bylemans et al., 2018; Foote et al.,

2012; Harper et al., 2019). Benzalkonium chloride is increasingly used as an antimicrobial agent, significantly reducing eDNA degradation (Takahara et al., 2020). However, keeping collected water samples at room temperature, storing in a fridge or on ice seem to have become favourable strategies (Takahashi et al., 2023). DNA copy numbers decrease significantly within 24 to 48 hours post-collection, highlighting the importance of filtering within 24 hour (Hinlo et al., 2017; Holman, Chng, & Rius, 2022).

Filtration method and filter type

In water samples, both cells containing eDNA and free-floating eDNA exists. eDNA is captured through filtration using pumps or syringes (Takahashi et al., 2023). Glass fibre and cellulose filters are common, with pore sizes typically less than 1.5 micrometres, but 0,2 and 0,45 micrometres are most common. These will collect cells containing eDNA, not free-floating eDNA. Filters with small pore sizes may become clogged by particles, like hummus, in the water, so the optimal pore size depends on the specific study and site requirements (Takahashi et al., 2023).

Filters are typically stored in freezers at temperatures of -20°C and -80°C (Friebertshauser et al., 2019). Alternatively, ethanol can be used for storage, although its flammable nature can pose transportation hazards (Marquina et al., 2021). Other methods include lysis buffers and biodegradable self-preserving filters (Thomas et al., 2019).

DNA extraction

The DNeasy Blood & Tissue and MoBioPowerWater kits from Qiagen are among the most frequently employed kit-based extraction methods (Takahashi et al., 2023). However, the PowerSoil kit from Qiagen has been noted for its ability to inhibit humic acids and has demonstrated promising results when used with water samples from turbid environments (Kumar, Eble, & Gaither, 2020).

Negative controls

A negativ control is a sample processed and analysed alongside the environmental samples in the same manner. Its purpose is to detect any contamination that may occur

during the sampling or analysis process. There is a lack of consensus on which negative controls should be included in the study. Apart from the widely recognized negative PCR-controls, various types of negative controls exist. These include sampling controls (clean water exposed at a site and filtered alongside samples), no-target controls (water collected from a location known not to contain target species), bleach controls (bleach used to clean filtration equipment), rinse water controls (water used for cleaning filtration equipment), and extraction controls (pure water processed alongside actual samples) (Takahashi et al., 2023). In real-time PCR studies, the results should be discarded if negative samples are amplified (Goldberg et al., 2016).

2. Objectives

This doctoral research project focus on examining some of the challenges associated with the degradation of freshwater ecosystems, particularly concerning the threats posed to endangered fish species and the proliferation of invasive species. The project comprises three distinct studies, each addressing different aspects of freshwater ecosystem conservation and management.

The European eel are considered as a single panmictic population (Palm et al., 2009), meaning each country where they're distributed bears the responsibility to set management plans aimed to reverse the declining trends, for example by fishing restrictions and facilitate for migration in freshwater systems. Norway's landscape features numerous steep waterfalls within its river systems, leading to the proliferation of hydroelectric power stations and dams. Such constructions could be a problem for fish migration. The first study (paper 1) aims to examine if there is a correlation between migration barriers and the decreasing occurrence of European eels (*Anguilla anguilla*) in distance from the sea along the southern Norwegian coast. The results could be important for the Norwegian management in making informed decisions about facilitating the upstream migration of European eels past human made barriers.

Estimating population size is also essential for managing endangered fish stocks, but it can be challenging to accurately determine. While various methods exist to gather this information, such as physical capture using nets, traps, or other equipment, these approaches can be time-consuming and potentially harmful to the organisms involved. Considering these limitations, the second study (paper 2) aims to examine if it is possible to use eDNA extracted from water samples to estimate the number of European eels present at specific locations.

In addition to human made barriers, invasive species can be a threat to native fish species in a freshwater ecosystem. The final study (paper 3) aims to investigate the ecological effects of a Northern pike (*Esox Lucius*) eradication effort in a lake. By examining the responses of some key fish species; Northern pike, European eel, Three-spined Stickleback (*Gasterosteus aculeatus*), and Brown trout (*Salmo trutta*), following a rotenone treatment, this study aims to contribute insights to guidance for the management of diverse aquatic ecosystems.

Through these interconnected studies, this doctoral research project aims to advance our understanding of freshwater ecosystem dynamics and contribute to the development of effective conservation strategies for vulnerable fish species.

3. Results and discussion

3.1 Migration barriers are decreasing the occurrence of European eel in distance from the sea

We have shown that in general, as distance from the sea to a given location decreases the probability to record European eel eDNA increases (paper 1). In addition, the probability of detecting eDNA from European eel decreases significantly with number of hydroelectric power stations and their water basins, even when the effect of distance to sea is accounted for. The probability of detecting eDNA from eels upstream of seven of such barriers are close to zero. Therefore, these constructions seem to constitute upstream migration challenges for eels.

European eels may navigate some power stations and water basins upstream, but encounter barriers at others, likely influenced by structural variations and individual characteristics. Differences in design, including wall height in water basins, pose varying challenges for migrating eels. Additionally, certain power stations offer alternative routes, like nearby streams or fish ladders, while others do not. Individual factors, such as body size, further influence the ability of eels to overcome these barriers.

3.2 Estimating number of European eel individuals using environmental DNA and haplotype count

Knowledge about population size is important for managing fish stocks for conservation purposes, yet it can be challenging to estimate. However, a new approach focusing on the DNA sequence differences between individuals using eDNA has gained interest. In this study (paper 2) we experimentally tested whether eDNA haplotype information from the mitochondrial D-loop region collected from water samples with a known number of eels was consistent with genomic haplotype information obtained from tissue samples of different eel individuals. Additionally, we analysed eDNA from water samples collected in three rivers to estimate the number of European eel individuals based on their haplotypes.

We successfully identified all eel individuals in closed experiment (tank) by analysing eDNA and comparing it with the genomic haplotype information from tissue samples of the same individuals. Thereafter, examining eDNA from water samples, we were able to differentiate between European eel haplotypes in the mitochondrial D-loop region. In the three rivers (Lilleelv, Kleplandsbekken, and Moelva), we found seven, four, and two unique haplotypes, respectively, which probably represent different eel individuals. This supports earlier studies indicating that eDNA haplotyping of individual species in water samples is as effective as tissue sampling (Dugal et al., 2021). The Anguilla genus is highly diverse in the mitochondrial D-loop region (Ragauskas et al., 2014), and our method is therefore particularly feasible for this species.

3.3 Collateral damage: Understanding the response and recovery of non-targeted fish species after rotenone eradication of an invasive species

In this study (paper 3) we explored the effect and the ecological consequence of an invasive species eradication campaign in a temperate lake, and studied if and how native non-targeted fish species recover in the ecosystem within a two-year period. Based on samples collected from several sites within each habitat at each time, and triplicate analysis of each sample, we assumed that the probability of a positive eDNA sample is a proxy of overall density of a target species. Our results indicate that that the rotenone treatment was overall very efficient in eradicating the invasive pike from the lake and that all three studied native fish species reestablished in the lake within a year after the treatment. However, the rate and nature of the recovery process greatly differed between species.

For northern pike, the eDNA-signal in the lake quickly dissipated post-treatment, indicating successful eradication. However, eDNA signals from pike reappeared approximately 540 days post-treatment in three distinct locations spread within the lake, suggesting that pike was present yet again. The most likely explanation is that the pike was reintroduced into the lake by humans. Fortunately, after this initial reappearance of pike, subsequent eDNA samples have not shown any signs of pike presence in the lake, sea or rivers, and we conclude that pike most probably is absent.

The eDNA-signal from three-spined stickleback had only a brief period of absence before it reached a higher level than pre-treatment. This suggests that the absence of the predatory pike might have resulted in an increased population density, a so called mesopredator release (Ritchie and Johnson, 2009).

The seasonal pattern for brown trout and European eel eDNA-signals in the lake was strikingly similar to each other and seemed to be repeated on a yearly basis (with a reduction in the signal in spring and an increase in autumn), and this pattern is possibly the cause of a seasonal migratory pattern of the two species. The levels of eDNA signals from trout seem higher compared to that of pre-treatment levels, but there was no long-term increase in eDNA signal for eel in the lake.

Future research using metabarcoding could significantly enhance our understanding of complex interactions between multiple species, as such methods allows for the

simultaneous identification of multiple species from environmental eDNA samples. Applying this technique to our existing time series data would give a broader understanding of the biodiversity and trophic relationships in this ecosystem.

4. References

Andruszkiewicz, E. A., Starks, H. A., Chavez, F. P., Sassoubre, L. M., Block, B. A., & Boehm, A. B. (2017). Biomonitoring of marine vertebrates in Monterey Bay using eDNA metabarcoding. *Plos One*, *12*(4), e0176343.

Antognazza, C. M., Britton, J. R., Potter, C., Franklin, E., Hardouin, E. A., Gutmann Roberts, C., Aprahamian, M., & Andreou, D. (2019). Environmental DNA as a non-invasive sampling tool to detect the spawning distribution of European anadromous shads (Alosa spp.). *Aquatic Conservation: Marine and Freshwater Ecosystems*, *29*(1), 148-152.

Arthington, A. H., Dulvy, N. K., Gladstone, W., & Winfield, I. J. (2016). Fish conservation in freshwater and marine realms: status, threats and management. *Aquatic Conservation-Marine and Freshwater Ecosystems*, *26*(5), 838-857. https://doi.org/10.1002/aqc.2712

Baras, E., Jeandrain, D., Serouge, B., & Philippart, J. C. (1998). Seasonal variations in time and space utilization by radio-tagged yellow eels Anguilla anguilla (L.) in a small stream. *Hydrobiologia*, *372*, 187-198. https://doi.org/10.1023/a:1017072213791

Barbarossa, V., Schmitt, R. J., Huijbregts, M. A., Zarfl, C., King, H., & Schipper, A. M. (2020). Impacts of current and future large dams on the geographic range connectivity of freshwater fish worldwide. *Proceedings of the National Academy of Sciences*, *117*(7), 3648-3655.

Barrett, M., Belward, A., Bladen, S., Breeze, T., Burgess, N., Butchart, S., Clewclow, H., Cornell, S., Cottam, A., & Croft, S. (2018). Living planet report 2018: Aiming higher.

Beaulieu, J., Trépanier-Leroux, D., Fischer, J., Olson, M., Thibodeau, S., Humphries, S., Fraser, D., & Derry, A. (2021). Rotenone for exotic trout eradication: nontarget impacts on aquatic communities in a mountain lake. *Lake and Reservoir Management*, *37*(3), 323-338.

Beck, K. G., Zimmerman, K., Schardt, J. D., Stone, J., Lukens, R. R., Reichard, S., Randall, J., Cangelosi, A. A., Cooper, D., & Thompson, J. P. (2008). Invasive species defined in a policy context: Recommendations from the Federal Invasive Species Advisory Committee. *Invasive Plant Science and Management*, *1*(4), 414-421.

Belpaire, C. G. J., Goemans, G., Geeraerts, C., Quataert, P., Parmentier, K., Hagel, P., & De Boer, J. (2009). Decreasing eel stocks: survival of the fattest? *Ecology of Freshwater Fish*, *18*(2), 197-214. https://doi.org/10.1111/j.1600-0633.2008.00337.x

Beng, K. C., & Corlett, R. T. (2020). Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. *Biodiversity and Conservation*, *29*(7), 2089-2121. https://doi.org/10.1007/s10531-020-01980-0

Bernery, C., Bellard, C., Courchamp, F., Brosse, S., Gozlan, R. E., Jarić, I., Teletchea, F., & Leroy, B. (2022). Freshwater fish invasions: A comprehensive review. *Annual Review of Ecology, Evolution, and Systematics*, *53*, 427-456.

Bornarel, V., Lambert, P., Briand, C., Antunes, C., Belpaire, C., Ciccotti, E., Diaz, E., Diserud, O., Doherty, D., Domingos, I., Evans, D., de Graaf, M., O'Leary, C., Pedersen, M., Poole, R., Walker, A., Wickstrom, H., Beaulaton, L., & Drouineau, H. (2018). Modelling the recruitment of European eel (Anguilla anguilla) throughout its European range. *Ices Journal of Marine Science*, *75*(2), 541-552. https://doi.org/10.1093/icesjms/fsx180

Bylemans, J., Gleeson, D. M., Hardy, C. M., & Furlan, E. (2018). Toward an ecoregion scale evaluation of eDNA metabarcoding primers: A case study for the freshwater fish biodiversity of the Murray–Darling Basin (Australia). *Ecology and evolution*, *8*(17), 8697-8712.

Capoccioni, F., Lin, D. Y., Iizuka, Y., Tzeng, W. N., & Ciccotti, E. (2014). Phenotypic plasticity in habitat use and growth of the E uropean eel (A nguilla anguilla) in transitional waters in the M editerranean area. *Ecology of Freshwater Fish*, *23*(1), 65-76.

Cappo, M., Speare, P., & De'ath, G. (2004). Comparison of baited remote underwater video stations (BRUVS) and prawn (shrimp) trawls for assessments of fish biodiversity in inter-reefal areas of the Great Barrier Reef Marine Park. *Journal of Experimental Marine Biology and Ecology*, *302*(2), 123-152.

Castonguay, M., Hodson, P. V., Moriarty, C., Drinkwater, K. F., & Jessop, B. M. (1994). Is there a role of ocean environment in American and European eel decline? *Fisheries Oceanography*, *3*(3), 197-203.

Clavero, M., Hermoso, V., Levin, N., & Kark, S. (2010). Biodiversity research: geographical linkages between threats and imperilment in freshwater fish in the Mediterranean Basin. *Diversity and Distributions*, *16*(5), 744-754.

Consuegra, S., Phillips, N., Gajardo, G., & de Leaniz, C. G. (2011). Winning the invasion roulette: Escapes from fish farms increase admixture and facilitate establishment of non-native rainbow trout. *Evolutionary Applications*, *4*(5), 660-671.

Cowx, I. (2015). Characterisation of inland fisheries in Europe. *Fisheries Management and Ecology*, *22*(1), 78-87.

Doi, H., Uchii, K., Takahara, T., Matsuhashi, S., Yamanaka, H., & Minamoto, T. (2015). Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. *Plos One*, *10*(3), e0122763.

Drouineau, H., Durif, C., Castonguay, M., Mateo, M., Rochard, E., Verreault, G., Yokouchi, K., & Lambert, P. (2018). Freshwater eels: A symbol of the effects of global change. *Fish and fisheries*, *19*(5), 903-930. https://doi.org/10.1111/faf.12300

Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z. I., Knowler, D. J., Leveque, C., Naiman, R. J., Prieur-Richard, A. H., Soto, D., Stiassny, M. L. J., & Sullivan, C. A. (2006). Freshwater biodiversity: importance, threats, status and conservation challenges. *Biological Reviews*, *81*(2), 163-182. https://doi.org/10.1017/s1464793105006950

Dufour, S., Burzawa-Gerard, E., Le Belle, N., Sbaihi, M., & Vidal, B. (2003). Reproductive endocrinology of the European eel, Anguilla anguilla. *Eel biology*, 373-383.

Dugal, L., Thomas, L., Jensen, M. R., Sigsgaard, E. E., Simpson, T., Jarman, S., Thomsen, P. F., & Meekan, M. (2021). Individual haplotyping of whale sharks from seawater environmental DNA. *Molecular Ecology Resources*, *22*(1), 56-65.

Durif, C. M. F., Arts, M., Bertolini, F., Cresci, A., Daverat, F., Karlsbakk, E., Koprivnikar, J., Moland, E., Olsen, E. M., Parzanini, C., Power, M., Rohtla, M., Skiftesvik, A. B., Thorstad, E., Vollestad, L. A., & Browman, H. (2023). The evolving story of catadromy in the European eel (Anguilla anguilla). *Ices Journal of Marine Science*. https://doi.org/10.1093/icesjms/fsad149

Durif, C. M. F., Stockhausen, H. H., Skiftesvik, A. B., Cresci, A., Nyqvist, D., & Browman, H. I. (2022). A unifying hypothesis for the spawning migrations of temperate anguillid eels. *Fish and fisheries*, *23*(2), 358-375. https://doi.org/10.1111/faf.12621

Eschmeyer, W. (2016). Species by family/subfamily in the Catalog of Fishes. *California Academy of Sciences*.

Evans, N. T., Shirey, P. D., Wieringa, J. G., Mahon, A. R., & Lamberti, G. A. (2017). Comparative cost and effort of fish distribution detection via environmental DNA analysis and electrofishing. *Fisheries*, *42*(2), 90-99.

Ferreira, T., Globevnik, L., & Schinegger, R. (2019). Water stressors in Europe: New threats in the old world. In *Multiple stressors in river ecosystems* (pp. 139-155). Elsevier.

Feunteun, E. (2002). Management and restoration of European eel population (Anguilla anguilla): An impossible bargain. *Ecological Engineering*, *18*(5), 575-591, Article Pii s0925-8574(02)00021-6. https://doi.org/10.1016/s0925-8574(02)00021-6

Ficetola, G. F., Taberlet, P., & Coissac, E. (2016). How to limit false positives in environmental DNA and metabarcoding? In: Wiley Online Library.
Finlayson, C. M., de Groot, R. S., Hughes, F. M., & Sullivan, C. A. (2018). Freshwater ecosystem services and functions. *Freshwater Ecology and Conservation: Approaches and Techniques*, *321*.
Fletcher, C. M., Collins, S. F., Nannini, M. A., & Wahl, D. H. (2019). Competition during early ontogeny: Effects of native and invasive planktivores on the growth, survival, and habitat use of bluegill. *Freshwater Biology*, *64*(4), 697-707. https://doi.org/10.1111/fwb.13255
Foote, A. D., Thomsen, P. F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L. A., Salling, A. B., Galatius, A., Orlando, L., & Gilbert, M. T. P. (2012). Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals.
Friebertshauser, R., Shollenberger, K., Janosik, A., Garner, J. T., & Johnston, C. (2019). The effect of bivalve filtration on eDNA-based detection of aquatic organisms. *Plos One*, *14*(11), e0222830.
Friedland, K. D., Miller, M. J., & Knights, B. (2007). Oceanic changes in the Sargasso Sea and declines in recruitment of the European eel. *Ices Journal of Marine Science*, *64*(3), 519-530. https://doi.org/10.1093/icesjms/fsm022
Føre, M., Frank, K., Dempster, T., Alfredsen, J. A., & Høy, E. (2017). Biomonitoring using tagged sentinel fish and acoustic telemetry in commercial salmon aquaculture: a feasibility study. *Aquacultural Engineering*, *78*, 163-172.
Gilbert, M. A., & Granath Jr, W. O. (2003). Whirling disease of salmonid fish: life cycle, biology, and disease. *Journal of Parasitology*, *89*(4), 658-667.
Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., Spear, S. F., McKee, A., Oyler-McCance, S. J., & Cornman, R. S. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in ecology and evolution*, *7*(11), 1299-1307.
Gozlan, R. E., Britton, J., Cowx, I., & Copp, G. (2010). Current knowledge on non-native freshwater fish introductions. *Journal of fish biology*, *76*(4), 751-786.
Grooten, M., & Almond, R. E. (2018). *WWF. Living planet report-2018: aiming higher* (2940529906).
Halvorsen, S., Korslund, L., Gustavsen, P. Ø., & Slettan, A. (2020). Environmental DNA analysis indicates that migration barriers are decreasing the occurrence of European eel (Anguilla anguilla) in distance from the sea. *Global Ecology and Conservation*, *24*, e01245.
Halvorsen, S., Korslund, L., Mattingsdal, M., & Slettan, A. (2023). Estimating number of European eel (Anguilla anguilla) individuals using environmental DNA and haplotype count in small rivers. *Ecology and evolution*, *13*(2), e9785.
Harper, L. R., Buxton, A. S., Rees, H. C., Bruce, K., Brys, R., Halfmaerten, D., Read, D. S., Watson, H. V., Sayer, C. D., & Jones, E. P. (2019). Prospects and challenges of environmental DNA (eDNA) monitoring in freshwater ponds. *Hydrobiologia*, *826*, 25-41.
Hata, H., Uemura, Y., & Ouchi, K. (2021). Decline of unionid mussels enhances hybridisation of native and introduced bitterling fish species through competition for breeding substrate. *Freshwater Biology*, *66*(1), 189-201. https://doi.org/10.1111/fwb.13629
Hinlo, R., Gleeson, D., Lintermans, M., & Furlan, E. (2017). Methods to maximise recovery of environmental DNA from water samples. *Plos One*, *12*(6), e0179251.
Holman, L. E., Chng, Y., & Rius, M. (2022). How does eDNA decay affect metabarcoding experiments? *Environmental DNA*, *4*(1), 108-116.
Itakura, H., Wakiya, R., Yamamoto, S., Kaifu, K., Sato, T., & Minamoto, T. (2019). Environmental DNA analysis reveals the spatial distribution, abundance, and biomass of Japanese eels at the river-basin scale. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 1-13. https://doi.org/10.1002/aqc.3058

Jacoby, D., & Gollock, M. (2014). Anguilla anguilla. *The IUCN red list of threatened species*, *2014*.

Jeunen, G. J., Knapp, M., Spencer, H. G., Taylor, H. R., Lamare, M. D., Stat, M., Bunce, M., & Gemmell, N. J. (2019). Species-level biodiversity assessment using marine environmental DNA metabarcoding requires protocol optimization and standardization. *Ecology and evolution*, *9*(3), 1323-1335.

Kettle, A. J., Vollestad, L. A., & Wibig, J. (2011). Where once the eel and the elephant were together: decline of the European eel because of changing hydrology in southwest Europe and northwest Africa? *Fish and fisheries*, *12*(4), 380-411. https://doi.org/10.1111/j.1467-2979.2010.00400.x

Knights, B. (2003). A review of the possible impacts of long-term oceanic and climate changes and fishing mortality on recruitment of anguillid eels of the Northern Hemisphere. *Science of the Total Environment*, *310*(1-3), 237-244. https://doi.org/10.1016/s0048-9697(02)00644-7

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

Lacoursière-Roussel, A., Howland, K., Normandeau, E., Grey, E. K., Archambault, P., Deiner, K., Lodge, D. M., Hernandez, C., Leduc, N., & Bernatchez, L. (2018). eDNA metabarcoding as a new surveillance approach for coastal Arctic biodiversity. *Ecology and evolution*, *8*(16), 7763-7777.

Layton, A., McKay, L., Williams, D., Garrett, V., Gentry, R., & Sayler, G. (2006). Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. *Applied and environmental microbiology*, *72*(6), 4214-4224.

Ling, N. (2003). Rotenoneôa review of its toxicity and use for fisheries management. *Science for conservation*, *211*, 1-40.

Mace, G. M., Barrett, M., Burgess, N. D., Cornell, S. E., Freeman, R., Grooten, M., & Purvis, A. (2018). Aiming higher to bend the curve of biodiversity loss. *Nature Sustainability*, *1*(9), 448-451.

Majaneva, M., Diserud, O. H., Eagle, S. H., Boström, E., Hajibabaei, M., & Ekrem, T. (2018). Environmental DNA filtration techniques affect recovered biodiversity. *Scientific Reports*, *8*(1), 4682.

Marquina, D., Buczek, M., Ronquist, F., & Łukasik, P. (2021). The effect of ethanol concentration on the morphological and molecular preservation of insects for biodiversity studies. *PeerJ*, *9*, e10799.

Martellini, A., Payment, P., & Villemur, R. (2005). Use of eukaryotic mitochondrial DNA to differentiate human, bovine, porcine and ovine sources in fecally contaminated surface water. *Water Research*, *39*(4), 541-548. https://doi.org/10.1016/j.watres.2004.11.012

Mena, J. L., Yagui, H., Tejeda, V., Bonifaz, E., Bellemain, E., Valentini, A., Tobler, M. W., Sánchez-Vendizú, P., & Lyet, A. (2021). Environmental DNA metabarcoding as a useful tool for evaluating terrestrial mammal diversity in tropical forests. *Ecological Applications*, *31*(5), e02335.

Miqueleiz, I., Bohm, M., Ariño, A. H., & Miranda, R. (2020). Assessment gaps and biases in knowledge of conservation status of fishes. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *30*(2), 225-236.

Miranda, R., Hermoso, V., & Hassall, C. (2019). Ecological quality and conservation status of inland waters. In (Vol. 9, pp. 275-277): Taylor & Francis.

Moore, A., & Riley, W. (2009). Magnetic particles associated with the lateral line of the European eel Anguilla anguilla. *Journal of fish biology*, *74*(7), 1629-1634.

Moorhouse, T. P., & Macdonald, D. W. (2015). Are invasives worse in freshwater than terrestrial ecosystems? *Wiley Interdisciplinary Reviews: Water*, *2*(1), 1-8.
Morin, P. A., Chambers, K. E., Boesch, C., & Vigilant, L. (2001). Quantitative polymerase chain reaction analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (Pan troglodytes verus). *Molecular Ecology*, *10*(7), 1835-1844.
Muha, T. P., Robinson, C. V., Garcia de Leaniz, C., & Consuegra, S. (2019). An optimised eDNA protocol for detecting fish in lentic and lotic freshwaters using a small water volume. *Plos One*, *14*(7), e0219218.
Nyqvist, D., Greenberg, L. A., Goerig, E., Calles, O., Bergman, E., Ardren, W. R., & Castro-Santos, T. (2017). Migratory delay leads to reduced passage success of Atlantic salmon smolts at a hydroelectric dam. *Ecology of Freshwater Fish*, *26*(4), 707-718.
Ogden, L. E. (2022). The Emergence of eDNA: An interdisciplinary tool helps monitor biodiversity and health. *BioScience*, *72*(1), 5-12.
Ogram, A., Sayler, G. S., & Barkay, T. (1987). THE EXTRACTION AND PURIFICATION OF MICROBIAL DNA FROM SEDIMENTS. *Journal of Microbiological Methods*, *7*(2-3), 57-66. https://doi.org/10.1016/0167-7012(87)90025-x
Palm, S., Dannewitz, J., Prestegaard, T., & Wickstrom, H. (2009). Panmixia in European eel revisited: no genetic difference between maturing adults from southern and northern Europe. *Heredity (Edinb)*, *103*(1), 82-89. https://doi.org/10.1038/hdy.2009.51
Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2013). Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Sciences*, *70*(8), 1123-1130.
Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2014). Factors influencing detection of eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources*, *14*(1), 109-116. https://doi.org/10.1111/1755-0998.12159
Piper, A. T., Svendsen, J. C., Wright, R. M., & Kemp, P. S. (2017). Movement patterns of seaward migrating European eel (Anguilla anguilla) at a complex of riverine barriers: implications for conservation. *Ecology of Freshwater Fish*, *26*(1), 87-98.
Porcher, J. P. (2002). Fishways for eels. *Bulletin Francais De La Peche Et De La Pisciculture*(364), 147-155. https://doi.org/10.1051/kmae/2002099
Ragauskas, A., Butkauskas, D., Sruoga, A., Kesminas, V., Rashal, I., & Tzeng, W. N. (2014). Analysis of the genetic structure of the European eel Anguilla anguilla using the mtDNA D-loop region molecular marker. *Fisheries Science*, *80*(3), 463-474. https://doi.org/10.1007/s12562-014-0714-1
Rahel, F. J., & Olden, J. D. (2008). Assessing the effects of climate change on aquatic invasive species. *Conservation Biology*, *22*(3), 521-533.
Recsetar, M. S., & Bonar, S. A. (2015). Effectiveness of two commercial rotenone formulations in the eradication of virile crayfish Orconectes virilis. *North American Journal of Fisheries Management*, *35*(3), 616-620.
Reid, A. J., Carlson, A. K., Creed, I. F., Eliason, E. J., Gell, P. A., Johnson, P. T. J., Kidd, K. A., MacCormack, T. J., Olden, J. D., Ormerod, S. J., Smol, J. P., Taylor, W. W., Tockner, K., Vermaire, J. C., Dudgeon, D., & Cooke, S. J. (2019). Emerging threats and persistent conservation challenges for freshwater biodiversity. *Biological Reviews*, *94*(3), 849-873. https://doi.org/10.1111/brv.12480
Righton, D., Westerberg, H., Feunteun, E., Okland, F., Gargan, P., Amilhat, E., Metcalfe, J., Lobon-Cervia, J., Sjoberg, N., Simon, J., Acou, A., Vedor, M., Walker, A., Trancart, T., Bramick, U., & Aarestrup, K. (2016). Empirical observations of the spawning migration of European eels: The long and dangerous road to the Sargasso Sea. *Science Advances*, *2*(10), Article UNSP e1501694. https://doi.org/10.1126/sciadv.1501694
Sandodden, R., Aune, S., Bardal, H., Adolfsen, P., & Nost, T. (2022). Rotenone application and degradation following eradication of invasive roach (Rutilus rutilus) in three Norwegian

lakes. *Management of Biological Invasions*, *13*(1), 233-245. https://doi.org/10.3391/mbi.2022.13.1.14

Schwarzbach, M., Laiacker, M., Mulero-Pazmany, M., & Kondak, K. (2014). Remote water sampling using flying robots. 2014 International Conference on Unmanned Aircraft Systems (ICUAS),

Silva, A. T., Lucas, M. C., Castro-Santos, T., Katopodis, C., Baumgartner, L. J., Thiem, J. D., Aarestrup, K., Pompeu, P. S., O'Brien, G. C., & Braun, D. C. (2018). The future of fish passage science, engineering, and practice. *Fish and fisheries*, *19*(2), 340-362.

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillott, A. D., Hines, H. B., & Kenyon, N. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*, *4*(2), 125-134.

Song, J. W., Small, M. J., & Casman, E. A. (2017). Making sense of the noise: The effect of hydrology on silver carp eDNA detection in the Chicago area waterway system. *Science of the Total Environment*, *605*, 713-720. https://doi.org/10.1016/j.scitotenv.2017.06.255

Strayer, D. L., & Dudgeon, D. (2010). Freshwater biodiversity conservation: recent progress and future challenges. *Journal of the North American Benthological Society*, *29*(1), 344-358.

Takahara, T., Iwai, N., Yasumiba, K., & Igawa, T. (2020). Comparison of the detection of 3 endangered frog species by eDNA and acoustic surveys across 3 seasons. *Freshwater Science*, *39*(1), 18-27.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., & Kawabata, Z. i. (2012). Estimation of fish biomass using environmental DNA. *Plos One*, *7*(4), e35868.

Takahashi, M., Saccò, M., Kestel, J. H., Nester, G., Campbell, M. A., van der Heyde, M., Heydenrych, M. J., Juszkiewicz, D. J., Nevill, P., Dawkins, K. L., Bessey, C., Fernandes, K., Miller, H., Power, M., Mousavi-Derazmahalleh, M., Newton, J. P., White, N. E., Richards, Z. T., & Allentoft, M. E. (2023). Aquatic environmental DNA: A review of the macro-organismal biomonitoring revolution. *Science of the Total Environment*, *873*, Article 162322. https://doi.org/10.1016/j.scitotenv.2023.162322

Takasaki, K., Aihara, H., Imanaka, T., Matsudaira, T., Tsukahara, K., Usui, A., Osaki, S., & Doi, H. (2021). Water pre-filtration methods to improve environmental DNA detection by real-time PCR and metabarcoding. *Plos One*, *16*(5), e0250162.

Tesch, F. (1977). The eel: biology and management of anguillid eels.(J. Greenwood, translator). In: John Wiley & Sons, New York, New York, USA.

Thomas, A. C., Nguyen, P. L., Howard, J., & Goldberg, C. S. (2019). A self-preserving, partially biodegradable eDNA filter. *Methods in ecology and evolution*, *10*(8), 1136-1141.

Thomsen, P. F., Kielgast, J., Iversen, L. L., Moller, P. R., Rasmussen, M., & Willerslev, E. (2012). Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples. *Plos One*, *7*(8), Article e41732. https://doi.org/10.1371/journal.pone.0041732

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA - An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4-18. https://doi.org/10.1016/j.biocon.2014.11.019

Thorstad, B. E., Larsen, M., Hesthagen, T., Næsje, F. T., Poole, R., Aarestrup, K., Pedersen, I. M., Hanssen, F., Østborg, G., Økland, F., Aasestad, I., & Sandlund, T. O. (2010). *Ål og konsekvenser av vannkraftutbygging* (Norges vassdrags- og energidirektorat Issue.

Tickner, D., Opperman, J. J., Abell, R., Acreman, M., Arthington, A. H., Bunn, S. E., Cooke, S. J., Dalton, J., Darwall, W., & Edwards, G. (2020). Bending the curve of global freshwater biodiversity loss: an emergency recovery plan. *BioScience*, *70*(4), 330-342.

Tsukamoto, K., & Aoyama, J. (1998). Evolution of freshwater eels of the genus Anguilla: a probable scenario. *Environmental Biology of Fishes*, *52*(1-3), 139-148. https://doi.org/10.1023/a:1007427724175

Tsukamoto, K., & Arai, T. (2001). Facultative catadromy of the eel Anguilla japonica between freshwater and seawater habitats. *Marine Ecology Progress Series*, *220*, 265-276.

Van Kessel, N., Dorenbosch, M., De Boer, M. R. M., Leuven, R., & Van der Velde, G. (2011). Competition for shelter between four invasive gobiids and two native benthic fish species. *Current Zoology*, *57*(6), 844-851. <Go to ISI>://WOS:000298293800021

Van Leeuwen, C. H., Dalen, K., Museth, J., Junge, C., & Vøllestad, L. A. (2018). Habitat fragmentation has interactive effects on the population genetic diversity and individual behaviour of a freshwater salmonid fish. *River Research and Applications*, *34*(1), 60-68.

Vøllestad, L. A. (1992). *Ålefisker*. J.W. Cappelens forlag.

West, K. M., Stat, M., Harvey, E. S., Skepper, C. L., DiBattista, J. D., Richards, Z. T., Travers, M. J., Newman, S. J., & Bunce, M. (2020). eDNA metabarcoding survey reveals fine-scale coral reef community variation across a remote, tropical island ecosystem. *Molecular Ecology*, *29*(6), 1069-1086.

Wonham, M. J., Carlton, J. T., Ruiz, G. M., & Smith, L. D. (2000). Fish and ships: relating dispersal frequency to success in biological invasions. *Marine Biology*, *136*, 1111-1121.

Woodford, D. J., Barber-James, H. M., Bellingan, T. A., Day, J. A., de Moor, F. C., Gouws, J., & Weyl, O. L. (2013). Immediate impact of piscicide operations on a Cape Floristic Region aquatic insect assemblage: a lesser of two evils? *Journal of Insect Conservation*, *17*, 959-973.

Wysujack, K., Westerberg, H., Aarestrup, K., Trautner, J., Kurwie, T., Nagel, F., & Hanel, R. (2014). The migration behaviour of European silver eels (Anguilla anguilla) released in open ocean conditions. *Marine and Freshwater Research*, *66*(2), 145-157.

Zerebecki, R. A., & Sorte, C. J. (2011). Temperature tolerance and stress proteins as mechanisms of invasive species success. *Plos One*, *6*(4), e14806.

5. Paper 1.

Environmental DNA analysis indicates that migration barriers are decreasing the occurrence of European eel (Anguilla anguilla) in distance from the sea

Silje Halvorsen, Lars Korslund, Per Ø. Gustavsen, Audun Slettan. 2020.

Global Ecology and Conservation. Volume 24, December 2020, e01245.

(doi: https://doi.org/10.1016/j.gecco.2020.e01245)

Global Ecology and Conservation 24 (2020) e01245

Contents lists available at ScienceDirect

Global Ecology and Conservation

journal homepage: http://www.elsevier.com/locate/gecco

Original Research Article

Environmental DNA analysis indicates that migration barriers are decreasing the occurrence of European eel (*Anguilla anguilla*) in distance from the sea

Silje Halvorsen [a, *], Lars Korslund [a], Per Ø. Gustavsen [b], Audun Slettan [a]

[a] Centre of Coastal Research, Department of Natural Sciences, University of Agder, P.O. Box 422, Kristiansand, NO-4604, Norway
[b] Gustavsen Naturanalyser, Varpevegen 26, 3728, Skien, Norway

ARTICLE INFO

Article history:
Received 19 March 2020
Received in revised form 23 August 2020
Accepted 23 August 2020

Keywords:
Environmental DNA
Anguilla Anguilla
Passage barriers
Conservation
Real-time PCR

ABSTRACT

The European eel (*Anguilla* L.) is considered critically endangered by the IUCN Red List, and recruitment remains low. One of the challenges for the species today is migration barriers that limit their habitat. Along the Norwegian coast, like in other countries, the abundance of eels appears to decrease with distance from the sea. This pattern may be a result of factors like water temperature, water quality, competition, and habitat suitability and availability. This study aims to use environmental DNA (eDNA) analysis to investigate the potential relationship between migration barriers and the decreasing occurrence of eels in distance from the sea by the coast of southern Norway. Sixty locations with potential migration barriers are investigated by collecting water samples upstream and downstream from each construction before eDNA from each sample is isolated and analyzed by real-time PCR with specific primers and probes matching *A. anguilla*. The results reveal that the probability of detecting eel eDNA decreases significantly with number of hydroelectric power stations and their associated basins, even when the effect of distance to sea is accounted for. In addition, there is a clear border at which eel eDNA could no longer be detected upstream of the major watercourses. Therefore, it is likely that the migration of eels is prohibited by these constructions, which seem to constitute a much greater challenge than every other type of potential migration barrier investigated in this study.

1. Introduction

Freshwater ecosystems provide homes for a considerable number of the world's species but are threatened by human activities that change the environment on local and global scales. Many of these alterations have led to dramatic changes in biodiversity, and few fish stocks have been more affected than the European eel (*Anguilla* L.) (Dekker and Beaulaton, 2016). The European eel stock has been reduced by approximately 90% since the 1980s, and they are now considered critically endangered by thssee IUCN Red List (Jacoby and Gollock, 2014). The negative development has been attributed to a combination of factors like overfishing and aquaculture (Castonguay et al., 1994), parasites (Feunteun, 2002), poisoning (Belpaire et al., 2009), seaic changes and global warming (Drouineau et al., 2018; Friedland et al., 2007), and the destruction of habitat and building of dams and barriers (Kettle et al., 2011). In 2007, the European Union (EU) proposed a regulation plan regarding

* Corresponding author.
E-mail address: silje.halvorsen@uia.no (S. Halvorsen).

https://doi.org/10.1016/j.gecco.2020.e01245

the restoration of the eel stock (Council regulation No 1100/2007). Every member country of the EU imposed secure conservation plans to reduce human-made mortality with the aim of increasing the stock by 40% of the historical biomass of silver eels (Anonymous, 2007). The conservation effort has contributed to a stop in the decreasing trend of the stock since 2011, and time-series from 1980 to 2019 reveal an increasing trend from 2011 to 2019 (ICES, 2019). However, in 2019, the recruitment of glass eel from the sea was only 1.4% of the historical level in the North Sea and 6.0% elsewhere in Europe compared to 1960–1979 (ICES, 2019). Considering the low recruitment in 2019, improvements and continuous conservation efforts are still needed.

The European eel larvae migrate over 5000 km from the spawning area in the Sargasso Sea to Europe and North Africa and metamorphose into glass eel (Righton et al., 2016). Most of the eels migrate upstream to freshwater systems, but some stay in marine and brackish water. Here they start their pigmentation and are called "elvers" until fully pigmented into yellow eels. The eels undergo the last metamorphosis into silver eels on their final migration back to the spawning area after approximately 20 years in the coastal habitats (Vøllestad, 1992).

In the freshwater systems, the probability of eel occurrence decreases significantly with increasing distance from the sea (Degerman et al., 2019; Ibbotson et al., 2002). In Norway, 46% of every registered area with an abundance of eels is located within 5 km from the sea, and 42% of lakes with eels are found less than 50 m above sea level (Thorstad et al., 2010). Distance is acknowledged as an explanation for the distribution pattern, in addition to factors like temperature (Ogden, 1970) and water quality (Degerman et al., 2019). Competition can also explain the pattern in which low population density leads to low competition for resources, thus failing to drive individuals further upstream (Arai, 2016; Ibbotson et al., 2002). Also, suitability and accessibility of habitats defines the inland abundance of eels (Laffaille et al., 2009). Habitat accessibility depends on free-flowing rivers and streams and could be restricted by migration barriers. The number of migration barriers has shown to have a more significant reduction effect on eel abundance than distance alone (White and Knights, 1997). Construction in freshwater systems escalated in the 1950–1960s, leading to about 50–90% habitat loss for eels in Europe (Feunteun, 2002; Tesch, 1977). Migration barriers taller than 50–60% of the eels' body length, or barriers that cause high flow rate or velocity, could prohibit further upstream migration (Porcher, 2002; Thorstad et al., 2010). Examples of such barriers are fish ladders, pipelines, basins, and hydroelectric power stations. Migration downstream is especially threatened by turbines connected to power stations. The results of several studies indicate that 52% of eels passing the turbines suffer injury or death (Thorstad et al., 2010).

Human-made modifications of freshwater systems constitute a threat to the European eel that should be possible to reduce. The first step in the conservation effort is to investigate possible connections between eel distribution and potential barriers. Traditional methods for species detection in aquatic environments are fishing, traps, or observation of the organisms. These methods can be challenging when investigating species with different life stages (like eggs or larvae), especially for rare species. Studies of environmental DNA (eDNA) could solve the challenges associated with traditional methods. eDNA is DNA that organisms shed into the environment, such as cells or tissue, and analyses of water for species-specific eDNA have increasingly become a tool for the detection of aquatic organisms (Rees et al., 2014). Because eDNA is shed into the water, the organism can potentially be detected in an analysis of a water sample with polymerase chain reaction (PCR) with specific primers and probes (Thomsen and Willerslev, 2015). The method gives more precise and objective results than many of the traditional methods (Strickler et al., 2015; Thomsen and Willerslev, 2015). It is also suited for the detection of rare species like the European eel, which could be difficult to catch or observe. eDNA could, therefore, be an advisable tool for investigating the abundance and distribution of the European eel.

This study aims to use eDNA analysis to investigate the potential relationship between migration barriers and the decreasing occurrence of eels in distance from the sea by the coast of southern Norway.

2. Materials and methods

2.1. Water sampling

We sampled water at 60 locations with potential migration barriers in the county Agder, in southern Norway. The sites were sorted into five categories of potential migration barriers—pipelines, tunnels and bridges; fish ladders; basins (separated with small dams, such as those for drinking water); hydroelectric power stations with associated water basins; or natural/unknown barriers. The positions for each site are shown in Fig. 2; GPS coordinates are listed in Table 1, Appendix A. We collected samples 1–26 in June 2018, the remaining in June–August 2019, both upstream and downstream of each potential barrier. One sample consists of 1 L of water, collected by combining 20 samples of each 50 ml from surface water. Each sample is collected within an area of 1–50 m along the river, stream, or lake. New 50 ml sterile Falcon centrifuge tubes and gloves were used for each sampling to prohibit contamination. The samples were stored on ice until filtration, which was performed within 5 h. The 1 L bottles were rinsed in 10% chlorine for 10 min followed by several washes with water before sampling. We used water from the location where samples were collected if the bottles were reused in the field. We filtered 150–900 ml water (as much as possible) from the samples through a cellulose nitrate filter with 0.45 μm pore size (Thermo Scientific Nalgene) by an ILMVAC vacuum pump (GmbH). After filtration, the filters were folded three times via tweezers and stored in a 1.5 ml Microcentrifuge tube at −20 °C until DNA was isolated.

2.2. eDNA isolation

We extracted eDNA from the filters by DNeasy® Blood and Tissue Kit (Qiagen) and bead beating, using the method described by Thomsen et al. (2012). After isolation, the eDNA was stored in microcentrifuge tubes at −20 °C. We performed the eDNA isolation in a separate room from the PCR amplification. Every sample was analyzed by a spectrophotometer (NanoDrop™ One, Thermofisher) after isolation to examine purity and eDNA-concentration.

2.3. PCR amplification

We examined eDNA extract for *A. anguilla* DNA using a real-time PCR assay with TaqMan®-probe and primers specific for a section of the *cytb* gene in the mitochondria. The primers were designed with Primer-BLAST at the web page of the National Center for Biotechnology Information (NCBI) and the program Primer Express 3.0.1 (Thermo Fisher). The sequences were as follows: "Alcyt forward": 5′-CACCCATACTTCTCCTACAAAGACCTA-3′, and "Alcyt reverse": 5′-TCTGGGTCTCCAAGCAGGTT-3' (101 bp product) and the probe: 5-FAM-TTCATTATCATGCTCACC-MGBEQ-3'. The primers and probe were tested for species-specificity by searching for homology to DNA sequences from species that could be found in the same area by using Clustal Omega (European Bioinformatics Institute) and NCBI's GenBank.

PCR-mix had the following ingredient concentrations: 1 x TaqMan Environmental Master Mix (Applied Biosystems), 0.9 μM Alcyt forward, 0.9 μM Alcyt reverse, and 0.55 μM probe. 20 μl PCR mix with 5 μl template was transferred to a 0.1 ml Micro Fast Tube Strips (Thermo Fisher). We conducted real-time PCR on a StepOnePlus™ Real-time PCR System (Applied Biosystem) with a temperature profile of 50 °C in 2 min and 95 °C in 10 min, followed by 60 cycles of 96 °C in 15 s, 57 °C in 30 s, and 72 °C in 30 s, with fluorescence detection after each cycle. We analyzed every sample in triplicates. A sample containing tissue of genomic DNA from *A. anguilla* was used as a positive control, and PCR-grade H_2O was used as a negative control. Positive and negative controls were included in all runs. A selected number (26) of the locations were analyzed for the abundance of brown trout (*Salmo trutta*) as an additional control for false negatives, as trout are expected to be present in almost every location. If a sample is negative for eel DNA and positive for trout DNA, the result is not a false negative. These analyses had the same PCR conditions as for European eel but with species-specific primers and probes matching *S. trutta*. The primers, specific for a section of the *cytb* gene in the mitochondria, and probe were designed with the same tools as for European eel. The sequences were as follows: Stcyt-F: 5′-CCACCCCTACTTCTCATA-3′, Stcytb-R: 5′-GGAGGTTGGGTGCGAA-TAGA-3' (88 bp product), and probe: 5′- FAM-CTTGGATTCGTAGCTAT-MGBEQ -3'.

The real-time PCR results were analyzed by the software provided by the StepOnePlus™ Real-time PCR System.

To conclude that the analyzed sample contains *A. anguilla* DNA, at least one of the PCR triplicates needs to be positive. One amplicon from a selected sample was Sanger-sequenced (by Eurofins Genomics, Germany) to verify that *A. anguilla* is detected.

2.4. Statistical analysis

To investigate if the probability of *A. Anguilla* eDNA presence at each location could be explained by the occurrence of migration barriers, we tested a set of logistic regression models, and a stepwise forward model selection procedure was applied. We started with the null-model (with no variables) and tested if the addition of each new variable improved the model sufficiently. We wanted to separate the effect of barriers from the effect of the distance eels needed to migrate to reach a location. Therefore, the distance from the location to the sea, in km, was included as a co-variate and kept in all subsequent models. The cumulative number of each of the five types of barriers (see Table 1) downstream of the location (i.e. the number of each type of barrier an eel would have to pass to reach the location) was used as potential variables in the model selection. The separate effect of each type of barrier was tested by including each of these variables in the model, one by one, and comparing the performance of the model with and without the variable in question, by comparing the residual deviance and degrees of freedom of the two models using a Chi-square test and a significance level of 0.05). This procedure was repeated until all variables that significantly improved the model fit was included in the model. The statistical analysis was performed in R ("R Core Team," 2017).

Table 1
Locations where eDNA from European eel is detected and not detected, sorted by the different types of migration barriers.

Type of barrier	Detected upstream and downstream	Not detected upstream, but detected downstream	Not detected upstream or downstream
Pipelines, tunnels, and bridges	1, 4, 5, 6, 8, 9, 10, 11, 14, 16, 18, 19, 20, 21, 22, 23, 24, 25, 26, 30, 49	15, 50	2, 13, 17
Fish ladders	3, 7, 12		
Basins	27, 43, 45, 46		
Hydroelectric power stations and associated water basins	34, 38, 59	28, 29, 31, 35, 58	32, 33, 36, 37, 57
Natural barriers/unknown	41, 42, 44, 47, 48, 51, 56, 60	39, 40, 52, 53, 54, 55	

3. Results

A summary of the results, where the detection of eDNA from *A. anguilla* is sorted by the different types of migration barriers, is provided in Table 1. In the five different categories of migration barriers, most locations are found in "Pipelines, tunnels and bridges" (26) followed by "Natural barriers/unknown" (14) and "Hydroelectric power stations and associated water basins" (13). There were few locations in the category of "Basins" (4) and "Fish ladders" (3).

As the distance to sea increased, the probability of detecting European eel eDNA decreased (Fig. 1A), and this effect was highly significant ($\chi^2 = 21.8$, df = 1, $p < 0.001$). The one category of barriers that had the strongest effect on the probability of detecting eel eDNA was hydroelectric power stations, even when the effect of distance to sea was accounted for. As the number of such barriers increased, the probability of detecting eel eDNA decreased significantly ($\chi^2 = 9.19$, df = 1, $p = 0.002$). The probability of detection dropped 26% going from 3 to 4 such barriers, and the probability of detection dropped close to zero when the number of such barriers reached seven (Fig. 1B). As the number of natural barriers increased, the probability of detecting eel eDNA decreased, but the pattern was not consistent (Fig. 1C) and this effect was not significant ($\chi^2 = 3.22$, df = 1, $p = 0.07$). There was a slight decrease in the probability of detecting eel eDNA with an increasing number of pipelines, tunnels or bridges (Fig. 1D), but this pattern was far from clear and the effect was not strong enough for this variable to be significant ($\chi^2 = 2.25$, df = 1, $p = 0.13$). In fact, at the only two locations positioned upstream of two such barriers, the water sample was positive (Fig. 1D). The number of basins downstream of a location had no effect on the probability of detecting eel eDNA ($\chi^2 = 0.01$, df = 1, $p = 0.95$). Only seven locations were positioned above one or two basins, and among these, only one was negative (Fig. 1E). The total number of fish ladders in the dataset was only three, and due to lack of data the models with number of fish ladders included as a variable did not converge. Therefore, only the observed data are presented (Fig. 1F).

The probability of detecting *A. Anguilla* eDNA was significantly reduced as one moved inward from the coast (Fig. 2). The water area upstream from site 34 is colored yellow in the figure, despite positive PCR-results (Appendix A), because eDNA

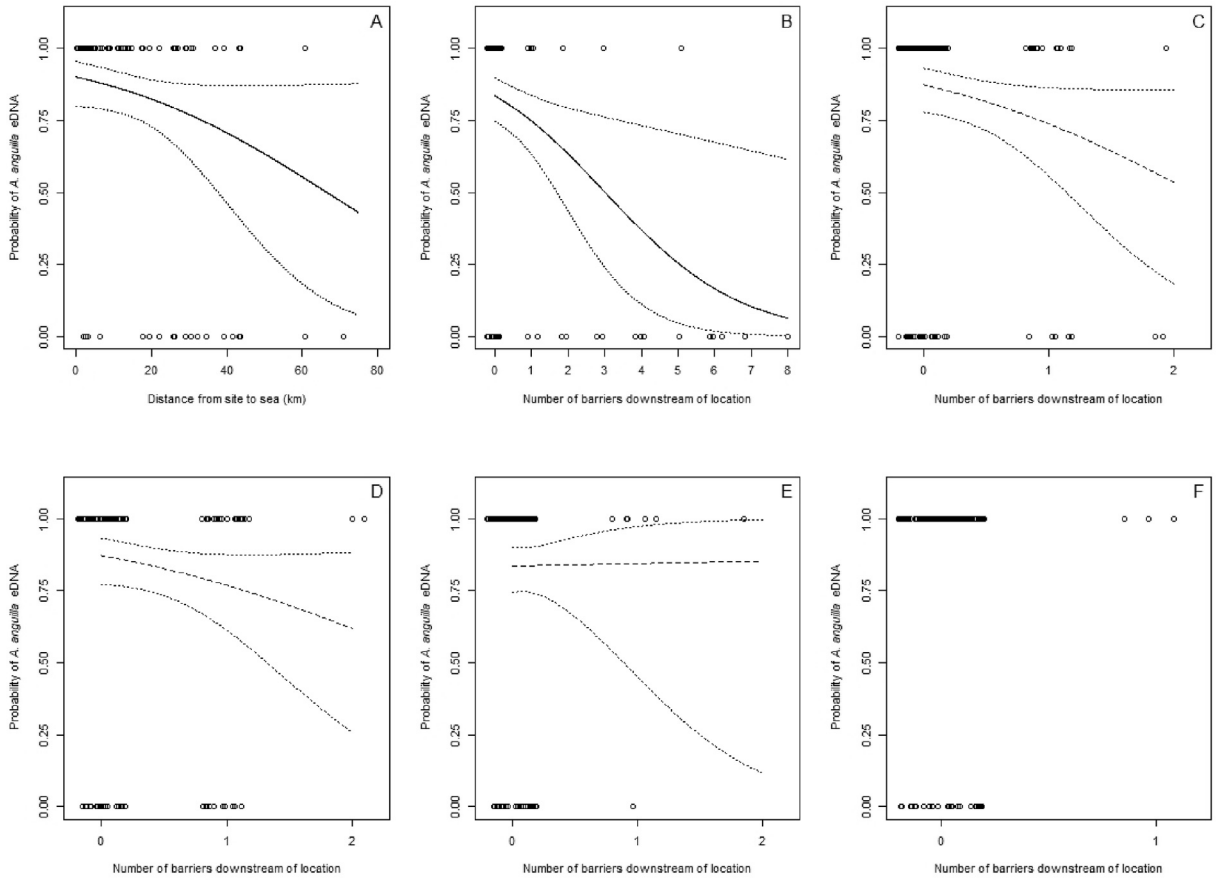

Fig. 1:. Predicted change in probability of detecting *A. anguilla* eDNA with the different predictor variables. Solid and dashed lines represent mean probability of significant and non-significant explanatory variables, respectively. Dotted lines represent the 95% confidence interval limits. Circles show observed data (positive or negative eDNA sample). A small amount of random noise is added to the observed data along the x-axis in order to prevent overplotting. A: Distance to sea in km, B: Number of hydroelectric power stations, C: Number of natural barriers, D: Number of pipelines, tunnels or bridges, E: Number of basins, F: Number of Fish ladders. Due to lack of data on fish ladders the model with this variable did not converge, and therefore only observed data are presented here.

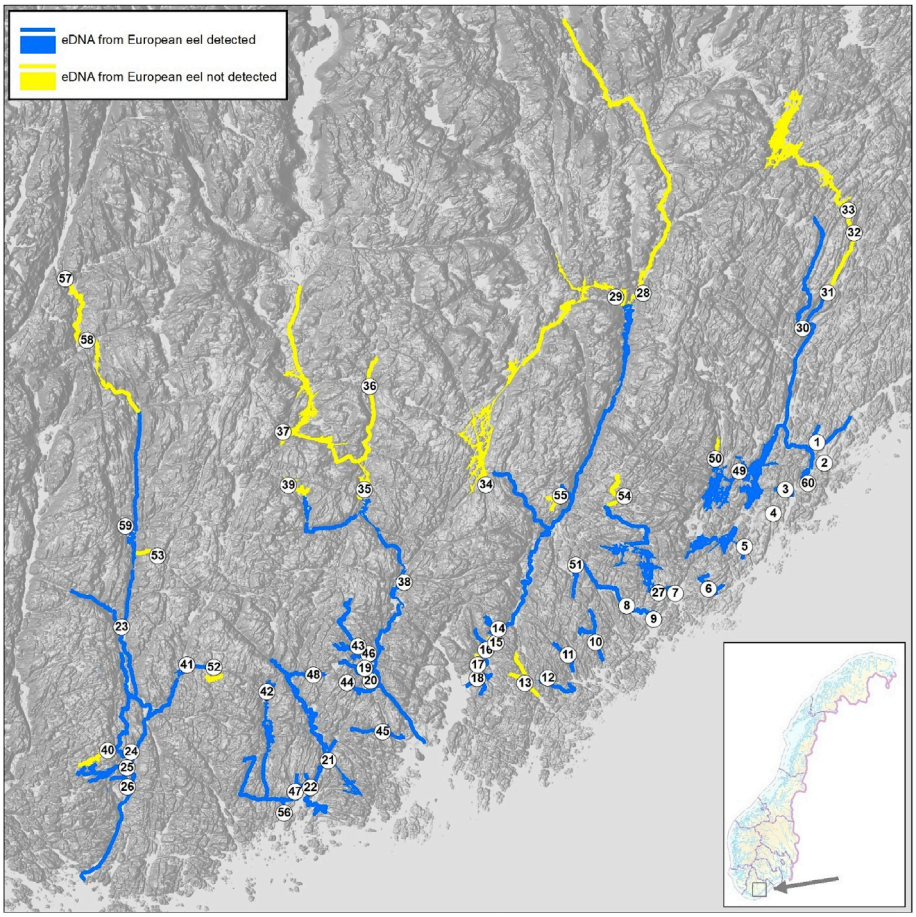

Fig. 2. Water samples collected at 60 locations in the county Agder, south of Norway. Watercourses where eDNA from European eel is detected are colored blue. Watercourses where eDNA from European eel is not detected are colored yellow. Illustration: Per Ø. Gustavsen. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

from European eel was not detected in the connecting waters upstream (at locality 29). Also, eDNA from eels was only recorded in one of the three samples in the PCR-triplicate upstream of location 34. Site 58 is near a basin at a hydroelectric power station. The downstream sample (eel detected) was collected downstream of the basin and upstream sample (eel not detected) was, due to difficult terrain, collected some hundred meters above the basin. The river was flowing slowly above the basin with no barriers. Therefore, the change in color at this site in Fig. 2 is placed by the barrier, not the upstream sample collection site. Detailed data for every location is found in Table 1, Appendix A.

The DNA sequence of a selected amplicon matched 100% with the corresponding region of the *cytb* gene of *A. anguilla* found in the NCBI GenBank (data not shown). There was a low degree of homology between the primers (Alcyt forward and Alcyt reverse) and probe with the corresponding sequence of other species that might be found in the same locations (Table 2, Appendix A) which secures a high degree of uniqueness. eDNA from trout was detected in 25 of the 26 water samples selected as controls. The concentration and purity of the samples analyzed in the spectrophotometer vary but had acceptable values (data not shown).

4. Discussion

4.1. Water basins connected to hydroelectric power stations constitute the greatest upstream migration challenge for European eel

We have shown that in general, as distance from the sea to a given location decreases the probability to record eel eDNA increases. In addition, the probability of detecting eDNA from European eel decreases significantly with number of hydroelectric power stations and their water basins, even when the effect of distance to sea is accounted for. The probability of detecting eDNA from eels upstream of seven of such barriers are close to zero. Therefore, these constructions seem to constitute upstream migration challenges for eels.

There are four major watercourses examined in this study in which eDNA from European eel is detected to a specific border upstream. Location 31 is a hydroelectric power station called Evenstad (~26 km from the sea) where eels are detected downstream but not upstream of the construction. This indicates that eel migration is prohibited. eDNA from European eel is not recorded in the upstream samples of locations 32 and 33 either, which are upstream from location 31.

In the second major watercourse, eDNA from eels is not detected in the upstream samples of sites 28 and 29. These sites are located upstream of the hydroelectric power station Hanefoss (~43 km from the sea), which therefore constitutes a passage barrier. Location 28 and 29, in addition to 34, empties into the same river downstream (Topdalselva). Even though there is approximately 6–9 km between sites 28–29 and 34, they are also connected by waterways upstream. Therefore, the power station Hanefoss also prohibits eel migration to location 34. However, it is also possible for eels to reach site 34 from the opposite direction (where the upstream sample is collected), which has possible natural barriers downstream. eDNA from European eel is detected in only one of three samples in the PCR-triplicate upstream of location 34, which may indicate low eel eDNA-concentration and a possible low abundance of eels. It is likely that some eels can migrate to this water area while most are unable.

At location 35, in the third major watercourse, eDNA from European eel is detected downstream of the power station Nomeland (~35 km from the sea). This indicates that eel migration is prohibited. There are, however, two additional stations further downstream the river system—Steinsfoss and Hunsfoss—where eDNA from eels is recorded upstream. These constructions are likely to cause challenges for migrating eels, even though some can pass.

In the fourth major watercourse in this study, we have detected a potential barrier hindering eel migration downstream of location 58. This location is linked to the power stations Bjelland and Håverstad (~61 km from the sea). eDNA from European eel is not recorded at location 57 either, which is upstream from location 58. Downstream of the mentioned power stations, there is an additional station called Laudal where eels can pass.

4.2. Constructions can constitute passage barriers even though eDNA from eels is recorded upstream

European eel can pass some of the power stations and water basins but are prohibited by others, probably due to differences between the constructions and between individual eels. The different designs of the structures, such as high or low walls of the water basins, can create distinct degrees of challenges for migrating eels. In addition, some power stations will have possibilities to pass by alternative routes, e.g., nearby streams or fish ladders, while some will not. Individual differences between eels, especially body size, will also affect their ability to migrate past the barriers. Obstacles creating high water velocity will prevent migration of the smallest individuals, and even barriers taller than 50–60% of the eel's body length is likely to prohibit migration (Thorstad et al., 2010). The differences between the constructions and between individual eels may be the reason why eel abundance decreases with number of hydroelectric power stations and their basins. Accordingly, some constructions limit migration despite the detection of eDNA from the species upstream. Knowledge regarding biomass of eels upstream of the installations could illuminate the degree of challenges connected to each migration barrier. Still, such information is not obtained in this study. Quantification of populations by eDNA has been performed by comparing eDNA concentration with biomass data from fishing or trapping methods. Such correlations are found in studies of different species, such as in the case of Japanese eel (*Anguilla japonica*), where a weak but significant correlation between eDNA concentration and biomass of eels was determined by electrofishing (Itakura et al., 2019). Another example is relationship between "eDNA rates" and six years of mark-recapture population estimates for eulachon (*Thaleichthys pacificus*) (Pochardt et al., 2020). However, this type of quantification is still debated (Lacoursiere-Roussel et al., 2016). Several studies do not find significant correlations between concentration and biomass, such as in a study of brown trout in the river Wehebach in Germany (Deutschmann et al., 2019). Estimating biomass by eDNA in water can be biased by multiple factors, e.g. differences in eDNA shedding among species, water flow, temperature, microbial activity, UV-light, in addition to different match with primers, and technical parameters (e.g., polymerase mixes and the number of PCR cycles) (Ficetola et al., 2019; Strickler et al., 2015). When estimating the abundance of a new set of species, it could be useful to calibrate the relationship between eDNA and species abundance, for instance by traditional methods (Ficetola et al., 2019). Future knowledge and development of eDNA-methods that could quantify populations would be highly valuable in conservation efforts connected to migration barriers.

4.3. Few migration barriers associated with pipelines, tunnels, bridges, basins and natural barriers

The probability of detecting eel eDNA decreases slightly with number of pipelines, tunnels or bridges, in addition to natural barriers, but not significantly. The number of basins had no effect on the probability of detecting eDNA from European eel, and there was not enough data regarding fish ladders in this study to draw a conclusion. Eel eDNA is detected in most locations upstream from the human made constructions (pipelines, tunnels, bridges and basins), which are mostly located about 0–25 km from the sea. Only in 5 of these 30 locations, eDNA from European eel is not detected. This means that the majority of these potential barriers do not prohibit the migration of eels upstream.

However, since eel spend approximately 20 years in the rivers and lakes, individuals might have migrated upstream from construction before it was built. Thus, eel might be recorded by eDNA analysis upstream of a potential barrier, and one may erroneously conclude that the barrier does not stop eel migration. European eel are, to a certain degree, able to migrate (horizontally and vertically) on a moist substrate (Vøllestad, 1992) and could therefore reach upstream waters by alternative

routes like wet grass or flood streams. In these cases, the constructions would probably prohibit the migration of other species, like Atlantic salmon (*Salmo salar*) and trout, which depend on flowing water.

Migration barriers could explain the 5 locations where eDNA from European eel is not detected. However, only at sites 15 and 50 in the category of pipelines, tunnels, and bridges is eDNA detected downstream of the specific barrier but not upstream. These locations could have barriers that prohibit the migration of eels. At the other locations in this category, eDNA could be detected neither upstream nor downstream from the constructions. Therefore, it is not possible to conclude whether these are migration barriers or not. Lack of suitable habitat could be the answer in locations 2 and 13 where the streams are tiny and do not lead to any lake upstream. The water quality could be the reason on site 17, where there is a known high concentration of aluminum (NVE, 2019).

The remaining locations (39, 40, 52, 53, 54, and 55) where eDNA from eels could not be detected are categorized as natural or unknown barriers. These are all located a bit further from the sea or main river and could have natural barriers downstream (e.g., steep waterfalls). The idea that low population density leads to low competition for resources and thus does not drive individuals further upstream (Ibbotson et al., 2002) could also explain why eDNA from eels is not detected in these locations. However, we have not focused on these issues in the study.

Trout is recorded in 25 of 26 selected locations, which indicates acceptable quality of the isolated eDNA. At the site where eDNA from trout and eel is not detected (site 2), the eDNA-concentration and purity were adequate, so the area was probably not suitable for these species.

Furthermore, European eel may be present in an area where eDNA is not recorded due to the long distance between eels and the location of sample collection, which can lead to low (not detectable) eDNA-concentrations. However, the eDNA-method is sensitive, and studies have detected eDNA 12 km downstream from the source (Shogren et al., 2017). Errors regarding potential low eDNA-concentrations are also minimized in this study because several sub-samples along a section of every location were collected (and pooled into one sample) at every possible site. Therefore, it is likely that eels are present in locations where eDNA is detected and not where eDNA is not detected. In future studies, one might collect samples both at a higher number of locations in a specific area, as well as regular samples from each site throughout the season.

5. Conclusions

The probability of detecting eDNA from European eel decreases with number of hydroelectric power stations and their associated water basins, even when distance to the sea is accounted for. There is a clear border to where eel eDNA could no longer be detected in the major watercourses along the coast of southern Norway, and this border seem to be associated with the presence of hydroelectric power stations and their associated water basins. Therefore, it is likely that these constructions prohibit the migration of eels. To continue with and improve the conservation effort for the European eel, we recommended facilitating migration past upstream and downstream human-made barriers. We also advise having a precautionary approach and facilitating passage where constructions are likely to limit migration, even if eels are recorded upstream.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A

Table 1
Localities where water samples are collected and results of real-time PCR-triplicate ("+" indicates that eDNA is detected, and "-" indicates that eDNA is not detected), location name, type of barrier, distance to sea and GPS-coordinates.

Location number, upstream/ downstream	Real-time PCR triplicate *A.anguilla*	Location name	Category/type of barrier	Distance to sea (km)	GPS-coordinates
1 downstream	+ + +	Nidelva			
1 upstream	+ + +		Pipelines, tunnels, and bridges	4,77	59.4099°N 8.6566°E
2 downstream	- - -	Birketveit			
2 upstream	- - -		Pipelines, tunnels, and bridges	2,49	58.3933°N 8.6673°E
3 downstream	+ + +	Sævelibekken			
3 upstream	+ + +		Fish ladder	1,12	58.3689°N 8.6047°E
4 downstream	+ + +	Frivoll			

(*continued on next page*)

Table 1 (*continued*)

Location number, upstream/ downstream	Real-time PCR triplicate *A.anguilla*	Location name	Category/type of barrier	Distance to sea (km)	GPS-coordinates
4 upstream	+ + +		Pipelines, tunnels, and bridges	2,51	58.3488°N 8.5855°E
5 downstream	+ + +	Reddalskanalen			
5 upstream	+ + +		Pipelines, tunnels, and bridges	3,42	58.3192°N 8.5388°E
6 downstream	+ + +	Pendalen			
6 upstream	+ + +		Pipelines, tunnels, and bridges	1,42	58.2822°N 8.4784°E
7 downstream	+ + +	Kaldvell			
7 upstream	+ + +		Fish ladder	0,20	58.2796°N 8.4200°E
8 downstream	+ + -	Moelva			
8 upstream	+ + -		Pipelines, tunnels, and bridges	5,21	58.263°N 8.344°E
9 downstream	+ + +	Tingsaker			
9 upstream	+ + +		Pipelines, tunnels, and bridges	0,04	58.2552°N 8.3881°E
10 downstream	+ + -	Fjelldalselva			
10 upstream	+ + -		Pipelines, tunnels, and bridges	2,36	58.2348°N 8.2936°E
11 downstream	+ + +	Steindalsbekken		1,92	
11 upstream	+ + +		Pipelines, tunnels, and bridges	1,92	58.2227°N 8.2503°E
12 downstream	+ + -	Urevann			
12 upstream	+ + +		Fish ladder	4,48	58.2032°N 8.2150°E
13 downstream	- - -	Studedalen			
13 upstream	- - -		Pipelines, tunnels, and bridges	1,94	59.1988°N 8.1758°E
14 downstream	+ + +	Blegehåla			
14 upstream	+ + +		Pipelines, tunnels, and bridges	6,75	58.236°N 8.128°E
15 downstream	+ - -	Østerbekk			
15 upstream	- - -		Pipelines, tunnels, and bridges	6,40	58.2367°N 8.1288°E
16 downstream	+ + +	Prestebekken			
16 upstream	+ + +		Pipelines, tunnels, and bridges	5,15	58.2290°N 8.1165°E
17 downstream	- - -	Bøen			
17 upstream	- - -		Pipelines, tunnels, and bridges	3,16	58.2°N 8.09°E
18 downstream	+ + +	Vesbekken			
18 upstream	+ + +		Pipelines, tunnels, and bridges	1,60	58.2031°N 8.0986°E
19 downstream	+ + +	Augland			
19 upstream	+ + +		Pipelines, tunnels, and bridges	8,89	58.20087°N 7.9254°E
20 downstream	+ + +	Øvre Strai			
20 upstream	+ + -		Pipelines, tunnels, and bridges	8,90	58.2055°N 7.9251°E
21 downstream	+ + +	Rosslandsbekken			
21 upstream	+ + +		Pipelines, tunnels, and bridges	12,97	58.1290°N 7.8554°E
22 downstream	+ + +	Kleplandsbekken			
22 upstream	+ + +		Pipelines, tunnels, and bridges	8,62	58.1045°N. 7.8232°E
23 downstream	+ + +	Mjålandsbekken			
23 upstream	+ + +		Pipelines, tunnels, and bridges	29,30	58.2420°N 7.5117°E
24 downstream	+ + +	Smelandsbekken			
24 upstream	+ + +		Pipelines, tunnels, and bridges	14,90	58.1296°N. 7.5300°E
25 downstream	+ - -	Fodnebøbekken			
25 upstream	+ + -		Pipelines, tunnels, and bridges	13,84	58.1197°N. 7.5255°E
26 downstream	+ + +	Vådnebekken			
26 upstream	+ + +		Pipelines, tunnels, and bridges	11,29	58.1022°N. 7.5307°E

Table 1 (*continued*)

Location number, upstream/ downstream	Real-time PCR triplicate *A.anguilla*	Location name	Category/type of barrier	Distance to sea (km)	GPS-coordinates
27 downstream	+ + +	Austre Grimevannet			
27 upstream	+ + +		Basin	1,82	58.2789°N 8.4017°E
28 downstream	+ - -	Herefoss			
28 upstream	- - -		Hydroelectric power stations and associated water basins	43,60	58.5386°N 8.3546°E
29 downstream	+ - -	Hanefoss			
29 upstream	- - -		Hydroelectric power stations and associated water basins	43,30	58.5356°N 8.3317°E
30 downstream	+ + +	Songeelva			
30 upstream	+ + +		Pipelines, tunnels, and bridges	21,94	58.5110°N 8.6330°E
31 downstream	+ + +	Evenstad			
31 upstream	- - -		Hydroelectric power stations and associated water basins	25,88	58.54°N 8.6738°E
32 downstream	- - -	Bøylefoss			
32 upstream	- - -		Hydroelectric power stations and associated water basins	32,40	58.5924°N 8.7185°E
33 downstream	- - -	Kilandsfjorden			
33 upstream	- - -		Hydroelectric power stations and associated water basins	34,70	58.6129°N 8.7074°E
34 downstream	+ + +	Oggevatn			
34 upstream	+ - -		Hydroelectric power stations and associated water basins	27,01	58.3703°N 8.1094°E
35 downstream	+ + +	Nomelandsdammen			
35 upstream	- - -		Hydroelectric power stations and associated water basins	30,42	58.3829°N 7.9103°E
36 downstream	- - -	Birketveitstjønna			
36 upstream	- - -		Hydroelectric power stations and associated water basins	41,66	58.4550°N 7.9127°E
37 downstream	- - -	Kilefjorden			
37 upstream	- - -		Hydroelectric power stations and associated water basins	43,30	58.4141°N 7.7711°E
38 downstream	+ + +	Venneslafjorden			
38 upstream	+ + +		Hydroelectric power stations and associated water basins	17,82	58.2852°N 7.9774°E
39 downstream	+ + +	Eikelandsvatn			
39 upstream	- - -		Natural barriers/unknown	39,35	58.3678°N 7.7808°E
40 downstream	+ + +	Kårstøylveien			
40 upstream	- - -		Natural barriers/unknown	17,53	58.1341°N 7.4980°E
41 downstream	+ + +	Høyevatnet			
41 upstream	+ + +		Natural barriers/unknown	26,48	58.2099°N 7.6204°E
42 downstream	+ + +	Birkelandsvannet			
42 upstream	+ + -		Natural barriers/unknown	14,01	58.1877°N 7.7521°E
43 downstream	+ + +	Sagtjønn			
43 upstream	+ - -		Basin	12,45	58.2262°N 7.9058°E
44 downstream	+ + +	Aurebekkvatnet			
44 upstream	+ + +		Natural barriers/unknown	10,90	58.1972°N 7.8839°E
45 downstream	+ + +	Grotjønn			
45 upstream	+ + +		Basin	3,01	58.1550°N 7.9421°E
46 downstream	+ + +	Stemmen			
46 upstream	+ + -		Basin	11,70	58.2240°N 7.9144°E
47 downstream	+ + +	Stemvann			
47 upstream	+ + +		Natural barriers/unknown	3,95	58.1078°N 7.8052°E
48 downstream	+ + +	Røyrvatnet			
48 upstream	+ + -		Natural barriers/unknown	31,34	58.2033°N 7.8291°E
49 downstream	+ + +	Syndle			

(*continued on next page*)

Table 1 (*continued*)

Location number, upstream/ downstream	Real-time PCR triplicate *A.anguilla*	Location name	Category/type of barrier	Distance to sea (km)	GPS-coordinates
49 upstream	+ - -		Pipelines, tunnels, and bridges	17,52	58.3849°N 8.5300°E
50 downstream	+ + +	Holvannet			
50 upstream	- - -		Pipelines, tunnels, and bridges	22,05	58.3957°N 8.4882°E
51 downstream	+ + -	Moelva			
51 upstream	+ + -		Natural barriers/unknown	9,03	58.3020°N 8.2577°E
52 downstream	+ + -	Heddekjerrvatnet			
52 uptream	- - -		Natural barriers/unknown	29,20	58.2084°N 7.6640°E
53 downstream	+ + +	Folltjørn			
53 upstream	- - -		Natural barriers/unknown	39,39	58.3041°N 7.5680°E
54 downstream	+ + +	Hundlandsvatnet			
54 upstream	- - -		Natural barriers/unknown	19,51	58.3622°N 8.3380°E
55 downstream	+ + +	Høgkleivvatnet			
55 upstream	- - -		Natural barriers/unknown	26,07	58.3620°N 8.2331°E
56 downstream	+ + +	Lundeelva			
56 upstream	+ + +		Natural barriers/unknown	0,47	58.0837°N 7.7846°E
57 downstream	- - -	Eikerapen			
57 upstream	- - -		Hydroelectric power stations and associated water basins	71,12	58.5440°N 7.4065°E
58 downstream	+ + +	Øre			
58 upstream	- - -		Hydroelectric power stations and associated water basins	60,86	58.4907°N 7.4431°E
59 downstream	+ + +	Mannflåvannet			
59 upstream	+ + +		Natural barriers/unknown	36,92	58.3306°N 7.5132°E
60 downstream	+ + +	Temse			
60 upstream	+ + +		Natural barriers/unknown	8,39	58.3822°N 8.6415°E

Table 2
Primers Alcyt-F, Alcyt-R and probe compared to mtDNA from species possible to find at location 1–60. The comparisons were conducted with Clustal Omega, and the sequences where found in the NCBI's Genbank. Grey areas in the sequences indicates nucleotides that are similar between the primers and probe of *A. anguilla* and the other species.

Species	Match between primers and probe of *A. anguilla* and mtDNA of the other species	
Brown trout	Probe	T**TCA**TTA**TCATGCTCACC**
Salmo trutta	*S. trutta*	G**TCC**TAT**TCCTGCTCACC**
GenBank: MF621760.1	Alcytb −F	**CACCCATACTTCTC**C**TACAAAGACCT**A
	S. trutta	**CACCCATACTTCTC**A**TACAAAGACCT**
	Alcytb −R	**TCTGGGTCTCCAAGCA**G**GTT**
	S. trutta	**AAGCAAGTT**
Arctic char	Probe	**TTCATT**ATCATGCTCACC
Salvelinus alpinus	*S. alpinus*	**TTCATT**TC**C**
GenBank: MF621743.1	Alcytb-F	**CACCC**A**TACTTCTC**C**TACAAAGACCT**A
	S. alpinus	**CACCCC**T**ACTTCTC**G**TACAAAGACCT**C
	Alcytb-R	**TCTGGG**T**CTCC**A**AGCAGGTT**
	S. alpinus	**TCTGG**C**TCTCCCA**
River lamprey	Probe	**TTCATT**A**TCATGCTCAC**C
Lampetra fluviatilis	*L. fluviatilis*	**TTCATT**T**TCATGATCAC**A
GenBank: Y18683.1	Alcytb −F	**CACCCATACTTCTCCTACAAAGACCTA**
	L. fluviatilis	**CACCCATACTTCTCTTTCAAAGACATT**
	Alcytb −R	**T**CT**GGG**T**CTCC**AAGCAGGTT
	L. fluviatilis	**T**TG**GGGCCTCC**
Common minnow	Probe	**TTCATT**ATC**ATGC**TCA**CC**
Phoxinus	*P. phoxinus*	**TTAATT**GCC**ATGC**CCT**CC**
GenBank: Y18683.1	Alcytb −F	**CA**CCCATA**C**TTCTCCTA**C**AAAGACCTA
	P. phoxinus	**CA**TCCATATTTTTCTTATAAAGACCTT

Table 2 (*continued*)

Species	Match between primers and probe of *A. anguilla* and mtDNA of the other species	
	Alcytb-R	TCTGGGTCT**CCAAGCAG**GTT
	P. phoxinus	**CCAAGCAG**TTA
European whitefish	Probe	T**TCATTATCATGCTCACC**
Coregonus lavaretus	*C. lavaretus*	G**TCCTTACCTTGCTCACC**
GenBank: AB034824.1	Alcyt-F	**CACCCATACTTCTCCTACAAAGACCT**A
	C. lavaretus	**CACCCCTACTTCTCATACAAAGACCT**G
	Alcyt-R	TCTG**GGTCTCCAAGCAGGTT**
	C. lavaretus	TAT**GGGCCCCCA**TGCCA**TT**
European smelt	Probe	T**TCATTATCATGCTCACC**
Osmerus eperlanus	*O. eperlanus*	**CTTATTATCCAGATCACC**
GenBank: MH238073.1	Alcyt-F	CACCCATAC**TTCTCCTACAA**AG**ACCT**A
	O. eperlanus	A**TTCAACTACAAGAACCT**
	Alcyt-R	**TCTGGGTCTCCAAGCAGGTT**
	O. eperlanus	**TCTTGCCCTAAAAGTGGGTT**
European perch	Probe	**TTCATTATCATGCTCA**CC
Perca fluviatilis	*P. fluviatilis*	**TTCATTTTCACGACCA**
GenBank: VHII01000304.1	Alcyt-F	**CACCCATACTTCTCCTACAAAGACCT**A
	P. fluviatilis	**CATCCTTATTTTTCTTACAAAGACCT**C
	Alcyt-R	T**CTGGGTCT**CCAAGCAGGTT
	P. fluviatilis	C**CGGGGTCT**A

References

Anonymous, 2007. Council Regulation (EC) No 1100/2007 of 18 September 2007 establishing measures for the recovery of the stock of European eel. Retrieved from Official Journal of the European Union L. http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/.

Arai, T., 2016. Biology and Ecology of Anguillid Eels. CRC Press.

Belpaire, C.G.J., Goemans, G., Geeraerts, C., Quataert, P., Parmentier, K., Hagel, P., De Boer, J., 2009. Decreasing eel stocks: survival of the fattest? Ecol. Freshw. Fish 18 (2), 197–214. https://doi.org/10.1111/j.1600-0633.2008.00337.x.

NVE (Cartographer), 2019. Vann-nett portal. Retrieved from. https://vann-nett.no/portal/#/mainmap.

Castonguay, M., Hodson, P.V., Moriarty, C., Drinkwater, K.F., Jessop, B.M., 1994. Is there a role of ocean environment in American and European eel decline? Fish. Oceanogr. 3 (3), 197–203.

Degerman, E., Tamario, C., Watz, J., Nilsson, P.A., Calles, O., 2019. Occurrence and habitat use of European eel (Anguilla anguilla) in running waters: lessons for improved monitoring, habitat restoration and stocking. Aquat. Ecol. 53 (4), 639–650. https://doi.org/10.1007/s10452-019-09714-3.

Dekker, W., Beaulaton, L., 2016. Climbing back up what slippery slope? Dynamics of the European eel stock and its management in historical perspective. ICES J. Mar. Sci. 73 (1), 5–13. https://doi.org/10.1093/icesjms/fsv132.

Deutschmann, B., Muller, A.K., Hollert, H., Brinkmann, M., 2019. Assessing the fate of brown trout (Salmo trutta) environmental DNA in a natural stream using a sensitive and specific dual-labelled probe. Sci. Total Environ. 655, 321–327. https://doi.org/10.1016/j.scitotenv.2018.11.247.

Drouineau, H., Durif, C., Castonguay, M., Mateo, M., Rochard, E., Verreault, G., Lambert, P., 2018. Freshwater eels: a symbol of the effects of global change. Fish Fish. 19 (5), 903–930. https://doi.org/10.1111/faf.12300.

Feunteun, E., 2002. Management and restoration of European eel population (Anguilla anguilla): an impossible bargain. Ecol. Eng. 18 (5), 575–591. https://doi.org/10.1016/s0925-8574(02)00021-6.

Ficetola, G.F., Manenti, R., Taberlet, P., 2019. Environmental DNA and metabarcoding for the study of amphibians and reptiles: species distribution, the microbiome, and much more. Amphibia-Reptilia 40 (2), 129–148. https://doi.org/10.1163/15685381-20191194.

Friedland, K.D., Miller, M.J., Knights, B., 2007. Oceanic changes in the Sargasso Sea and declines in recruitment of the European eel. ICES J. Mar. Sci. 64 (3), 519–530. https://doi.org/10.1093/icesjms/fsm022.

Ibbotson, A., Smith, J., Scarlett, P., Aprhamian, M., 2002. Colonisation of freshwater habitats by the European eel Anguilla anguilla. Freshw. Biol. 47 (9), 1696–1706. https://doi.org/10.1046/j.1365-2427.2002.00930.x.

ICES, 2019. Joint EIFAAC/ICES/GFCM Working Group on Eels (WGEEL) (2618-1371). Retrieved from.

Itakura, H., Wakiya, R., Yamamoto, S., Kaifu, K., Sato, T., Minamoto, T., 2019. Environmental DNA analysis reveals the spatial distribution, abundance, and biomass of Japanese eels at the river-basin scale. Aquat. Conserv. Mar. Freshw. Ecosyst. 1–13.

Jacoby, D., Gollock, M., 2014. Anguilla anguilla. The IUCN red list of threatened species. Retrieved from. http://www.iucnredlist.org/details/60344/0.

Kettle, A.J., Vollestad, L.A., Wibig, J., 2011. Where once the eel and the elephant were together: decline of the European eel because of changing hydrology in southwest Europe and northwest Africa? Fish Fish. 12 (4), 380–411. https://doi.org/10.1111/j.1467-2979.2010.00400.x.

Lacoursiere-Roussel, A., Dubois, Y., Normandeau, E., Bernatchez, L., 2016. Improving herpetological surveys in eastern North America using the environmental DNA method. Genome 59 (11), 991–1007. https://doi.org/10.1139/gen-2015-0218.

Laffaille, P., Lasne, E., Baisez, A., 2009. Effects of improving longitudinal connectivity on colonisation and distribution of European eel in the Loire catchment, France. Ecol. Freshw. Fish 18 (4), 610–619. https://doi.org/10.1111/j.1600-0633.2009.00378.x.

Ogden, J.C., 1970. Relative abundance, food habits, and age OF AMERICAN eel, Anguilla-rostrata (lesueur), IN certain new-Jersey streams. Trans. Am. Fish. Soc. 99 (1), 54. https://doi.org/10.1577/1548-8659(1970)99<54:Rafhaa>2.0.Co;2.

Pochardt, M., Allen, J.M., Hart, T., Miller, S.D.L., Yu, D.W., Levi, T., 2020. Environmental DNA facilitates accurate, inexpensive, and multiyear population estimates of millions of anadromous fish. Molecular Ecology Resources 20 (2), 457–467. https://doi.org/10.1111/1755-0998.13123.

Porcher, J.P., 2002. Fishways for Eels. Bulletin Francais De La Peche Et De La Pisciculture, vol. 364, pp. 147–155. https://doi.org/10.1051/kmae/2002099.

R Core Team, 2017. Vienna, Austria: R Foundation for Statistical Computing.

Rees, H.C., Maddison, B.C., Middleditch, D.J., Patmore, J.R.M., Gough, K.C., 2014. REVIEW: the detection of aquatic animal species using environmental DNA – a review of eDNA as a survey tool in ecology. J. Appl. Ecol. 51 (5), 1450–1459. https://doi.org/10.1111/1365-2664.12306.

Righton, D., Westerberg, H., Feunteun, E., Okland, F., Gargan, P., Amilhat, E., Aarestrup, K., 2016. Empirical observations of the spawning migration of European eels: the long and dangerous road to the Sargasso Sea. Science Advances 2 (10). https://doi.org/10.1126/sciadv.1501694.

Shogren, A.J., Tank, J.L., Andruszkiewicz, E., Olds, B., Mahon, A.R., Jerde, C.L., Bolster, D., 2017. Controls on eDNA movement in streams: transport, retention, and resuspension. Sci. Rep. 7 https://doi.org/10.1038/s41598-017-05223-1.

Strickler, K.M., Fremier, A.K., Goldberg, C.S., 2015. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biol. Conserv. 183, 85–92. https://doi.org/10.1016/j.biocon.2014.11.038.

Tesch, F., 1977. The Eel: Biology and Management of Anguillid eels.(J. Greenwood, Translator). John Wiley & Sons, New York, New York, USA.
Thomsen, P.F., Willerslev, E., 2015. Environmental DNA - an emerging tool in conservation for monitoring past and present biodiversity. Biol. Conserv. 183, 4–18. https://doi.org/10.1016/j.biocon.2014.11.019.
Thomsen, P.F., Kielgast, J., Iversen, L.L., Moller, P.R., Rasmussen, M., Willerslev, E., 2012. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PloS One 7 (8). https://doi.org/10.1371/journal.pone.0041732.
Thorstad, B.E., Larsen, M., Hesthagen, T., Næsje, F.T., Poole, R., Aarestrup, K., Sandlund, T.O., 2010. Ål Og Konsekvenser Av Vannkraftutbygging (Retrieved from).
Vøllestad, L. A. (1992). Ålefisker. I Jonsson, B. & Semb-Johansen, A. (red) Norges dyr: J.W. Cappelens forlag.
White, E.M., Knights, B., 1997. Dynamics of upstream migration of the European eel, Anguilla anguilla (L.), in the Rivers Severn and Avon, England, with special reference to the effects of man-made barriers. Fish. Manag. Ecol. 4 (4), 311–324. https://doi.org/10.1046/j.1365-2400.1997.00050.x.

6. Paper 2

Estimating number of European eel (Anguilla anguilla) individuals using environmental DNA and haplotype count in small rivers

Silje Halvorsen, Lars Korslund, Morten Mattingsdal, Audun Slettan. 2023.

Ecology and Evolution. Volume 13, Issue 2, e9785.

(doi: https://doi.org/10.1002/ece3.9785)

Received: 11 May 2022 | Revised: 21 December 2022 | Accepted: 16 January 2023

DOI: 10.1002/ece3.9785

Ecology and Evolution Open Access WILEY

RESEARCH ARTICLE

Estimating number of European eel (*Anguilla anguilla*) individuals using environmental DNA and haplotype count in small rivers

Silje Halvorsen | **Lars Korslund** | **Morten Mattingsdal** | **Audun Slettan**

Faculty of Engineering and Science, University of Agder, Kristiansand, Norway

Correspondence
Silje Halvorsen, Faculty of Engineering and Science, University of Agder, Universitetsveien 25, 4630 Kristiansand, Norway.
Email: silje.halvorsen@uia.no

Abstract

Knowledge about population genetic data is important for effective conservation management. Genetic research traditionally requires sampling directly from the organism, for example tissue, which can be challenging, time-consuming, and harmful to the animal. Environmental DNA (eDNA) approaches offer a way to sample genetic material noninvasively. In attempts to estimate population size of aquatic species using eDNA, researchers have found positive correlations between biomass and eDNA concentrations, but the approach is debated because of variations in the production and degrading of DNA in water. Recently, a more accurate eDNA-approach has emerged, focusing on the genomic differences between individuals. In this study, we used eDNA from water samples to estimate the number of European eel (*Anguilla anguilla*) individuals by examining haplotypes in the mitochondrial D-loop region, both in a closed aquatic environment with 10 eels of known haplotypes and in three rivers. The results revealed that it was possible to find every eel haplotype in the eDNA sample collected from the closed environment. We also found 13 unique haplotypes in the eDNA samples from the three rivers, which probably represent 13 eel individuals. This means that it is possible to obtain genomic information from European eel eDNA in water; however, more research is needed to develop the approach into a possible future tool for population quantification.

KEYWORDS
Anguilla anguilla, conservation, environmental DNA, haplotype count, population quantification

TAXONOMY CLASSIFICATION
Conservation ecology

1 | INTRODUCTION

The decline in global freshwater biodiversity is greater than in most affected terrestrial ecosystems (Grooten & Almond, 2018). Effective conservation management of species relies on population genetic data, which can be challenging to collect. Genetic research traditionally requires collecting biological samples from the organism of interest after using fishing and trapping methods, which can be stressful and cause harm, discomfort, or death (Bearzi, 2000; Romero & Reed, 2005). These approaches can also be time-consuming

and challenging when examining rare or elusive species (Jerde et al., 2011). Environmental DNA (eDNA) approaches offer a way to sample genetic material noninvasively, causing no significant damage to the species or the habitats (Antognazza et al., 2019), often being more sensitive and economically beneficial (Itakura et al., 2019; Thomsen & Willerslev, 2015), while also being capable of detecting rare and elusive species (Takahara et al., 2020).

There have been several attempts to examine the detection probabilities of eDNA where the conclusion often is that eDNA provides a snapshot of the species composition in space and time. In the sea, killer whale (*Orcinus orca*) eDNA are detected up to 2 h after individuals were observed in the sampled area (Baker et al., 2018) and eDNA from caged white trevally (*Pseudocaranx dentex*) are detected 30 m from the source (Murakami et al., 2019). In freshwater systems, eDNA from caged trout in fishless streams is detected 239.5 m downstream from the source (Jane et al., 2015).

In the effort to estimate species population size, positive correlations are found between, for example, eDNA concentration and biomass of common carp (*Cyprinnus carpio*) (Takahara et al., 2012) or eDNA concentration and biomass of eels (*Anguilla japonica*) (Itakura et al., 2019). However, the effectiveness of such approaches is debated because of variations in eDNA production and degradation rates (Lacoursiere-Roussel et al., 2016), and several studies do not find significant correlations between observed biomass and eDNA quantity (Deutschmann et al., 2019). The explanation for the deviating results can be connected to the various factors affecting the eDNA concentration in water—both the shedding of eDNA by different species and individuals, the transport of eDNA in different environments, and the degrading of eDNA that varies with temperatures, microbial activities, and UV-radiation (Shogren et al., 2017; Strickler et al., 2015), as well as different sampling methods.

However, a new approach focusing on the DNA sequence differences between individuals using eDNA is emerging. Uchii et al. (2016) estimated the degree of invasion of non-native genotypes of common carp by eDNA, and Sigsgaard et al. (2016) used eDNA from seawater to study mitochondrial haplotypes of whale sharks (*Rhincodon typus*) and assessed the population structure. In addition, Parsons et al. (2018) developed an approach for generating population-specific mitochondrial sequence data from eDNA using seawater samples, and Adams et al. (2022) recovered haplotypes from New Zealand blackfoot pāua (*Haliotis iris*) from marine eDNA samples. Concerning the challenges associated with eDNA concentration surveys, more accurate information about population size can be estimated by examining the DNA sequence differences between individuals. Determining the number of haplotypes in DNA-regions with high genetic variability could be a tool for quantifying populations given sufficient genetic variation between individuals (Yoshitake et al., 2019). The D-loop (regulatory) region in the mitochondrial genome is a variable area with intraspecific mutations (Sigsgaard et al., 2016). Recently, Yoshitake et al. (2019) examined haplotype diversity in Japanese eels (*Anguilla japonica*) and estimated the number of individuals in a population by sequencing the mitochondrial D-loop region. It has been found that the D-loop region of the related species European eel (*Anguilla anguilla*) has a haplotype diversity of h = 0.995 (0.996 in the North Sea) (Ragauskas et al., 2014), which means that almost every individual has a unique DNA sequence in this region. Studying this DNA region is therefore suitable when aiming to distinguish between haplotypes and thus counting individuals in the area where water samples are collected.

Anguillid eels are catadromous and inhabit rivers, lakes, brackish water, the coast, and the sea (Thorstad et al., 2010). The European eel is currently labeled as critically endangered by the International Union for Conservation of Nature (IUCN) Red List (Pike & Gollock, 2020) and the Norwegian Red List. The species is affected by threats at numerous developmental phases of its complex life, including overfishing, illegal trade and aquaculture (Castonguay et al., 1994; Shiraishi & Crook, 2015), habitat loss and destruction (Halvorsen et al., 2020; Kettle et al., 2011), freshwater parasites (Feunteun, 2002), poisoning (Belpaire et al., 2009) in addition to ocean changes and global warming (Drouineau et al., 2018; Friedland et al., 2007). Despite conservation efforts by EU member countries in response to the European Commission Regulation EC 110/2007 (ICES, 2019), the stock is currently decreasing (Pike & Gollock, 2020), and protection of the species is needed.

In this study, we aim to use eDNA from water samples in an effort to estimate the number of *A. anguilla* individuals by examining haplotypes in the mitochondrial D-loop region and to experimentally examine whether eDNA-haplotype information collected from a water sample is compliant with the genomic haplotype information obtained from tissue samples of each individual. Second, we aim to examine how many different *A. anguilla* haplotypes we can find at selected locations in three different rivers.

2 | MATERIALS AND METHODS

2.1 | Genomic DNA and eDNA from 10 eel individuals

Ten eels were caught by electrofishing in the river Lilleelv, September 20, 2018. A tissue sample of each individual was gathered by a small fin-clip from the caudal fin and preserved in 96% ethanol. We then transferred every individual to an 80-L tank with approximately 60 L of well-oxygenated water from the river. After the 10 eels had been in the tank for 1 h, without any water replacement, a water sample from the tank (for now on referred to as TANK) was collected, and from the river itself (see description below), before the eels were released back into the river.

2.2 | Water sampling

We sampled water in the rivers Kleplandsbekken (for now on referred to as KLE, 58,1045°N. 7,8232°E) and Moelva (for now on referred to as MOE, 58,2552°N 8,3881°E) in June 2018 and Lilleelv (for now on referred to as LIL, 58,4429°N. 8,6908°E) in September

2018 in the county of Agder, in South Norway. The sampling and processing of samples followed method described by Halvorsen et al. (2020). Each sample consisted of 1 L surface water, which was stored on ice until filtration (within 5 h). New gloves were used for each sampling, and the bottles were rinsed in 10% chlorine followed by tap water before each sampling. Back in the laboratory, 300–1000 mL water (as much as possible) was filtrated through a 0.45 μm pore size cellulose nitrate filter (Thermo Scientific Nalgene) by an ILMVAC vacuum pump (GmbH). The filters were folded and stored at −20°C after filtration.

2.3 | DNA extraction

We used DNeasy® Blood and Tissue Kit (Qiagen) and bead beating to extract DNA from water samples using a method described by Thomsen et al. (2012). The same kit was used to extract DNA from the tissue samples following the protocol of the producer (Qiagen). After isolation, the eDNA was stored in microcentrifuge tubes at −20°C. We performed the eDNA isolation in a separate room from the PCR amplification. Every sample was analyzed by a spectrophotometer (NanoDrop™ One, Thermo Scientific) after isolation to examine purity and eDNA-concentration.

2.4 | PCR amplification

We amplified a 731 bp section of the mitochondrial D-loop region from *A. anguilla* from every DNA sample using PCR and specific primers (Table 1). The PCR products from the eDNA-samples were additionally amplified by a nested PCR with nested primers (Table 1) to improve sensitivity and specificity. The primers were designed with Primer-BLAST at the web page of the National Center for Biotechnology Information (NCBI) and the program Primer Express 3.0.1 (Thermo Fisher). The primers were tested for species-specificity by searching for homology to DNA sequences from species that could be found in the same area using Clustal Omega (European Bioinformatics Institute) and NCBI's GenBank. In order to avoid false haplotypes caused by erroneous inserted nucleotides in the PCR, a high-fidelity DNA polymerase was used.

The PCR-mix had the following ingredient concentrations: 1 x Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher), 0.9 μM AaD-F, and 0.9 μM AaD-R. Fifteen microliters PCR mix with 5 μL eDNA-template or 19 μL PCR mix with 1 μL genomic DNA template was transferred to a 0.1 mL Micro Fast Tube Strip (Thermo Fisher). We conducted PCR in a Veriti 96 Well Thermal Cycler PCR System (Applied Biosystem). The thermal condition of the PCR for the genomic DNA samples was as follows: 1 incubation of 98°C in 3 min, 40 cycles of 98°C in 3 s, 59°C in 30 s, 72°C in 30 s, and 1 incubation of 72°C in 5 m, and the thermal condition for the eDNA-samples was as follows: 1 incubation of 95°C in 5 min, 40 cycles of 95°C in 15 s, 57°C in 15 s, 72°C in 30 s, and 1 incubation of 72°C in 7 m. PCR-grade H_2O was used as template in a negative control reaction, and genomic DNA from *A. anguilla* was used as template in a positive control reaction. eDNA samples were amplified by nested PCR to increase the specificity and secure enough product for sequencing. The PCR mix had the same ingredient concentrations as described above, but with 1 μL of the PCR-products instead of the eDNA-templates, and the nested primers AaDN-F and AaDN-R replaced the original primers (Table 1, Appendix A). The nested PCR had the same temperature profile, but the initial denaturation step of 95°C lasted 10 min instead of 5 min.

Following amplification, we transferred 10 μL PCR product to a 1% agarose gel for electrophoresis in 30 m at 90 V to confirm a successful amplification.

TABLE 1 Primers and nested primers in 5′ to 3′ direction for the sequence in the D-loop region for *A. anguilla* (731 bp product).

Primers	AaD-F: CCTAGCGCTAAAAATCAGAGAGG AaD-R: TGGCAAACTTTTTAGAAGGTGTCT
Nested primers	**AaDN-F:** CGCTAAAAATCAGAGAGGAAAGATTT **AaDN-R:** ACTTTTTAGAAGGTGTCTCACATGTAA

2.5 | Preparation and sequencing

The PCR-templates were purified by PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen) following the producer's protocol and quantified using the Qubit dsDNA broad-range assay (Invitrogen). The amplicons from genomic DNA were Sanger sequenced by Eurofins Scientific (Ebersberg, Germany). The amplicons from eDNA-samples were pair-end sequenced (2×250 bp) with the Illumina MiSeq platform by Norwegian Sequencing Centre (Oslo, Norway).

2.6 | Analysis of Illumina sequencing data

We used cutadapt (Martin, 2011) to remove primers and nested primers. We used DADA2 to determine haplotype variants using denoising (Callahan et al., 2016). Briefly, the denoising algorithm estimates the sample-specific error rates for every possible nucleotide transversions and transition from the data and infers the sequence composition of the samples after convergence of the algorithm. In both methods, we used the default values (Tsuji, Miya, et al., 2020). In DADA2, we defined the following parameters when filtering reads and learning error rates: default expected error rate (maxEE = 2), minimum read length to 250 (minLen = 250), and truncated reads if any base had a quality score of 2 or less (truncQ = 2), and subsequently removed any truncated reads less than 250 (truncLen = 250). As the reads had mixed-orientation, the option "orient.fwd" was used specifying the five first bases of the forward primer. Chimeras were removed and the sequence table was constructed.

The effect of sequencing errors and false haplotypes can be mitigated by assessing sequence abundance per haplotype. We ignored haplotypes with less than 1% of total reads per sample (Tsuji, Maruyama, et al., 2020).

The four samples were processed simultaneously. The reads were filtered and trimmed using default settings. Approximately ~40% of the river data did not contain the nested primer and was excluded. Then, error rates were estimated using the learnErrors function and reads were corrected and assigned to the representative sequence community. Then, the forward and reverse reads were merged using mergePairs. As the primer design dot does not allow for overlapping reads, the justConcatenate option was set to true. Finally, chimeras were identified using the pooled strategy and removed. Here, the TANK sample contained 54% chimeras which were excluded.

2.7 | Sequence analysis

Sequences were aligned using default MAFFT (Katoh et al., 2002) and visualized in PopArt (Leigh & Bryant, 2015) using median joining. We also obtained 56 complete *Anguilla anguilla* mitogenomes (Jacobsen et al., 2014). Again, we aligned these using default MAFFT settings and edited the resulting alignment in JalView (Waterhouse et al., 2009) to remove gapped regions introduced by our study, leaving the D-region. We did not include sequences from Ragauskas et al. (2014) as our reverse sequences did not overlap with their reported sequences.

3 | RESULTS

3.1 | Sequencing data

In total, there were 592,761 reads, from the LIL (103,788), MOE (112,289), KLE (99,289) rivers, and the TANK (277,395) sample. After filtering and cleaning the data, 26,645 (MOE), 21,514 (LIL), 24,403 (KLE), and 46,341 (TANK) reads survived and were assigned to one of the 1059 representative sequences. Of these, 1059 representative sequences did 23 capture 99% of all quality-controlled reads.

3.2 | Haplotypes observed in the tank

Ten haplotypes were detected in the tank water, where 10 eel individuals from the river Lilleelv had been kept (Figure 1). Each of the 10 haplotypes had an identical match to the haplotype detected in the genomic DNA sample from each of the 10 eel-individuals.

3.3 | Haplotypes observed in the rivers

In total, 18 eel haplotypes were detected in the three water samples from rivers. Seven were detected in the eDNA sample from the river Lilleelv, and of these seven, five had an identical match among the 10 haplotypes found in the tank water (see haplotypes colored red in Figures 1 and 2). Four *A. anguilla* haplotypes were detected in the eDNA sample from Kleplandsbekken, and two in Moelva.

3.4 | Sequence analysis

Aligning detected D-region sequences against previously published mitogenomes revealed 81 segregating sites, 52 parsimony-informative sites, and a nucleotide diversity $n = 0.024$ (for access to dataset, see Mattingsdal (2022)). The alignment of the 18 unique haplotypes detected in the water sample from each of the three the rivers and in the water sample from the tank showed that there is no obvious genetic structure in the population. Diversity is high and

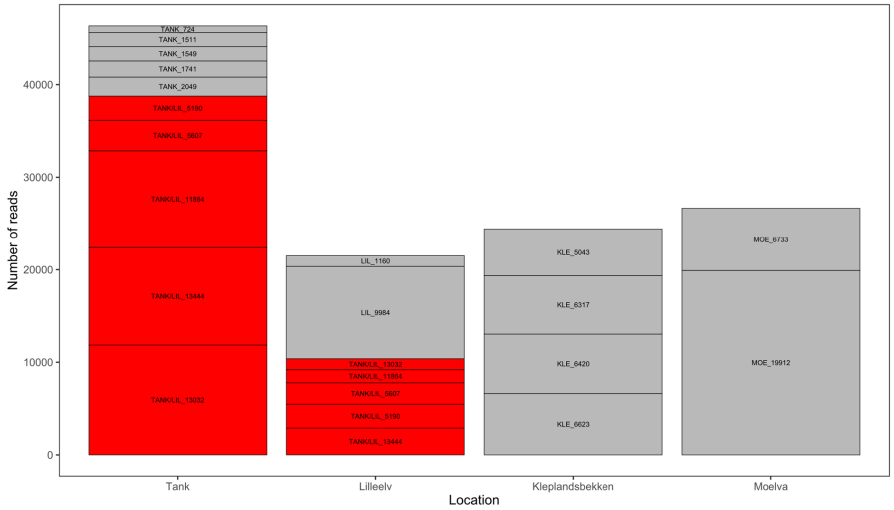

FIGURE 1 Detection of *Anguilla anguilla* haplotypes from four eDNA samples (tank with 10 eel individuals, and the rivers Lilleelv, Kleplandsbekken, and Moelva). Y-axis shows the number of reads assigned to the haplotypes. Each segment of a stacked bar represents a haplotype, and the height of the segment is proportional to the number of reads of that sample. The haplotype notation is "site"_"total number of reads" Ten unique haplotypes were observed in the tank (TANK), seven in the river Lilleelv (LIL), four in Kleplandsbekken (KLE), and two in Moelva (MOE). The five haplotypes that were detected both in the tank and in the Lilleelv sample are colored red.

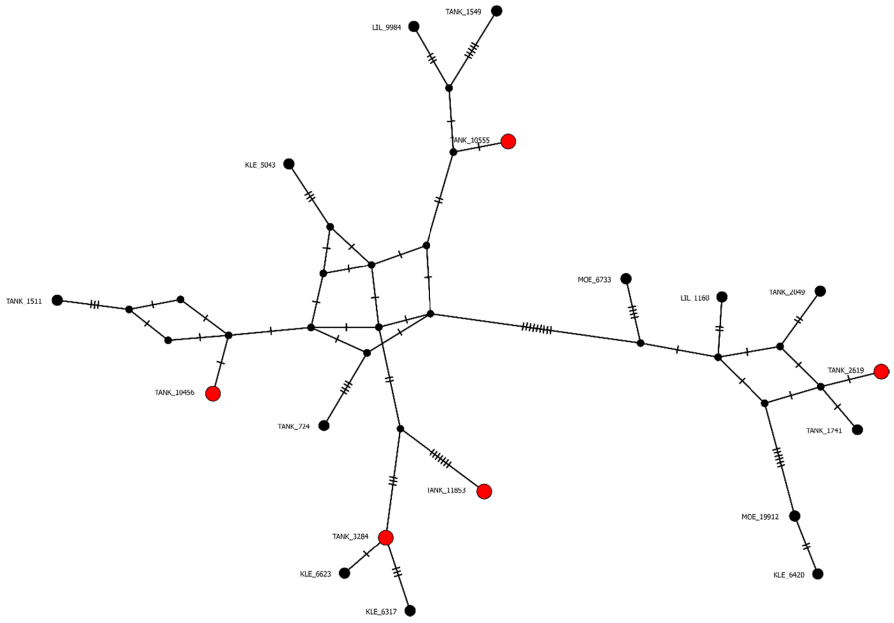

FIGURE 2 Haplotype network with the inferred and aligned sequences. Colors and notations are similar to that of Figure 1, and ticks on edges represent sequence changes.

haplotypes originating from the same river are not grouped together (see haplotype network in Figure 2). Of the 18 unique haplotypes identified, four were found to be identical to four of the 56 previously described *Anguilla anguilla* mitogenomes (Jacobsen et al., 2014).

4 | DISCUSSION

4.1 | Haplotype count

By examining eDNA from water samples, we were able to differentiate between *A. anguilla* haplotypes in the mitochondrial D-loop region. In the three rivers Lilleelv, Kleplandsbekken, and Moelva, we found seven, four, and two unique haplotypes, respectively, which probably represent different eel individuals. In addition, it was possible to identify every 10 eel individuals in the tank water by analyzing eDNA from a tank water sample and comparing it with the genomic haplotype information obtained from tissue samples of the same 10 eel individuals. This supports earlier studies that eDNA haplotyping of individual species in water samples is as effective as tissue sampling (Dugal et al., 2021). The *Anguilla* genus is highly diverse in the mitochondrial D-loop region (Ragauskas et al., 2014), and our method is therefore particularly feasible for this species. If studying other species with lower haplotype diversity, such as fish species with kinship in the same river system, one might need to examine nuclear DNA (nDNA) to distinguish between individuals. Because of higher effective population size of nuclear genome than mitochondria genome and the possibility to include higher number of loci/haplotypes using nuclear DNA markers, it might be favorable to analyze nDNA in such cases. However, the high copy number of mitochondrial genome compared with nDNA in cells increases the possibility of detection in eDNA analyses. Development of long-read sequencing approaches and nDNA markers would be beneficial for population genetic studies (Adams et al., 2019).

4.2 | Detectable eDNA after removal of eel individuals

Haplotypes from five of the 10 individuals in the tank water were also detected in the river water sample, which means that eDNA from these five individuals were detectable in the river one hour after they were caught and removed from their environment. Given that this is running water and that the discharge was about 0.48 m^3/second (i.e., 1720 m^3/hour) at the time of sampling, it is interesting to find that DNA from these individuals was still detectable one hour later. Various studies have shown that eDNA can be detected in standing water days after the source of the DNA has been removed, see, for example, Barnes et al. (2014), but in running water samples of several liters has often been necessary to compensate for the reduced probability of detection caused by removal of eDNA by water flow (Rees et al., 2014). Field experiments in rivers with low discharge (<100 liters/second) has shown that eDNA concentrations are relatively stable the first 24 h after the source of DNA has been removed, but that eDNA concentration decreases with increased discharge (Jane et al., 2015; Nevers et al., 2020). The discharge when we collected the sample was much higher, and this should have reduced the probability of detection even more. However, in aquatic environments, eDNA is found to easily bind to the sediment, which has the capability to store eDNA for days or weeks (Sakata et al., 2020; Strickler et al., 2015; Turner et al., 2015; Wei et al., 2018). The bottom substrate at the sample site in Lilleelv consists of mainly silt, sand, and some gravel, and it is likely that eDNA <>stored in this sediment was continuously released into the flowing water and detected in our sample 1 h after removal of the individual eels, despite the higher discharge. The five haplotypes found in both the river and the tank sample were the five that were most abundant in the tank sample. This might indicate that these are originating from individuals that, for some reason, shed more eDNA into the surrounding

than the average individual does. If so, it is not surprising that eDNA from these five were the still present in the river water 1 h later.

4.3 | Eel haplotype diversity and detection rates

Of the 18 unique haplotypes detected, four haplotypes were identical in the D-loop region to four of 56 previously described haplotypes (Jacobsen et al., 2014). This suggests that the haplotype diversity could be lower than previously described (h = 0.996 in the North Sea) (Ragauskas et al., 2014). Two of the four haplotypes that were identical to previously described haplotypes pertained to eels in Lilleelv and the tank which were Sanger-sequenced, but the other two were only Illumina-sequenced. The PCR products were 730 bp, but the Illumina pair-end sequencing only covered 250 bp from each end. That is, the forward and reverse read in the Illumina sequencing did not overlap, which means that there is a section in the middle of the amplicon that is excluded. This section may inhabit nucleotide variations that could distinguish the two Illumina-sequenced haplotypes from the previously described haplotypes they seem identical to. In addition, the illustration of our haplotype network (Figure 2) shows that there is no sign of genetic similarities between individuals in the rivers.

The analysis of the sequence data revealed that one of the haplotypes (in the river Moelva, Figure 1) had a much higher detection rate than other haplotypes from the river samples. The explanation can be spatial heterogeneity of eDNA: The water sample might have been collected close to, in space and time, the individual, leading to a high number of reads. It is also possible that one individual was larger or more active than the others, and shed more eDNA to the water. Regardless of the cause, this is not affecting the total number of unique haplotypes detected, and therefore neither the inferred number of individuals at the site. If we had studied eDNA-concentration per se in order to estimate the number of eel individuals, these factors could easily have led to misleading results. This supports the suggestion that population structure estimates based on eDNA from water samples should origin from the presence/absence of haplotypes (Azarian et al., 2021). In addition, a high copy number of one haplotype would also be observed if two individuals share the same haplotype. That would cause an underestimation of the number of individuals. However, the probability of two eel individuals sharing a haplotype of the studied D-loop region is low considering the estimates of Ragauskas et al. (2014). Even if haplotype diversity is somewhat lower, our approach will still provide an estimate of the minimum number of individuals present, as we can be certain that there must at least be as many individuals present as haplotypes detected. If we assume equal haplotype diversity in time and space, this will still be a valuable way to estimate the minimum number of individuals present. The phenomenon heteroplasmy, which is the presence of more than one mitochondrial haplotype within a cell or individual, could theoretically influence our estimates of number of individuals at a location. However, the frequency of the extra haplotype in heteroplasmy is in general low and will probably not influence these estimates notably.

4.4 | Challenges with the study

One of the challenges with this approach is the error rates in PCR and sequencing, which can be mistaken as natural mutations in the sequences. To secure the most accurate results, we used High-Fidelity DNA polymerase in the PCR. In the analysis of our sequences, we found that the natural difference between haplotypes of eel generally was larger than expected PCR and sequencing errors.

Determining the set of representative sequences (OTUs) is nontrivial and can be achieved by clustering, denoising, or both (Antich et al., 2021; Brandt et al., 2021). However, determining which OTUs to consider true or false remains somewhat challenging, as often hundreds of OTUs are identified, in which some have a very low abundance. Informed by our experimental setup, we set the lower threshold to the rate in which all known individuals were recalled (valid OTU >1% abundance), and applied that threshold to the samples from the field, similarly as Tsuji, Miya, et al. (2020). The same values were then applied to the rivers. In this case, 99% of all reads were included, while the remaining 1% were counted as sequencing errors. The output of the sequencing analysis, and consequently the haplotype estimates, is of course sensitive to the cutoff values that are set. However, in this study, the number of haplotypes found in the rivers was the same with a cutoff value of 1% or 5%, indicating that the conclusions are robust to the chosen cutoff and that real haplotypes can likely be distinguished from false haplotypes (sequencing errors) based on large relative number of reads. Still, using consistent cutoff values when studying a species over time would likely give the most accurate and consistent haplotype estimates.

A potential weakness of the study could be the lack of field and extraction controls. Ideally, we should have brought with us distilled water in field, or collected water samples in a lake with guaranteed absence of eels, and treated them the same way as the water samples. However, every water sample we collected was sequenced, and a contamination between water samples should therefore be disclosed as one haplotype detected in more than one river sample. No haplotype was found in more than one river, and this indicates that there have been no cross contaminations between water samples during filed or lab work.

Considering the estimated number of eels in the three rivers, we are not able to define the size of the area which the water samples cover, and thus which part of the river the individuals pertain to. Further work would be to collect a sufficient number of samples along a river to detect the highest number of haplotypes possible, and to see to what extent the haplotype signatures is allocated in space (e.g., along a river or within a lake).

5 | CONCLUSIONS

By examining the mitochondrial D-loop region in eDNA samples collected from three rivers, we found 18 unique *A. anguilla* haplotypes. Because of the high haplotype diversity of the species, the haplotypes probably represent 18 individuals. In a closed environment with 10 eels, we also found that the eDNA haplotype information collected from the water sample was compliant with the D-loop haplotype information obtained from tissue samples of each individual. Our results reveal that it is possible to obtain haplotype information from European eel eDNA in water, which could be the initial phase of a possible future quantification method for this species, but also for other aquatic species. However, more research is needed to develop the approach into a future tool for population quantitation.

AUTHOR CONTRIBUTIONS

Silje Halvorsen: Conceptualization (equal); investigation (equal); methodology (equal); project administration (lead); resources (lead); writing – original draft (lead); writing – review and editing (lead). **Lars Mørch Korslund:** Formal analysis (equal); supervision (supporting); writing – review and editing (supporting). **Morten Mattingsdal:** Data curation (lead); formal analysis (equal); writing – review and editing (supporting). **Audun Slettan:** Conceptualization (equal); methodology (supporting); project administration (equal); supervision (equal); writing – review and editing (supporting).

ACKNOWLEDGMENTS

We would like to thank Kurt Johansen for his help with fieldwork. This work funded by University of Agder and the County Governor in Agder, and the latter also gave permission for catching and handling of the eels.

CONFLICT OF INTEREST STATEMENT

We have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Figshare at https://doi.org/10.6084/m9.figshare.19208214.v1.

ORCID

Silje Halvorsen https://orcid.org/0000-0001-6484-7751

REFERENCES

Adams, C. I. M., Hepburn, C., Jeunen, G.-J., Cross, H., Taylor, H. R., Gemmell, N. J., Bunce, M., & Knapp, M. (2022). Environmental DNA reflects common haplotypic variation. *Environmental DNA*, 1–14.

Adams, C. I. M., Knapp, M., Gemmell, N. J., Jeunen, G.-J., Bunce, M., Lamare, M. D., & Taylor, H. R. (2019). Beyond biodiversity: Can environmental DNA (eDNA) cut it as a population genetics tool? *Genes*, *10*(3), 192.

Antich, A., Palacin, C., Wangensteen, O. S., & Turon, X. (2021). To denoise or to cluster, that is not the question: Optimizing pipelines for COI metabarcoding and metaphylogeography. *BMC Bioinformatics*, *22*, 177.

Antognazza, C. M., Britton, J. R., Potter, C., Franklin, E., Hardouin, E. A., Gutmann Roberts, C., Aprahamian, M., & Andreou, D. (2019). Environmental DNA as a non-invasive sampling tool to detect the spawning distribution of European anadromous shads (Alosa spp.). *Aquatic Conservation: Marine and Freshwater Ecosystems*, *29*, 148–152.

Azarian, C., Foster, S., Devloo-Delva, F., & Feutry, P. (2021). Population differentiation from environmental DNA: Investigating the potential of haplotype presence/absence-based analysis of molecular variance. *Environmental DNA*, *3*, 541–552.

Baker, C. S., Steel, D., Nieukirk, S., & Klinck, H. (2018). Environmental DNA (eDNA) from the wake of the whales: Droplet digital PCR for detection and species identification. *Frontiers in Marine Science*, *5*, 133.

Barnes, M. A., Turner, C. R., Jerde, C. L., Renshaw, M. A., Chadderton, W. L., & Lodge, D. M. (2014). Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science & Technology*, *48*, 1819–1827.

Bearzi, G. (2000). First report of a common dolphin (Delphinus delphis) death following penetration of a biopsy dart. *Journal of Cetacean Research and Management*, *2*, 217–222.

Belpaire, C. G. J., Goemans, G., Geeraerts, C., Quataert, P., Parmentier, K., Hagel, P., & De Boer, J. (2009). Decreasing eel stocks: Survival of the fattest? *Ecology of Freshwater Fish*, *18*, 197–214.

Brandt, M. I., Trouche, B., Quintric, L., Günther, B., Wincker, P., Poulain, J., & Arnaud-Haond, S. (2021). Bioinformatic pipelines combining denoising and clustering tools allow for more comprehensive prokaryotic and eukaryotic metabarcoding. *Molecular Ecology Resources*, *21*, 1904–1921.

Callahan, B. J., Mcmurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*, 581–583.

Castonguay, M., Hodson, P. V., Moriarty, C., Drinkwater, K. F., & Jessop, B. M. (1994). Is there a role of ocean environment in American and European eel decline? *Fisheries Oceanography*, *3*, 197–203.

Deutschmann, B., Muller, A. K., Hollert, H., & Brinkmann, M. (2019). Assessing the fate of brown trout (Salmo trutta) environmental DNA in a natural stream using a sensitive and specific dual-labelled probe. *Science of the Total Environment*, *655*, 321–327.

Drouineau, H., Durif, C., Castonguay, M., Mateo, M., Rochard, E., Verreault, G., Yokouchi, K., & Lambert, P. (2018). Freshwater eels: A symbol of the effects of global change. *Fish and Fisheries*, *19*, 903–930.

Dugal, L., Thomas, L., Jensen, M. R., Sigsgaard, E. E., Simpson, T., Jarman, S., Thomsen, P. F., & Meekan, M. (2021). Individual haplotyping of whale sharks from seawater environmental DNA. *Molecular Ecology Resources*, *22*, 56–65.

Feunteun, E. (2002). Management and restoration of European eel population (*Anguilla anguilla*): An impossible bargain. *Ecological Engineering*, *18*, 575–591.

Friedland, K. D., Miller, M. J., & Knights, B. (2007). Oceanic changes in the Sargasso Sea and declines in recruitment of the European eel. *ICES Journal of Marine Science*, *64*, 519–530.

Grooten, M., & Almond, R. E. (2018). WWF. Living planet report-2018: Aiming higher. Gland, Switzerland.

Halvorsen, S., Korslund, L., Gustavsen, P. Ø., & Slettan, A. (2020). Environmental DNA analysis indicates that migration barriers are decreasing the occurrence of European eel (*Anguilla anguilla*) in distance from the sea. *Global Ecology and Conservation*, *24*, e01245.

ICES. (2019). Joint EIFAAC/ICES/GFCM working group on eels (WGEEL).

Itakura, H., Wakiya, R., Yamamoto, S., Kaifu, K., Sato, T., & Minamoto, T. (2019). Environmental DNA analysis reveals the spatial distribution, abundance, and biomass of Japanese eels at the river-basin scale. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *29*, 361–373.

Jacobsen, M. W., Pujolar, J. M., Gilbert, M. T., Moreno-Mayar, J. V., Bernatchez, L., Als, T. D., Lobon-Cervia, J., & Hansen, M. M. (2014).

Speciation and demographic history of Atlantic eels (*Anguilla anguilla* and *A. rostrata*) revealed by mitogenome sequencing. *Heredity (Edinb)*, *113*, 432–442.

Jane, S. F., Wilcox, T. M., Mckelvey, K. S., Young, M. K., Schwartz, M. K., Lowe, W. H., Letcher, B. H., & Whiteley, A. R. (2015). Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources*, *15*, 216–227.

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, *4*, 150–157.

Katoh, K., Misawa, K., Kuma, K., & Miyata, T. (2002). MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, *30*, 3059–3066.

Kettle, A. J., Vollestad, L. A., & Wibig, J. (2011). Where once the eel and the elephant were together: Decline of the European eel because of changing hydrology in Southwest Europe and Northwest Africa? *Fish and Fisheries*, *12*, 380–411.

Lacoursiere-Roussel, A., Dubois, Y., Normandeau, E., & Bernatchez, L. (2016). Improving herpetological surveys in eastern North America using the environmental DNA method. *Genome*, *59*, 991–1007.

Leigh, J. W., & Bryant, D. (2015). Popart: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, *6*, 1110–1116.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. Journal*, *17*, 10–12.

Mattingsdal, M. (2022). *Anguilla anguilla* genomes vs eDNA. figshare. Dataset.

Murakami, H., Yoon, S., Kasai, A., Minamoto, T., Yamamoto, S., Sakata, M. K., Horiuchi, T., Sawada, H., Kondoh, M., Yamashita, Y., & Masuda, R. (2019). Dispersion and degradation of environmental DNA from caged fish in a marine environment. *Fisheries Science*, *85*, 327–337.

Nevers, M. B., Przybyla-Kelly, K., Shively, D., Morris, C. C., Dickey, J., & Byappanahalli, M. N. (2020). Influence of sediment and stream transport on detecting a source of environmental DNA. *PLoS One*, *15*, e0244086.

Parsons, K. M., Everett, M., Dahlheim, M., & Park, L. (2018). Water, water everywhere: Environmental DNA can unlock population structure in elusive marine species. *Royal Society Open Science*, *5*, 180537.

Pike, C. C. V., & Gollock, M. (2020). *Anguilla anguilla. The IUCN Red List of Threatened Species* [Online]. https://doi.org/10.2305/IUCN.UK.2020-2.RLTS.T60344A152845178.en

Ragauskas, A., Butkauskas, D., Sruoga, A., Kesminas, V., Rashal, I., & Tzeng, W. N. (2014). Analysis of the genetic structure of the European eel *Anguilla anguilla* using the mtDNA D-loop region molecular marker. *Fisheries Science*, *80*, 463–474.

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., & Gough, K. C. (2014). REVIEW: The detection of aquatic animal species using environmental DNA – A review of eDNA as a survey tool in ecology. *Journal of Applied Ecology*, *51*, 1450–1459.

Romero, L. M., & Reed, J. M. (2005). Collecting baseline corticosterone samples in the field: Is under 3 min good enough? *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *140*, 73–79.

Sakata, M. K., Yamamoto, S., Gotoh, R. O., Miya, M., Yamanaka, H., & Minamoto, T. (2020). Sedimentary eDNA provides different information on timescale and fish species composition compared with aqueous eDNA. *Environmental DNA*, *2*, 505–518.

Shiraishi, H., & Crook, V. (2015). *Eel market dynamics: An analysis of Anguilla production*. TRAFFIC.

Shogren, A. J., Tank, J. L., Andruszkiewicz, E., Olds, B., Mahon, A. R., Jerde, C. L., & Bolster, D. (2017). Controls on eDNA movement in streams: Transport, retention, and resuspension. *Scientific Reports*, *7*, 5065.

Sigsgaard, E. E., Nielsen, I. B., Bach, S. S., Lorenzen, E. D., Robinson, D. P., Knudsen, S. W., Pedersen, M. W., Al Jaidah, M., Orlando, L., & Willerslev, E. (2016). Population characteristics of a large whale shark aggregation inferred from seawater environmental DNA. *Nature Ecology & Evolution*, *1*, 1–5.

Strickler, K. M., Fremier, A. K., & Goldberg, C. S. (2015). Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation*, *183*, 85–92.

Takahara, T., Iwai, N., Yasumiba, K., & Igawa, T. (2020). Comparison of the detection of 3 endangered frog species by eDNA and acoustic surveys across 3 seasons. *Freshwater Science*, *39*, 18–27.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., & Kawabata, Z. I. (2012). Estimation of fish biomass using environmental DNA. *PLoS One*, *7*, e35868.

Thomsen, P. F., Kielgast, J., Iversen, L. L., Moller, P. R., Rasmussen, M., & Willerslev, E. (2012). Detection of a diverse marine fish Fauna using environmental DNA from seawater samples. *PLoS One*, *7*, e41732.

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA - an emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4–18.

Thorstad, B. E., Larsen, M., Hesthagen, T., Næsje, F. T., Poole, R., Aarestrup, K., Pedersen, I. M., Hanssen, F., Østborg, G., Økland, F., Aasestad, I., & Sandlund, T. O. (2010). Ål og konsekvenser av vannkraftutbygging. *Norges vassdrags- og energidirektorat* NINA.

Tsuji, S., Maruyama, A., Miya, M., Ushio, M., Sato, H., Minamoto, T., & Yamanaka, H. (2020). Environmental DNA analysis shows high potential as a tool for estimating intraspecific genetic diversity in a wild fish population. *Molecular Ecology Resources*, *20*, 1248–1258.

Tsuji, S., Miya, M., Ushio, M., Sato, H., Minamoto, T., & Yamanaka, H. (2020). Evaluating intraspecific genetic diversity using environmental DNA and denoising approach: A case study using tank water. *Environmental DNA*, *2*, 42–52.

Turner, C. R., Uy, K. L., & Everhart, R. C. (2015). Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation*, *183*, 93–102.

Uchii, K., Doi, H., & Minamoto, T. (2016). A novel environmental DNA approach to quantify the cryptic invasion of non-native genotypes. *Molecular Ecology Resources*, *16*, 415–422.

Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M., & Barton, G. J. (2009). Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, *25*, 1189–1191.

Wei, N., Nakajima, F., & Tobino, T. (2018). A microcosm study of surface sediment environmental DNA: Decay observation, abundance estimation, and fragment length comparison. *Environmental Science & Technology*, *52*, 12428–12435.

Yoshitake, K., Yoshinaga, T., Tanaka, C., Mizusawa, N., Reza, M. S., Tsujimoto, A., Kobayashi, T., & Watabe, S. (2019). HaCeD-Seq: A novel method for reliable and easy estimation about the fish population using haplotype count from eDNA. *Marine Biotechnology*, *21*, 813–820.

How to cite this article: Halvorsen, S., Korslund, L., Mattingsdal, M., & Slettan, A. (2023). Estimating number of European eel (*Anguilla anguilla*) individuals using environmental DNA and haplotype count in small rivers. *Ecology and Evolution*, *13*, e9785. https://doi.org/10.1002/ece3.9785

APPENDIX A

Primers AaDN-F and AaDN-R compared with mtDNA from species possible to find at the locations where water samples were collected. The sequences are the best match between the primers and the mtDNA from the other fish species. Hyphens "-" in the sequences represent identical base pairs. The comparisons were conducted with Clustal Omega, and the sequences were found in the NCBI's GenBank.

Salmo trutta GenBank: LC137015.1	AaDN-F	CGCTAAAAATCAGAGAGGAAAGATTT
	S.trutta	--G-T---G--C---CA-G--TTA-G
	AaDN-R	TTACATGTGAGACACCTTCTAAAAAGT
	S.trutta	C--AC-C---TT-T------GTG-G--
Salvelinus alpinus GenBank: MF621743.1	AaDN-F	CGCTAAAAATCAGAGAGGAAAGATTT
	S.alpinus	-C---GTGC--------AGG------
	AaDN-R	TTACATGTGAGACACCTTCTAAAAAGT
	S.alpinus	---AC-T-A-A-A-----A---TGCC-
Lampreta fluviatilis GenBank: FP929026.1	AaDN-F	CGCTAAAAATCAGAGAGGAAAGATTT
	L.fluv.	--T--C----TCT-C--C-GCA----
	AaDN-R	TTACATGTGAGACACCTTCTAAAAAGT
	L.fluv.	GC-GC--A--T-A-A-G-T-----T--
Phoxinus phoxinus GenBank: AB671170.1	AaDN-F	CGCTAAAAATCAGAGAGGAAAGATTT
	P. phoxinus	--T----GC--G--C--A--GA----
	AaDN-R	TTACATGTGAGACACCTTCTAAAAAGT
	P. phoxinus	A-GA--C----G-GG-----C-GT--A
Coregonus lavaretus GenBank: AB034824.1	AaDN-F	CGCTAAAAATCAGAGAGGAAAGATTT
	C. lavaretus	-C---GTGC--------AGG------
	AaDN-R	TTACATGTGAGACACCTTCTAAAAAGT
	C. lavaretus	C--GC-A--G--A-TA----T-T-CT-
Osmerus eperlanus GenBank: MH238073.1	AaDN-F	CGCTAAAAATCAGAGAGGAAAGATTT
	O. eperlanus	--T----GC-----C--C-C---CC-
	AaDN-R	TTACATGTGAGACACCTTCTAAAAAGT
	O. eperlanus	A-C-TA---T-G--GGA---GG-----
Perca fluviatilis GenBank: KM410088.1	AaDN-F	CGCTAAAAATCAGAGAGGAAAGATTT
	P. fluv	A-TG--GCC-A-A-A--C-G------
	AaDN-R	TTACATGTGAGACACCTTCTAAAAAGT
	P. fluv.	A-GG--C----G-GG-----C-GT--A

7. Paper 3

Collateral damage: Understanding the response and recovery of non-targeted fish species after rotenone eradication of an invasive species.

Silje Halvorsen, Audun Slettan, Elise Rustad, Lars Korslund. 2024.

Collateral damage: Understanding the response and recovery of non-targeted fish species after rotenone eradication of an invasive species

Silje Halvorsen, Audun Slettan, Elise Rustad, Lars Korslund

Abstract: While several studies have examined the efficiency of rotenone treatment on invasive fish species and its effects on non-targeted macroinvertebrate communities, research specifically addressing the impact of such treatment on non-targeted native fish species is notably lacking. Understanding the potential impact of rotenone exposure on these species and their natural recovery is essential for comprehensive ecosystem management and conservation efforts. This study explores the ecological consequences of a Northern pike (*Esox lucius*) eradication campaign and investigates the recovery of native non-targeted fish species. In addition to the pike, we focused on three key fish species with contrasting life histories: the European eel (*Anguilla anguilla),* the three-spined stickleback (*Gasterosteus aculeatus*), and the brown trout (*Salmo trutta*). Using environmental DNA (eDNA) from water samples collected across multiple stations in various habitats (including upstream and downstream of the treated lake), we monitored these species over a two-year period (before, during, and after treatment). Our findings indicate a successful unassisted recovery of all three native fish species, but the speed and rate of recovery vary. The ability of a species to recover after an eradication campaign likely depends on migratory capabilities and local access to refuges. Pike, unable to migrate into upstream rivers and the saline sea, failed to naturally reestablish. Conversely, trout, eel, and stickleback, capable of inhabiting both marine and freshwater environments, recovered within a year or less. The ecological consequence of using a broad-scale pesticide to eradicate an invasive species are many and affect several trophic levels. Future research should utilize metabarcoding techniques to gain a more comprehensive understanding of ecosystem effects of rotenone treatment, allowing for simultaneous identification of multiple species from eDNA samples and providing a more holistic view of biodiversity and trophic relationships.

1. Introduction

The transport of species beyond their native habitats has increased during the last centuries (Seebens et al., 2017). The release and establishment of such species (from here on referred to as invasive species) has profound implications, contributing to the human-induced biodiversity crisis (Clavero & García-Berthou, 2005). Invasive species can outcompete native species for resources such as food, habitat, and breeding sites, leading to declines or extinctions (Fletcher, Collins, Nannini, & Wahl, 2019; Hata, Uemura, & Ouchi, 2021; Van Kessel, Dorenbosch, De Boer, Leuven, & Van der Velde, 2011). They can also alter ecosystem dynamics and processes, such as nutrient cycling and water flow patterns, which can have cascading effects on ecosystem health and function (Capps & Flecker, 2013; Strayer & Dudgeon, 2010). Furthermore, invasive species may serve as hosts for parasites or diseases that can harm native wildlife (Gilbert & Granath Jr, 2003; Groocock et al., 2007; Skerratt et al., 2007). Over time, the presence of invasive species can thus cause biotic homogenization, a process causing the diversity of species across different geographic regions to become increasingly alike, resulting in reduced biodiversity and the loss of distinctive ecological characteristics that once defined an area (Hulme, 2009; Simberloff et al., 2013; Winter et al., 2009). Together these impacts impose significant economic burdens through the costs associated with controlling and managing invasive species and restoring degraded ecosystems (Pimentel, Zuniga, & Morrison, 2005).

Freshwater ecosystems are among the most highly invaded ecosystems in the world (Ricciardi & MacIsaac, 2011), and in many freshwater habitats the spread of invasive species is one of the major threats to biodiversity loss (McGeoch et al., 2010; Reid et al., 2019). Invasions in freshwater ecosystems pose particular challenges due to their impact on essential ecosystem services such as water supply and fisheries (Carpenter, Stanley, & Vander Zanden, 2011). Establishment of invasive fish species results from escape from aquaculture (Consuegra, Phillips, Gajardo, & de Leaniz, 2011), ornamental trade (Beyer, 2004), fishing and angling (Gozlan, Britton, Cowx, & Copp, 2010), introducing nonnative fish species as biological control for pests (Beisel & Lévêque, 2010), stocking for fisheries (Gozlan et al., 2010), ballast transport (Wonham, Carlton, Ruiz, & Smith, 2000), and the disruption of natural barriers by humans (Galil, Nehring, & Panov, 2007). The release of fish for sport fishing, game fishing, or as bait for

angling is a significant pathway for introducing fish species (Gozlan et al., 2010), and top predator species are often introduced to new sites (Donaldson et al., 2011). Despite the significant consequences of invasive fish spreading, there appears to be a tendency among policymakers and the public to underestimate the consequences, leading to delayed or limited efforts to prevent its escalation (Meyerson & Reaser, 2003).

When action is taken to eradicate invasive fish species, chemical treatments are often used, and rotenone is a commonly applied piscicide (Beaulieu et al., 2021; Britton, Gozlan, & Copp, 2011; Recsetar & Bonar, 2015; Sandodden, Brazier, Sandvik, Moen, Wist, & Adolfsen, 2018). Rotenone is a broad-spectrum toxin that blocks the reoxidation of nicotinamide adenine dinucleotide (NADH), which inhibits cellular respiration (Horgan, Singer, & Casida, 1968). Fish are acutely sensitive to rotenone poisoning, quickly absorbing the toxin across the gill surface and dying within hours (Ling, 2003). Aquatic invertebrates are generally less sensitive than fish, but rotenone treatment will also cause significant loss of invertebrate fauna (Ling, 2003). The use of rotenone for eradication of a fish species is controversial due to its impact on non-targeted organisms (Finlayson, Somer, & Vinson, 2010; Kjærstad & Arnekleiv, 2011). However, the consequence of invasive fish species can be considerable, and if native species can recover, by spreading from nearby non-treated areas, or by reintroduction, eradication can be regarded as the "lesser of two evils" (Woodford et al., 2013).

Several studies have examined the efficiency of rotenone treatment on the target invasive fish species (Coleman et al., 2024; Jordaan & Weyl, 2013; Recsetar & Bonar, 2015) and the effect on non-targeted macroinvertebrate communities (Bellingan, Hugo, Woodford, Gouws, Villet, & Weyl, 2019; Gaute Kjærstad, Jo Vegar Arnekleiv, Gaute Velle, & Anders Gravbrøt Finstad, 2022; Pham, Jarvis, West, & Closs, 2018), there is a remarkable absence of research specifically addressing the effect of rotenone treatment on non-targeted native fish species, and this represents a significant knowledge gap. Understanding the potential impact of rotenone exposure on non-targeted native fish species, and their ability to recover naturally, is essential for comprehensive ecosystem management and conservation efforts. The vulnerability of a fish species to rotenone treatment, and its population's ability to recover, is likely influenced by its spatial distribution (relative to the rotenone treatment area), habitat use, migration strategy and -capability and its general life history.

In this study we evaluate the effect of rotenone treatment on the invasive piscivore northern pike (*Esox lucius*, from here on referred to as pike), and on native non-targeted fish species, over a two-year period in Southern Norway. Pike is a freshwater top-predator naturally occurring in the North-Eastern and South-Eastern parts of Norway, but it has been widely introduced to other regions, and is now found in all counties (Figure 1) (Forsgren, Bærum, Finstad, Hesthagen, Knutsen, & Wienerroither, 2023). This fish species can have severe negative impacts as an invader (Hesthagen et al., 2020). Functioning as an opportunistic predator (Sepulveda, Rutz, Ivey, Dunker, & Gross, 2013) with a relatively good migratory ability (Koed, Balleby, Mejlhede, & Aarestrup, 2006), thereby posing a substantial threat to the species and trophic composition of aquatic systems (Byström, Karlsson, Nilsson, Van Kooten, Ask, & Olofsson, 2007). An invasion of pike can have a cascading effect on food webs (Byström et al., 2007; Haught & von Hippel, 2011; Sepulveda, Rutz, Dupuis, Shields, & Dunker, 2015). In the lake Gillsvannet, pike was first reported in 2018, and the population quickly increased. The lake is located only meters from the sea and has a surface less than 1 meter above sea level, and the proximity to the sea leads to influx of saltwater during high tide, and thus partially brackish water conditions. Local management were in 2021 authorized to treat the lake with rotenone to eradicate pike. The basis for the decision was that pike, as an invasive species with a large invasion potential, had severe ecological impacts (Forsgren et al., 2023) and it was considered important to protect salmon (*Salmo salar*) populations in nearby tributaries, as well as local populations of stationary and anadromous brown trout (*Salmo trutta*). As the ecological consequences of rotenone treatment is severe, with all fish species being affected, the decision was subject of considerable controversy in the local community.

The aim of this study is to explore the ecological consequence of a pike eradication campaign and to study how native non-targeted fish species recover in the ecosystem. We focus on four key fish species with contrasting life-histories: The invasive pike, the European eel (*Anguilla anguilla*, from here on referred to as eel), the three-spined stickleback (*Gasterosteus aculeatus*, from here on referred to as stickleback) and the brown trout (from here on referred to as trout), and use environmental DNA (from here on referred to as eDNA) of water sampled in space (multiple stations in several habitats, including upstream and downstream of the treated lake) and time (before, during and after treatment) to monitor the effect on the four species.We assume that the probability

of a positive eDNA sample is a proxy of overall density of a target species at any given time and that positive eDNA samples at many stations within a given habitat can be used as a relative measure of spatial distribution of the species. Given effective eradication we expect that pike will be a permanently removed from the lake as it is not expected to inhabit the outside fjord, and thus not expected to reinvade. Conversely, stickleback, commonly found both in freshwater and marine habitats, is expected to be only temporarily absent and to relatively quickly reestablish in the lake by migration from nearby marine refuges. The anadromous trout and the catadromous eel are also expected to reappear, but possibly at a slower rate, as seasonal migration from the sea progresses. We also expect that one or more of the native fish species might exceed pre-treatment population sizes, as pike disappears from the lake.

2. Materials and methods

2.1 The lake

Gillsvannet (58°11′17″N 8°01′21″Ø) is a lake situated near the fjord Topdalsfjorden in Southern Norway, with a total area of 1.15 km², a catchment area of 10.6 km^2 and an estimated water volume of 7,314,030 m³. It is characterized by two short (approximately 200 meters) outlet streams leading to the sea, along with two major and several smaller inlet streams contributing to the lake's water flow. All inlet streams come from smaller lakes, and all have natural migration barriers, impassable for pike, but not for eel and trout. The shores consist of a mix of bedrock, small sandy beaches and reed (Phragmites australis) beds. With a surface less than 1 meter above sea level influx of saltwater regularly occurs during high tide, and this has major effect on the water conditions. Maximum depth is 24 meters, and the water below 8 meters is anoxic and saline (15-20 ppt) , thus only the surface layer is habitable for fish (Oredalen, 2002).

2.2 The fish community and study species

Nine species of fish has been reported from the lake. In addition to the four species in question in this study (see details below), three other non-native species, Tench (*Tinca tinca*), Goldfish (*Carassius auratus*) and Crucian carp (*Carassius carassius*), the native

freshwater Perch (*Perca fluviatilis*) and the marine and brackish European Flounder (*Platichthys flesus*) was present prior to the rotenone treatment.

The pike is a top predator that can reach more than 15 kg and is primarily found in freshwater, but it is also present in brackish habitats (Sunde, Tamario, Tibblin, Larsson, & Forsman, 2018). It can spawn at salinities up to 6.5 ppt (Westin & Limburg, 2002) and adults can tolerate salinity up to 12–15 ppt (Raat, 1988) but in Norway lakes and slow-running rivers is the primary habitat and no major spawning migration occur. They are often inhabiting shallow water with aquatic macrophytes lining lake shores (Kobler, Klefoth, Wolter, Fredrich, & Arlinghaus, 2008; A. Vøllestad, Skurdal, & Qvenild, 1986). The spawning typically occurs between March and May, with the eggs hatching in the subsequent months from March to June (Tibblin, Forsman, Borger, & Larsson, 2016).

The critically endangered European eel can normally reach a maximum weigh of 5 kg and is known for its adaptability to various environmental conditions (C. M. F. Durif et al., 2023). Despite being catadromous, often spending years of its life in freshwater, it can complete its lifecycle entirely in the sea and can also undergo multiple migrations between salt, brackish, and freshwater environments (Tsukamoto & Arai, 2001; Tzeng & Tsai, 1994). The eels migrate to the Sargasso Sea for spawning and juvenile elvers migrate up coastal rivers usually between May and September (A. Vøllestad, Skurdal, & Qvenild, 1986).

Brown trout exhibit several life histories. Populations can be potadromous (migrating within freshwater systems and usually spawning in rivers) or anadromous, and potadromous and anadromous populations can be sympatric in the same coastal catchment (Ferguson, Reed, Cross, McGinnity, & Prodöhl, 2019). Spawning occurs exclusively in freshwater, and anadromous spawning migration from the sea peaks around September-October (Aarestrup & Jepsen, 1998). Anadromous kelts (spent individuals after spawning) may immediately return to sea in late autumn/early winter or may remain in freshwater over the winter before returning to sea (Thorstad et al., 2016) and potadromous individuals may stay in the river or migrate to a nearby lake after spawning (Ferguson et al., 2019).

The threes-pined stickleback is a small (<10 cm) fish with a wide distribution in coastal regions in the northern hemisphere (Wootton, 2009), and which has several ecotypes

with different morphology and life-history strategy (Clavero, Pou-Rovira, & Zamora, 2009). It can be stationary or migratory, and populations can be anadromous (spawning in fresh or brackish water) or permanent residents of either marine or freshwater habitats (Wootton, 2009). The spawning season vary with latitude and between ecotypes, but it usually starts spawning in May-June (Ishikawa & Kitano, 2020).

2.3 The rotenone treatment

The lake, including surface layers, deeper layers, inlet streams, shores and nearby bogs and wet habitat, was treated with rotenone at an average concentration of 23 µg/L during the period November 2 to 5, 2021 (Aune & Kielland, 2023). Water samples showed that the level of rotenone was below measurable levels (<2 µg/liter) January 22, 2022, 78 days after the treatment. All deceased fish observed were collected and removed from the lake within a 14-day period spanning from November 4 to November 23.

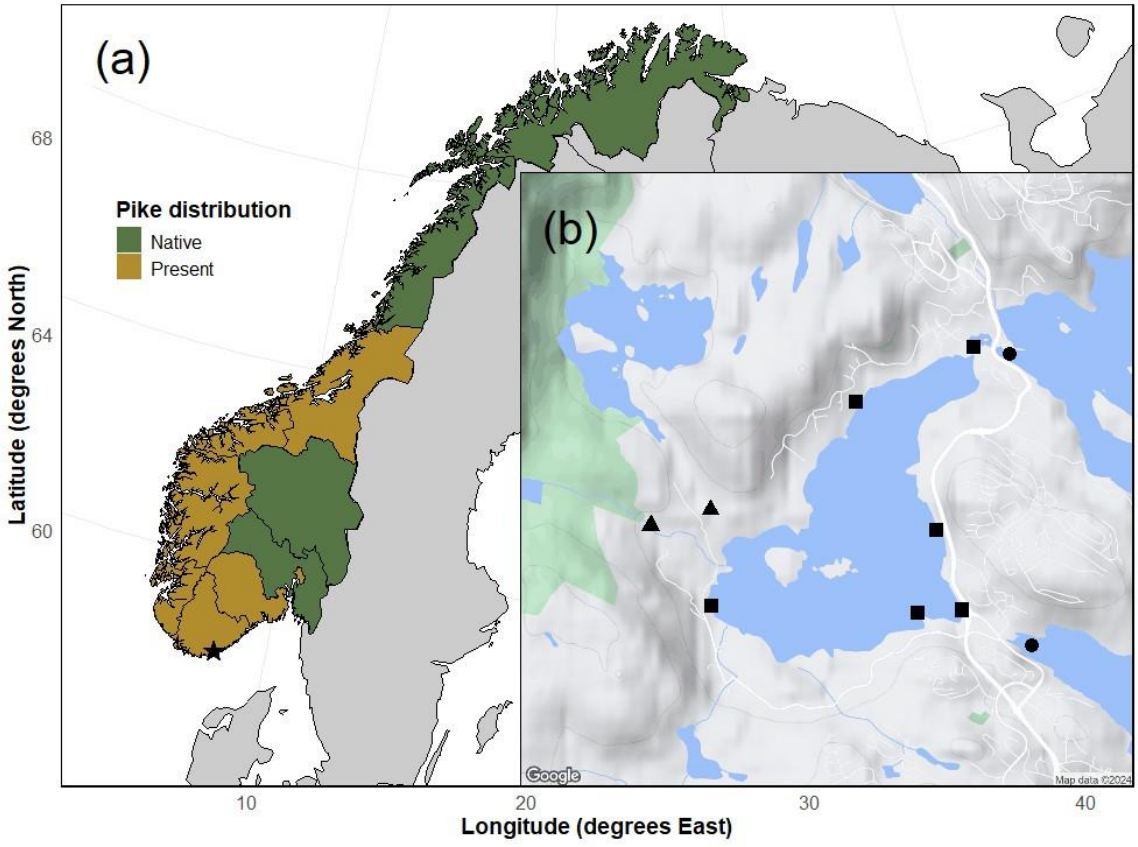

Figure 1: Map of Norway (a) with counties having native populations (green) and counties having invasive populations of northern pike (dark golden) and map of the rotenone treated Gillsvannet (b) located close to the fjord (seawater) to the east, several inlet streams leading from other smaller nearby lakes to the south and east and eDNA water sampling stations. The star represents the location of the lake in Norway and triangles squares and circles represent sampling locations upstream, lake and sea habitat respectively. (Map source, panel b: © Google, 2024).

2.4 eDNA water sampling

Water was sampled from 10 different locations in three different habitat types: Lotic water in major inlet streams between upstream smaller lakes and the Gillsvannet lake, located above natural pike migration barriers (hereafter referred to as *Upstream*), lentic water in the lake (hereafter referred to as *Lake*) and lentic water in the sea, approximately 150 meters outside of the lake outlet (hereafter referred to as *Sea*). The number of sampling stations in each habitat type was two, six and two respectively (se figure 1 for details).

The water samples were collected once every month from September 2021 to November 2022 and thereafter once every third month until October 2023. Water samples were also collected in October 2023 from five adjacent lakes. It should be noted that we were unable to collect samples from some specific locations in the lake during certain winter periods due to ice coverage, and a few samples were depleted during laboratory analyses (Appendix A).

Every water sample consisted of 1 Liter of surface water, placed on ice for preservation until filtration within a 5-hour timeframe. For each sampling event, a new set of nitrile gloves were used, and all sample bottles underwent rinsing with a 10% chlorine solution, followed by tap water and water from the location before being used. As negative field controls, we brought 1-liter bottles with distilled water in the field and treated them with the same procedure in the lab. In the laboratory setting, a volume ranging from 400 to 1000 mL of water (as much as possible before clogging of the filter), underwent filtration through a 0.45 µm pore size cellulose nitrate filter (Thermo Scientific Nalgene) using an ILMVAC vacuum pump (GmbH). Post-filtration, the filters were folded and stored at −20°C.

2.5 DNA isolation and PCR amplification

The extraction of environmental DNA (eDNA) from the filters and genomic DNA from *A. anguilla, S. trutta, E. lucius* and *G. aculeatus* collected from tissue samples was carried out utilizing bead beating and the DNeasy® Blood and Tissue Kit (Qiagen), following the procedure outlined by Thomsen, Kielgast, Iversen, Moller, Rasmussen, and Willerslev (2012). Following isolation, the DNA was preserved in microcentrifuge tubes at −20 °C. The eDNA isolation process was conducted in a distinct room separate

from the PCR amplification. Subsequent to isolation, each sample underwent analysis for purity and eDNA concentration using a spectrophotometer (NanoDrop™ One, Thermofisher). We examined eDNA extract for *A. anguilla, S. trutta, E. lucius* and *G. aculeatus* DNA using a real-time PCR assay with TaqMan®-probe and primers (Appendix B). The *A. anguilla* and *S. trutta* primers and probe were designed with Primer-BLAST by Halvorsen, Korslund, Gustavsen, and Slettan (2020) at the web page of the National Center for Biotechnology Information (NCBI) and the program Primer Express 3.0.1 (Thermo Fisher). *G. aculeatus* primers and probes were designed by Thomsen et al. (2012) but were modified (shortened and extended, respectively), for optimal Tm (melting temperature) by using the software Primer express 3.0.1 (Thermofisher). The *E. lucius* primers and probe were designed by Olsen, Lewis, Massengill, Dunker, and Wenburg (2015). The primers and probe were tested for species-specificity by searching for homology to DNA sequences from species that could habit the same area by using Clustal Omega (European Bioinformatics Institute) and NCBI's GenBank. PCR-reaction had the following ingredient concentrations: 1 x TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 0.9 µM forward primer, 0.9 µM reverse primer, and 0.55 µM probe. 20 µl PCR mix with this concentration, including 2,5 µl template, were used in the PCR-reaction. The mix was transferred to a 0.1 ml Micro Fast Tube Strips (Thermo Fisher) before we conducted the real-time PCR on a StepOnePlus™ Real-time PCR System (Applied Biosystem).

The real-time PCR temperature profile was as follows for the different species:
A. anguilla and *S. trutta:* 50 °C in 2 min and 95 °C in 10 min, followed by 60 cycles of 96 °C in 15 s, 57 °C in 30 s, and 72 °C in 30 s. *E. lucius*: 50 °C in 2 min and 95°C in 10 min, followed by 60 cycles of 95 °C in 15 sec, 57°C in 1 min, and 60°C for 30 sec. G. aculeatus: 50 °C in 2 min and 95°C in 10 min, followed by 60 cycles of 95 °C in 15 sec and 60°C in 1 min. Fluorescence was measured during the last step of every cycle for all species.
We analysed every DNA-sample in triplicates. A sample containing tissue of genomic DNA from each species was used as a positive control, and PCR-grade H_2O was used as a negative control. Positive and negative controls were included in all runs. We also analysed the negative field control that had been brought with us in the field work.

2.6 Statistical analysis

Statistical analysis and production of figures were performed in R (R-Core team 2024) The response variable was defined as the probability of getting a positive eDNA water sample. To model the change in the response variable over time we ran a generalized additive mixed model for each fish species separately, with time (days relative to rotenone treatment start date) as predictor, a binomial family and a separate spline smoother with maximum seven nots and a separate intercept for each habitat type (Upstream, Lake and Sea), using the *mgcv* (Wood, 2017) package in R. To account for repeated sampling and triplicate real-time PCR analysis, we included a random intercept per station and to account for autocorrelation in time within habitat type we allowed for an autoregressive correlation structure within each habitat type (Pinheiro & Bates 2000). We used a Metropolis Hastings sampler to generate prediction intervals, by sampling from the probability distribution. This was based on the *fitted samples*-function in the *gratia*-package (Simpson, 2024). Maps were created using the *ggmap* (Kahle, 2013), *csmaps* (R. A. White & Zhang, 2023) and *csdata* (R. A. Z. White, C. , 2024) packages.

3. Results

3.1 General results

In every field and lab control conducted, only negative results were observed. The concentration of the DNA samples ranged between 2 and 294 ng/µL. eDNA from pike was not detected in any of the water samples collected in the five connected lakes or water bodies upstream. Eel and trout were detected in all of these upstream locations, and stickleback were detected in one of the upstream lakes, called Grovannet. All models produced reliable outputs, except for the pike model, where only non-positive eDNA samples in both upstream stations caused a lack of conversion and unreliable estimates for this habitat (Appendix C).

Pike and perch dominated among the cadavers found in the lake after the rotenone treatment, with pike being the second most common in number and the largest in biomass (Frode Kroglund, personal communication). The models revealed extensive differences in the probability of positive eDNA samples, both over time, between species and between habitats (Figure 2, Appendix C).

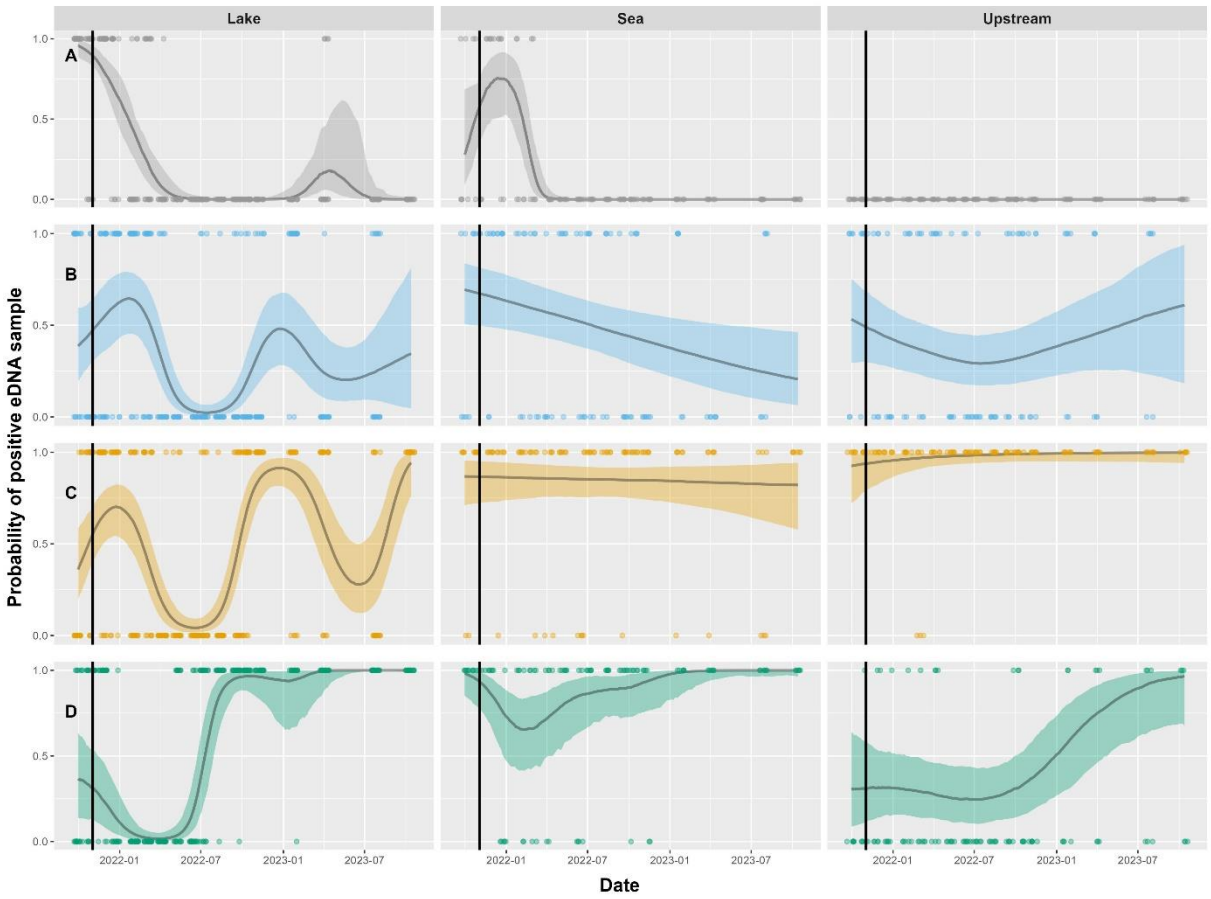

Figure 2: The probability of positive eDNA samples for A) Northern pike, B) European eel, C) Brown trout, and D) Three-spined stickleback in the lake, the sea outside and the upstream rivers over time, from September 2021 to October 2023. The dots at the top and bottom of each diagram represents observed positive and negative samples respectively, and these are shifted a small random amount in the horizontal plane to reduce overfitting. The curves show the predicted probability of positive samples, based on the GAMM-models and the colored band shows the 95% prediction interval. The black vertical line marks the time of the rotenone treatment. All samples in upstream rivers were negative for pike DNA.

3.2 Northern pike

Samples were exclusively positive for pike in the lake at the beginning of the monitoring, 33 days before the treatment, but the probability of positive samples dropped rapidly after the treatment. Roughly half of the samples taken from the sea were positive at the beginning, but the probability of positive samples increased shortly after the treatment before it began to fall. eDNA from pike was not detected in any samples from the lake or the sea from mid-April 2022, approximately 170 days post-treatment (figure 2, A), and until April 2023, approximately 550 days post-treatment. However, traces of pike eDNA reappeared in each of three distinct locations in the lake (1, 1, and 2 positive triplicates, respectively). After this, and until the end of the sampling period, no more samples were positive. Samples were exclusively negative at both upstream stations.

3.3 European eel

Roughly half of the samples collected from the lake were positive for European eel eDNA before the treatment. After a slight increase in positive samples during the first three months post-treatment, the number of positive samples decreased until June 2022, approximately 250 days post-treatment, when no samples were positive (Figure 2, B). Eel eDNA quickly reappeared and increased to pre-treatment levels by the end of the year. The pattern in 2023 mirrored that of 2022, with a decrease in detections from winter to summer and an increase in detection afterward. Throughout the observation period, eDNA signals consistently appeared in both the sea and upstream locations in moderate proportions, although with a slight decline observed in the sea. The signals in the streams experienced a slight decrease post-treatment and gradually increased to above pre-treatment levels by the end of the time series. However, compared to the changes in the lake, the probability of eDNA detection remained relatively stable.

3.4 Brown trout

The eDNA signals from trout in the lake exhibit a pattern strikingly similar to, but more pronounced than, that of the eel (figure 2, C). Initially, there was an increase in detection during the first two months post-treatment, followed by a decline the coming spring, and a period of almost exclusively negative samples from April to September 2022, approximately 150 to 300 days post-treatment. The subsequent change in eDNA detection probability manifest as two distinct waves, one each fall, when the detection probability increases rapidly, and a decline the spring in between. Ultimately, the probability of eDNA detection surpasses pre-treatment levels. The eDNA signals from trout in both the upstream rivers and the sea remain high and stable throughout the study period.

3.5 Three-spined stickleback

The three-spined stickleback eDNA detection probability in the lake were moderate prior to the treatment and diminish quickly post-treatment (figure 2, D). From around new year 2022, approximately 60 days after the rotenone treatment, and late spring no lake samples were positive However, positive samples reappeared within 200 days post-treatment and the probability of a positive eDNA sample increased rapidly during the fall of 2022, resulting in much higher probability than observed before the treatment.

Concurrently, there was an observed decline in the eDNA signals from the sea, eventually returning to pre-treatment levels. Both lake- and sea signals were positive for every location at the end of the time series. Furthermore, the eDNA signals were consistently present in one of the two studied streams, exhibiting a significant increase from 300 days post-treatment onwards. This results in a substantially higher quantity of signals post-treatment compared to pre-treatment levels.

4. Discussion

Recovery of native fish populations after piscicide treatment is an important, but surprisingly poorly studied part of invasive species management. In this study we aim to explore the effect and the ecological consequence of an invasive species eradication campaign in a temperate lake, and to study if and how native non-targeted fish species recover in the ecosystem within a two-year period. Based on samples collected from several sites within each habitat at each time, and triplicate analysis of each sample, we assume that the probability of a positive eDNA sample is a proxy of overall density of a target species. We also believe that positive eDNA samples at many stations within a given habitat can be used as a relative measure of spatial distribution of the species. Our results indicate that that the rotenone treatment was overall very efficient in eradicating the invasive pike from the lake and that all three studied native fish species reestablished in the lake within a year after the treatment. However, the rate and nature of the recovery process greatly differed between species.

4.1 Immediate response to rotenone treatment

The collection of dead fish floating in the lake showed that about 75% of all carcasses found was collected within the first week after the treatment was completed biomass (Frode Kroglund, personal communication), and we assume that most fish died within hours and days after the lake was treated with rotenone. However, it is likely that a substantial proportion of fish that died was never recovered, and that these carcasses decomposed in the lake. When fish die and decompose, they release cellular material and DNA into the water (Bessell et al., 2023), and we should therefore expect positive

eDNA samples for a period after the treatment, even if the rotenone is effective of killing all fish.

While samples positive for stickleback eDNA disappeared from the lake within 60 days, lake samples remained positive for pike, eel, and trout eDNA until approximately 150-250 days post-treatment. Interestingly, the proportion of positive samples for eel and trout increased during this period, indicating a long degradation time for the carcasses of these larger species. Dunker et al. (2016) found that eDNA from pike carcasses remained detectable for up to 35 days in water temperatures ranging from 10 to 20°C but was undetectable at 70 days. We expected the decomposition of stickleback to be relatively quick due to its small size, whereas larger species decompose more slowly. None the less, we detected eDNA from pike, trout and eel much longer than expected. This suggest that the decomposition time was long.

The decomposing of fish carcasses and the degradation of eDNA are both influenced by several environmental factors, with degradation occurring at a slower rate in colder or darker environments (Dejean et al., 2011). During the first winter post-treatment, the lake was ice-covered for several weeks, indicating water temperatures substantially lower than in the study of Dunker et al. (2016), and lower temperatures slow down decomposition rate, due to reduced microbial activity, (Passerini, Cunha-Santino, & Bianchini Jr, 2016; Wetzel, Likens, Wetzel, & Likens, 1991). Low oxygen concentrations in parts of the lake can also have resulted in slower organic matter breakdown (Sobek et al., 2009). In addition, scavenger species, usually accelerate carcass breakdown by feeding on dead tissue (Orihuela-Torres et al., 2024; Szaniawska & Szaniawska, 2018), but rotenone treatment also reduce the number of gill-breathing invertebrates (G. Kjærstad, J. V. Arnekleiv, G. Velle, & A. G. Finstad, 2022) and a reduction of invertebrate scavengers would probably further slow down the decomposition process.

Decomposition, and the resulting release of DNA, might also explain the initial increase in eel and trout positive eDNA samples post-treatment. If the net release of DNA from carcasses in this initial period exceeds the natural breakdown of free DNA in the environment, we should expect a temporary increase in detection probability, especially if the overall concentration of target DNA in the water prior to treatment is low or medium. Indeed, the proportion of positive eel and trout samples was at a medium level

prior to the treatment before it temporary increased. The proportion of pike positive eDNA samples, however, was already equal to 1 pre-treatment, and any preliminary increase in pike DNA concentration in the water post-treatment, due to decomposition, could not lead to a further increase in the proportion of positive samples any further. None the less, we would have expected that the proportion of pike positive eDNA samples would maintain at a high level as pike carcasses decomposed, but strangely enough the pike signal started to decrease quickly after the treatment, while the trout and eel signal increased. Despite a median proportion of positive stickleback samples pre-treatment there were no initial increase in detection of this species post-treatment, as was the case with trout and eel. It is possible that stickleback carcasses, due to their relatively small size, decomposed so quickly that most of the release of DNA in the water only lasted a short period. The fact that the level of positive stickleback samples was maintained the first sampling after the treatment (three weeks post-treatment), but then quickly dropped, supports this theory.

4.2 Pike eradication

Pike had the highest eDNA-signal in the lake of all monitored species prior to the treatment, with almost all samples being positive – suggesting it being common in all parts of the lake. However, the signal quickly dissipated after the treatment. By April 2022, five months post-treatment, no samples were positive, suggesting a very effective eradication campaign. No samples taken in the two upstream rivers showed sign of pike eDNA. This was as expected since each collection site was located above a small waterfall with a barrier steeper than what pike is found to be able to pass (Spens, Englund, & Lundquist, 2007). Pre-treatment, approximately half of the samples from both sea sites were positive, and the pike eDNA-signal in the sea temporarily increased after the treatment before it quickly disappeared. The ultimate lack of an eDNA-signal in the sea suggest that pike was not, and is not, inhabiting the fjord system outside of the lake, most probably because the salinity is too high. The medium level of positive samples in the sea pre-treatment, and the temporary increase of the signal post-treatment there as well, was probably not indicative of pike presence in the fjord, but rather a result of a downstream transportation of DNA from the lake, as described by Roussel, Paillisson, Tréguier, and Petit (2015), from live specimens pre-treatment and from

decomposing carcasses post-treatment, as this temporary increase in positive samples mimic that of trout and eel in the lake.

Despite that the eradication effort of pike seemed to be efficient, eDNA signals from pike reappeared approximately 540 days post-treatment in three distinct locations spread within the lake, suggesting that pike was present yet again, but probably at low numbers. Concurrently, one single pike individual was captured by a local fisherman, confirming the presence. However, despite consistent effort from the fisherman no more pike was captured thereafter (Vidar Christensen, personal communication). Three hypotheses may account for this reappearance of pike. Firstly, it is possible that one or more pike individuals despite all odds managed to survive the rotenone treatment within the lake, for example hiding in underground streams or water pockets unaffected by the piscicide. Alternatively, pike may have persisted in the nearby fjord or upstream river and entered the lake at a later stage. Lastly, the possibility remains that humans yet again released pike into the lake. The complete absence of positive samples in the lake in the months prior to and after the pike was captured oppose the alternative that one or more pike managed to survive in the lake. Similarly, the corresponding lack of positive samples in the sea and in the upstream rivers for more than a year before the pike was captured in the lake oppose that pike might have migrated into the lake from nearby habitats. However, false-negative eDNA samples can have several causes (Guillera-Arroita, Lahoz-Monfort, van Rooyen, Weeks, & Tingley, 2017) and is not a guarantee of absence, and thus we cannot exclude the possibility that pike managed to survive in the lake or to return from a refuge in the sea or in a steam. However, if pike was present, even in low numbers, in the lake, sea or streams we would expect sporadic positive samples in these habitats, and the consistent streak of negative samples in all habitats for almost a year therefore suggests that these two alternative explanations are less likely. The Norwegian Veterinary Institute collected samples from both the pike caught in April 2023, and dead pike collected after the rotenone treatment in 2021 and performed chemical element analysis that suggest that the pike caught in April 2023 indeed had spent time in a different body of water (Trapnes, Gjelstad, Pedersen, Garberg, & Moen, 2024). Since we have no indication of pike finding refuge in the sea or the rivers this suggests that the pike was reintroduced into the lake by humans. Fortunately, after this initial reappearance of pike, subsequent eDNA samples have not

shown any signs of pike presence in the lake, sea or rivers, and we conclude that pike most probably is absent.

4.3 Quick recovery of the stickleback

Less than two months after the rotenone treatment all samples were negative for stickleback eDNA, but less than four months later (May 2022) positive samples reoccurred, and the proportion of positive samples increased rapidly during spring and summer. Less than one year after the treatment, and up to the end of our study, almost all samples from the lake were positive. This increase and stable high level of positive samples suggests that after a brief period of absence sticklebacks recolonized the lake quickly and were thereafter present all over the lake and in higher numbers than before pike was removed. The quick recovery can have several causes. Firstly, one of the upstream rivers (but not the other) had samples positive of stickleback eDNA and stickleback was also present in the lake above. In addition, samples from the sea had a high proportion of positive samples, indicating sticklebacks being common here as well. The species is known for its flexible life-history, migratory ability and for being present in both freshwater and marine habitats (Wootton, 2009), and the presence of populations both upstream and in the fjord outside suggests that migration into the lake and a subsequent reestablishment should be very likely. In addition, the reappearance of positive samples cooccur with the spawning season in spring/early summer (Heins, Singer, & Baker, 2000) and it is possible that the quick return of positive eDNA samples was a result of spawning migration from the sea, with a higher number of, and more active, individuals present. It is also possible that the high level of positive eDNA samples was partially caused by the presence of large numbers of gametes and larva during and after spawning, as documented by Tsuji and Shibata (2021). The persistence of a higher level of positive samples, relative to what was observed pre-treatment is possibly the consequence of a numerical response in the stickleback lake population, where the absence of the predatory pike, and thus reduced predation pressure, have resulted in an increased population density, a so called mesopredator release (Ritchie & Johnson, 2009). Studies have shown that pike is a major predator on adult sticklebacks with the potential to suppress population densities (Donadi et al., 2017). However, sticklebacks can also predate on pike eggs (Nilsson, 2006) and larvae (Nilsson, Flink, & Tibblin, 2019) and hight densities of sticklebacks has been shown to be able to limit

recruitment of top-predator fishes like pike and perch (Donadi et al., 2020; Nilsson, Flink, & Tibblin, 2019). In the case of a reinvasion or a reintroduction of pike to this system it is possible that a large stickleback population in the lake can supress the growth of the pike population, but such reversal of predator–prey roles can also potentially limit the recovery of other native species in the lake, such as perch.

4.4 Seasonal patterns in trout and eel

Trout eDNA was detected at high levels throughout the whole study period in the sea and in the upstream rivers, and while eel was detected in relatively lower numbers in both these habitats there were no strong trends in the data for this species, except for a slow decrease in positive samples in the sea. Both eel and trout displayed similar and seemingly seasonal patterns in eDNA detections in lake samples. After a brief increase in the proportion of positive samples the winter and spring post-treatment the probability of detection decreased rapidly and both species was not detected or detected in very small proportions during the following summer.

However, during the autumn the proportion of positive samples for both these species rapidly increased, and by January 2023, approximately 400 days post-treatment, almost all samples were positive for trout, a level exceeding all prior sampling occasions, and almost half of the samples were positive for eel. This strikingly similar pattern for trout and eel in the lake are likely caused by a real seasonal change in presence and activity. Trout usually exhibits spawning migration in autumn (Finstad, Økland, Thorstad, & Heggberget, 2005; Östergren, Lundqvist, & Nilsson, 2011; Aarestrup & Jepsen, 1998) and it is likely that anadromous trout did migrate from the sea and into the lake, and further up into upstream rivers, to spawn in the autumn of 2022. The active migration of spawners and the presence of gametes and fish larvae in these spawning streams could very well have led to higher concentration of trout DNA in both the upstream river and the lake water samples. Although eels can be permanently resident in the sea, many individuals exhibit a catadromous behaviour, with elvers migrating from the sea to freshwater lakes and rivers (C. Durif et al., 2023), and although this migration can vary greatly in space and time it usually happen in summer or autumn (Naismith & Knights, 1988; L. A. Vøllestad & Jonsson, 1986). Therefore, it is possible that the increase in positive eel samples during autumn is a result of elver migration. However, we should

then also expect the elver migration to manifest as an increase in positive eDNA samples in the upstream rivers as well, and although there seem to have been a slight increase in positive samples during the same period upstream this increase is very slight.

Regardless, the seasonal pattern for trout and eel eDNA-signals in the lake is strikingly similar to each other, and seem to be repeated on a yearly basis (with a reduction in the signal in spring and an increase in autumn), and this pattern is likely the cause of a true seasonal migratory pattern of the two species. The migratory behaviour, and the various and flexible life-history, of the trout and eel have most probably been an important factor in the recolonisation of these two species in the lake. Despite the obvious seasonal pattern, in the fall of 2022 and 2023 the levels of eDNA signals from trout seem higher compared to that of pre-treatment levels in fall of 2021. This suggests a higher presence of brown trout in the lake post-treatment, and this might be another example of mesopredator release in the lake. Pike can be a significant predator of trout (Jepsen, Pedersen, & Thorstad, 2000) and the absence of pike have most likely led to a reduced mortality in trout in our study system. However, the increase in positive eDNA samples is less pronounced for trout, compared to that of the stickleback, and it is therefore less certain that the observed eDNA signal is a direct effect of the absence of pike. There is no such long-term increase in eDNA signal for eel in the lake.

4.5 The reliability of eDNA samples as a proxy for species presence

We are monitoring the probability of positive eDNA samples, and not the density of populations or number of individuals per se, but we assume that more positive samples, and especially more positive triplicates and positive samples in multiple sampling stations, represents situations when the species is present in higher numbers and over larger areas in the habitat in question. Several studies have explored using eDNA to estimate species population size. Species-specific DNA concentrations have been shown to be positively correlated with biomass or abundance (Itakura, Wakiya, Yamamoto, Kaifu, Sato, & Minamoto, 2019; Takahara, Minamoto, Yamanaka, Doi, & Kawabata, 2012), or haplotype diversity have been shown to be representative of the number of individuals present (Halvorsen, Korslund, Mattingsdal, & Slettan, 2023). However, it is important to acknowledge the potential limitations of this method (Roussel et al., 2015).

eDNA degradation rates can vary based on environmental conditions such as temperature, pH, and microbial activity, potentially affecting the detection of eDNA over time (Strickler, Fremier, & Goldberg, 2015). Furthermore, the spatial and temporal distribution of eDNA can be influenced by factors like water flow and fish movement patterns, which may lead to uneven eDNA distribution (Strickler, Fremier, & Goldberg, 2015). In our case, also the potential patchy distribution of fish carcasses, at least the first period after rotenone treatment, can cause uneven eDNA distribution and thus an unreliable link between eDNA signals and population size and distribution. Due to this we have not based our results on Ct-values or other measures of target DNA concentration, but rather treated positive samples as a binomial response variable and based on this calculated the proportion of positive samples. Given the spatial coverage of samples collected over a two-year period in this study, especially in the lake itself, we believe this approach provides reliable information about relative population trends in the rotenone treated lake for the fish species studied.

4.6 Ecological insights and future directions

In this study, we monitored a limited number of fish species, each representing different life histories. While this provides valuable insights, it does not capture the full ecological picture, including all trophic interactions. Some fish species in the lake lacked upstream or sea refuges and were eradicated following the rotenone treatment. Other species, such as cyprinids, have a higher rotenone tolerance (Marking & Bills, 1976) and did possibly survive the treatment. However, the fate of other species is beyond the scope of this study.

Moreover, our study focused solely on fish, excluding lower trophic levels such as invertebrates, which play critical roles in the ecosystem. Future research using metabarcoding, allowing for simultaneous identification of multiple species from environmental eDNA samples, could significantly enhance our understanding of these complex interactions. Applying this technique to our existing time series data would give a broader understanding of the biodiversity and trophic relationships in this ecosystem.

The reduced frequency of sampling towards the end of the study period may have limited our ability to detect fine-scale temporal patterns in the fish populations.

However, we assume that the ecosystem stabilizes over time, reducing the marginal benefit of frequent sampling. Initial phases might show rapid changes, while later phases exhibit slower, more predictable trends. We believe that the reduced sampling frequency towards the end of the study period would still capture the essential trends.

The recovery of native fish populations following rotenone treatment is surprisingly under-researched. While some studies have investigated restocking efforts to reintroduce native species after the eradication of invasive species (Lampton & Dunnigan, 2023), there remains a significant gap in understanding the natural recolonization and recovery processes of the native species. Natural recolonization is a critical yet understudied aspect that influence the success of post-treatment recovery.

4.7 Conclusion

In this study, we report the successful unassisted recovery of three native fish species after rotenone treatment. The ability of a species to recover without human intervention likely depends on its migratory capabilities and access to refuges. Our findings indicate that pike, which seem unable to persist in upstream rivers and the sea, is unable to naturally reestablish, but that trout, eel, and stickleback, capable of migrating between marine and freshwater environments, can recover within a year or less. Species less capable of inhabiting neighbour habitats, or with poorer migration capabilities, are expected to recover more slowly or to not recover without being restocked. Understanding species specific resilience to piscivore treatment is crucial for developing effective conservation and management strategies.

References

Aune, S., & Kielland, Ø. N. (2023). Bekjempelse av den regionale fremmede arten gjedde (Esox lucius) i Gillsvannet, Kristiansand.

Beaulieu, J., Trépanier-Leroux, D., Fischer, J., Olson, M., Thibodeau, S., Humphries, S., . . . Derry, A. (2021). Rotenone for exotic trout eradication: nontarget impacts on aquatic communities in a mountain lake. *Lake and Reservoir Management, 37*(3), 323-338.

Beisel, J., & Lévêque, C. (2010). Introductions of species in aquatic environments: should we be afraid of biological invasions? *Introductions of species in aquatic environments: should we be afraid of biological invasions?*

Bellingan, T. A., Hugo, S., Woodford, D. J., Gouws, J., Villet, M. H., & Weyl, O. L. (2019). Rapid recovery of macroinvertebrates in a South African stream treated with rotenone. *Hydrobiologia, 834*, 1-11.

Bessell, T. J., Appleyard, S. A., Stuart-Smith, R. D., Johnson, O. J., Ling, S. D., Heather, F. J., . . . Stuart-Smith, J. (2023). Using eDNA and SCUBA surveys for detection and monitoring of a threatened marine cryptic fish. *Aquatic Conservation: Marine and Freshwater Ecosystems, 33*(5), 431-442.

Beyer, K. (2004). Escapees of potentially invasive fishes from an ornamental aquaculture facility: the case of topmouth gudgeon Pseudorasbora parva. *Journal of fish biology, 65*, 326-327.

Britton, J., Gozlan, R. E., & Copp, G. H. (2011). Managing non-native fish in the environment. *Fish and fisheries, 12*(3), 256-274.

Byström, P., Karlsson, J., Nilsson, P., Van Kooten, T., Ask, J., & Olofsson, F. (2007). Substitution of top predators: effects of pike invasion in a subarctic lake. *Freshwater Biology, 52*(7), 1271-1280.

Capps, K. A., & Flecker, A. S. (2013). Invasive aquarium fish transform ecosystem nutrient dynamics. *Proceedings of the Royal Society B: Biological Sciences, 280*(1769), 20131520.

Carpenter, S. R., Stanley, E. H., & Vander Zanden, M. J. (2011). State of the world's freshwater ecosystems: physical, chemical, and biological changes. *Annual review of Environment and Resources, 36*, 75-99.

Clavero, M., & García-Berthou, E. (2005). Invasive species are a leading cause of animal extinctions. *Trends in ecology & evolution, 20*(3), 110.

Clavero, M., Pou-Rovira, Q., & Zamora, L. (2009). Biology and habitat use of three-spined stickleback (Gasterosteus aculeatus) in intermittent Mediterranean streams. *Ecology of Freshwater Fish, 18*(4), 550-559.

Coleman, T. S., Eckelbecker, R. W., Carlson, A. K., Devries, D. R., Wright, R. A., Staton, B. A., . . . Catalano, M. J. (2024). Evaluation of shoreline rotenone application to control Largemouth Bass recruitment in small impoundments. *North American Journal of Fisheries Management, 44*(1), 57-69. doi:10.1002/nafm.10953

Consuegra, S., Phillips, N., Gajardo, G., & de Leaniz, C. G. (2011). Winning the invasion roulette: Escapes from fish farms increase admixture and facilitate establishment of non-native rainbow trout. *Evolutionary Applications, 4*(5), 660-671.

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., & Miaud, C. (2011). Persistence of environmental DNA in freshwater ecosystems. *Plos One, 6*(8), e23398.

Donadi, S., Austin, Å. N., Bergström, U., Eriksson, B. K., Hansen, J. P., Jacobson, P., . . . Eklöf, J. S. (2017). A cross-scale trophic cascade from large predatory fish to algae in coastal ecosystems. *Proceedings of the Royal Society B: Biological Sciences, 284*(1859), 20170045.

Donadi, S., Bergström, L., Berglund, J. M. B., Anette, B., Mikkola, R., Saarinen, A., & Bergström, U. (2020). Perch and pike recruitment in coastal bays limited by stickleback predation and environmental forcing. *Estuarine, Coastal and Shelf Science, 246*, 107052.

Donaldson, M. R., O'Connor, C. M., Thompson, L. A., Gingerich, A. J., Danylchuk, S. E., Duplain, R. R., & Cooke, S. J. (2011). Contrasting global game fish and non-game fish species. *Fisheries, 36*(8), 385-397.

Dunker, K. J., Sepulveda, A. J., Massengill, R. L., Olsen, J. B., Russ, O. L., Wenburg, J. K., & Antonovich, A. (2016). Potential of environmental DNA to evaluate northern pike (Esox lucius) eradication efforts: an experimental test and case study. *PLoS One, 11*(9), e0162277.

Durif, C., Arts, M., Bertolini, F., Cresci, A., Daverat, F., Karlsbakk, E., . . . Parzanini, C. (2023). The evolving story of catadromy in the European eel (Anguilla anguilla). *ICES Journal of Marine Science*, fsad149.

Durif, C. M. F., Arts, M., Bertolini, F., Cresci, A., Daverat, F., Karlsbakk, E., . . . Browman, H. (2023). The evolving story of catadromy in the European eel (Anguilla anguilla). *Ices Journal of Marine Science*. doi:10.1093/icesjms/fsad149

Ferguson, A., Reed, T. E., Cross, T. F., McGinnity, P., & Prodöhl, P. A. (2019). Anadromy, potamodromy and residency in brown trout Salmo trutta: the role of genes and the environment. *Journal of fish biology, 95*(3), 692-718.

Finlayson, B., Somer, W. L., & Vinson, M. R. (2010). Rotenone toxicity to rainbow trout and several mountain stream insects. *North American Journal of Fisheries Management, 30*(1), 102-111.

Finstad, A., Økland, F., Thorstad, E., & Heggberget, T. (2005). Comparing upriver spawning migration of Atlantic salmon Salmo salar and sea trout Salmo trutta. *Journal of Fish Biology, 67*(4), 919-930.

Fletcher, C. M., Collins, S. F., Nannini, M. A., & Wahl, D. H. (2019). Competition during early ontogeny: Effects of native and invasive planktivores on the growth, survival, and habitat use of bluegill. *Freshwater Biology, 64*(4), 697-707. doi:10.1111/fwb.13255

Forsgren, E., Bærum, K., Finstad, A. G., KØ. , Hesthagen, T., Knutsen, H., & Wienerroither, R. (2023). Fisker: Vurdering av gjedde Esox lucius for Fastlands-Norge med havområder. Fremmedartslista 2023. Artsdatabanken. doi: http://www.artsdatabanken.no/lister/fremmedartslista/2023/1769

Galil, B. S., Nehring, S., & Panov, V. (2007). Waterways as invasion highways–Impact of climate change and globalization. *Biological Invasions, 193*, 59-74.

Gilbert, M. A., & Granath Jr, W. O. (2003). Whirling disease of salmonid fish: life cycle, biology, and disease. *Journal of Parasitology, 89*(4), 658-667.

Gozlan, R. E., Britton, J., Cowx, I., & Copp, G. (2010). Current knowledge on non-native freshwater fish introductions. *Journal of fish biology, 76*(4), 751-786.

Groocock, G., Getchell, R., Wooster, G., Britt, K., Batts, W., Winton, J., . . . Bowser, P. (2007). Detection of viral hemorrhagic septicemia in round gobies in New York State (USA) waters of Lake Ontario and the St. Lawrence River. *Diseases of aquatic organisms, 76*(3), 187-192.

Guillera-Arroita, G., Lahoz-Monfort, J. J., van Rooyen, A. R., Weeks, A. R., & Tingley, R. (2017). Dealing with false-positive and false-negative errors about species occurrence at multiple levels. *Methods in ecology and evolution, 8*(9), 1081-1091.

Halvorsen, S., Korslund, L., Gustavsen, P. Ø., & Slettan, A. (2020). Environmental DNA analysis indicates that migration barriers are decreasing the occurrence of European eel (Anguilla anguilla) in distance from the sea. *Global Ecology and Conservation, 24*, e01245.

Halvorsen, S., Korslund, L., Mattingsdal, M., & Slettan, A. (2023). Estimating number of European eel (Anguilla anguilla) individuals using environmental DNA and haplotype count in small rivers. *Ecology and evolution, 13*(2), e9785.

Hata, H., Uemura, Y., & Ouchi, K. (2021). Decline of unionid mussels enhances hybridisation of native and introduced bitterling fish species through competition for breeding substrate. *Freshwater Biology, 66*(1), 189-201. doi:10.1111/fwb.13629

Haught, S., & von Hippel, F. A. (2011). Invasive pike establishment in Cook Inlet Basin lakes, Alaska: diet, native fish abundance and lake environment. *Biological Invasions, 13*(9), 2103-2114. doi:10.1007/s10530-011-0029-4

Heins, D. C., Singer, S. S., & Baker, J. A. (2000). Virulence of the cestode Schistocephalus solidus and reproduction in infected threespine stickleback, Gasterosteus aculeatus. *Canadian Journal of Zoology, 77*(12), 1967-1974.

Hesthagen, T., Rikstad, A., Adolfsen, P., Sandlund, T. O., Bardal, H., Bergan, M. A., . . . Slettom, L. (2020). *Omfattende spredning og bekjempelse av gjedde i Trøndelag*. Retrieved from VANN:

Horgan, D. J., Singer, T. P., & Casida, J. (1968). Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase: XIII. Binding sites of rotenone, piericidin A, and amytal in the respiratory chain. *Journal of Biological Chemistry, 243*(4), 834-843.

Hulme, P. E. (2009). Trade, transport and trouble: managing invasive species pathways in an era of globalization. *Journal of Applied Ecology, 46*(1), 10-18.

Ishikawa, A., & Kitano, J. (2020). Diversity in reproductive seasonality in the three-spined stickleback, Gasterosteus aculeatus. *Journal of Experimental Biology, 223*(Suppl_1). doi:10.1242/jeb.208975

Itakura, H., Wakiya, R., Yamamoto, S., Kaifu, K., Sato, T., & Minamoto, T. (2019). Environmental DNA analysis reveals the spatial distribution, abundance, and biomass of Japanese eels at the river-basin scale. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 1-13. Retrieved from https://doi.org/10.1002/aqc.3058

Jepsen, N., Pedersen, S., & Thorstad, E. (2000). Behavioural interactions between prey (trout smolts) and predators (pike and pikeperch) in an impounded river. *Regulated Rivers: Research & Management: An International Journal Devoted to River Research and Management, 16*(2), 189-198.

Jordaan, M. S., & Weyl, O. L. F. (2013). Determining the minimum effective dose of rotenone for eradication of alien smallmouth bass Micropterus dolomieu from a South African river. *African Journal of Aquatic Science, 38*, 91-95. doi:10.2989/16085914.2013.784699

Kahle, D. W., H. . (2013). ggmap: Spatial Visualization with ggplot2. *The R Journal*(5(1)), 144–161. doi:https://journal.r-project.org/archive/2013-1/kahle-wickham.pdf

Kjærstad, G., & Arnekleiv, J. V. (2011). Effects of rotenone treatment on lotic invertebrates. *International Review of Hydrobiology, 96*(1), 58-71.

Kjærstad, G., Arnekleiv, J. V., Velle, G., & Finstad, A. G. (2022). Long-term responses of benthic invertebrates to rotenone treatment. *River Research and Applications, 38*(8), 1436-1449. doi:10.1002/rra.3919

Kjærstad, G., Arnekleiv, J. V., Velle, G., & Finstad, A. G. (2022). Long-term responses of benthic invertebrates to rotenone treatment. *River Research and Applications, 38*(8), 1436-1449.

Kobler, A., Klefoth, T., Wolter, C., Fredrich, F., & Arlinghaus, R. (2008). Contrasting pike (Esox lucius L.) movement and habitat choice between summer and winter in a small lake. *Hydrobiologia, 601*, 17-27.

Koed, A., Balleby, K., Mejlhede, P., & Aarestrup, K. (2006). Annual movement of adult pike (Esox lucius L.) in a lowland river. *Ecology of Freshwater Fish, 15*(2), 191-199.

Lampton, J. K., R, & Dunnigan, J. (2023). Efficacy of one-time rotenone application for Westslope Cutthroat Trout restoration in Boulder Creek, Montana. *North American Journal of Fisheries Management, 43*(4), 1081-1092.

Ling, N. (2003). Rotenoneôa review of its toxicity and use for fisheries management. *Science for conservation, 211*, 1-40.

Marking, L. L., & Bills, T. D. (1976). *Toxicity of rotenone to fish in standardized laboratory tests* (Vol. 72): Department of the Interior, Fish and Wildlife Service.

McGeoch, M. A., Butchart, S. H. M., Spear, D., Marais, E., Kleynhans, E. J., Symes, A., . . . Hoffmann, M. (2010). Global indicators of biological invasion: species numbers, biodiversity impact and policy responses. *Diversity and Distributions, 16*(1), 95-108. doi:10.1111/j.1472-4642.2009.00633.x

Meyerson, L. A., & Reaser, J. K. (2003). Bioinvasions, bioterrorism, and biosecurity. *Frontiers in Ecology and the Environment, 1*(6), 307-314.

Naismith, I., & Knights, B. (1988). Migrations of elvers and juvenile European eels, Anguilla anguilla L., in the River Thames. *Journal of Fish Biology, 33*, 161-175.

Nilsson, J. (2006). Predation of Northern Pike (Esox lucius L.) Eggs: A Possible Cause of Regionally Poor Recruitment in the Baltic Sea. *Hydrobiologia, 553*(1), 161-169. doi:10.1007/s10750-005-1949-8

Nilsson, J., Flink, H., & Tibblin, P. (2019). Predator–prey role reversal may impair the recovery of declining pike populations. *Journal of Animal Ecology, 88*(6), 927-939.

Olsen, J. B., Lewis, C. J., Massengill, R. L., Dunker, K. J., & Wenburg, J. K. (2015). An evaluation of target specificity and sensitivity of three qPCR assays for detecting environmental DNA from Northern Pike (Esox lucius). *Conservation Genetics Resources, 7*(3), 615-617.

Oredalen, T. J. (2002). *Tilstandsvurdering av Gillsvannet i Kristiansand kommune*. Retrieved from

Orihuela-Torres, A., Morales-Reyes, Z., Hermoso, V., Picazo, F., Sánchez Fernández, D., Pérez-García, J. M., . . . Sebastián-González, E. (2024). Carrion ecology in inland aquatic ecosystems: a systematic review. *Biological Reviews*.

Passerini, M., Cunha-Santino, M. d., & Bianchini Jr, I. (2016). Oxygen availability and temperature as driving forces for decomposition of aquatic macrophytes. *Aquatic Botany, 130*, 1-10.

Pham, L., Jarvis, M. G., West, D., & Closs, G. P. (2018). Rotenone treatment has a short-term effect on New Zealand stream macroinvertebrate communities. *New Zealand Journal of Marine and Freshwater Research, 52*(1), 42-54. doi:10.1080/00288330.2017.1330273

Pimentel, D., Zuniga, R., & Morrison, D. (2005). Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological economics, 52*(3), 273-288.

Recsetar, M. S., & Bonar, S. A. (2015). Effectiveness of two commercial rotenone formulations in the eradication of virile crayfish Orconectes virilis. *North American Journal of Fisheries Management, 35*(3), 616-620.

Reid, A. J., Carlson, A. K., Creed, I. F., Eliason, E. J., Gell, P. A., Johnson, P. T. J., . . . Cooke, S. J. (2019). Emerging threats and persistent conservation challenges for freshwater biodiversity. *Biological Reviews, 94*(3), 849-873. doi:10.1111/brv.12480

Ricciardi, A., & MacIsaac, H. J. (2011). Impacts of biological invasions on freshwater ecosystems. *Fifty years of invasion ecology: the legacy of Charles Elton, 1*, 211-224.

Ritchie, E. G., & Johnson, C. N. (2009). Predator interactions, mesopredator release and biodiversity conservation. *Ecology Letters, 12*(9), 982-998. doi:https://doi.org/10.1111/j.1461-0248.2009.01347.x

Roussel, J.-M., Paillisson, J.-M., Tréguier, A., & Petit, E. (2015). The downside of eDNA as a survey tool in water bodies. *Journal of Applied Ecology, 52*(4), 823-826. doi:https://doi.org/10.1111/1365-2664.12428

Raat, A. J. (1988). *Synopsis of biological data on the northern pike: Esox lucius Linnaeus, 1758*: Food & Agriculture Org.

Sandodden, R., Brazier, M., Sandvik, M., Moen, A., Wist, A. N., & Adolfsen, P. (2018). Eradication of Gyrodactylus salaris infested Atlantic salmon (Salmo salar) in the Rauma River, Norway, using rotenone. *Management of Biological Invasions, 9*(1), 67.

Seebens, H., Blackburn, T. M., Dyer, E. E., Genovesi, P., Hulme, P. E., Jeschke, J. M., . . . Arianoutsou, M. (2017). No saturation in the accumulation of alien species worldwide. *Nature Communications, 8*(1), 14435.

Sepulveda, A. J., Rutz, D. S., Dupuis, A. W., Shields, P. A., & Dunker, K. J. (2015). Introduced northern pike consumption of salmonids in Southcentral Alaska. *Ecology of Freshwater Fish, 24*(4), 519-531. doi:10.1111/eff.12164

Sepulveda, A. J., Rutz, D. S., Ivey, S. S., Dunker, K. J., & Gross, J. A. (2013). Introduced northern pike predation on salmonids in southcentral Alaska. *Ecology of Freshwater Fish, 22*(2), 268-279. doi:10.1111/eff.12024

Simberloff, D., Martin, J.-L., Genovesi, P., Maris, V., Wardle, D. A., Aronson, J., . . . Pascal, M. (2013). Impacts of biological invasions: what's what and the way forward. *Trends in ecology & evolution, 28*(1), 58-66.

Simpson, G. g. R. p. v. (2024). Graceful ggplot-Based Graphics and Other Functions for GAMs Fitted using mgcv. R package version 0.9.0.

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillott, A. D., . . . Kenyon, N. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth, 4*(2), 125-134.

Sobek, S., Durisch-Kaiser, E., Zurbrügg, R., Wongfun, N., Wessels, M., Pasche, N., & Wehrli, B. (2009). Organic carbon burial efficiency in lake sediments controlled by oxygen exposure time and sediment source. *Limnology and Oceanography, 54*(6), 2243-2254.

Spens, J., Englund, G., & Lundquist, H. (2007). Network connectivity and dispersal barriers: using geographical information system (GIS) tools to predict landscape scale distribution of a key predator (Esox lucius) among lakes. *Journal of Applied Ecology, 44*(6), 1127-1137. doi:https://doi.org/10.1111/j.1365-2664.2007.01382.x

Strayer, D. L., & Dudgeon, D. (2010). Freshwater biodiversity conservation: recent progress and future challenges. *Journal of the North American Benthological Society, 29*(1), 344-358.

Strickler, K. M., Fremier, A. K., & Goldberg, C. S. (2015). Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation, 183*, 85-92. doi:10.1016/j.biocon.2014.11.038

Sunde, J., Tamario, C., Tibblin, P., Larsson, P., & Forsman, A. (2018). Variation in salinity tolerance between and within anadromous subpopulations of pike (Esox lucius). *Scientific Reports, 8*(1), 22.

Szaniawska, A., & Szaniawska, A. (2018). Function and importance of crustaceans. *Baltic crustaceans*, 185-188.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., & Kawabata, Z. i. (2012). Estimation of fish biomass using environmental DNA. *Plos One, 7*(4), e35868.

Thomsen, P. F., Kielgast, J., Iversen, L. L., Moller, P. R., Rasmussen, M., & Willerslev, E. (2012). Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples. *Plos One, 7*(8). doi:10.1371/journal.pone.0041732

Thorstad, E. B., Todd, C. D., Uglem, I., Bjørn, P. A., Gargan, P. G., Vollset, K. W., . . . Finstad, B. (2016). Marine life of the sea trout. *Marine Biology, 163*, 1-19.

Tibblin, P., Forsman, A., Borger, T., & Larsson, P. (2016). Causes and consequences of repeatability, flexibility and individual fine-tuning of migratory timing in pike. *Journal of Animal Ecology, 85*(1), 136-145.

Trapnes, S. H., Gjelstad, S. K., Pedersen, R., Garberg, M., & Moen, V. (2024). *Geoelementanalyse av skjell fra gjedde fanget i Gillsvannet 2023. – Hvordan har gjedda som ble fanget i Gillsvannet i 2023 kommet tilbake?* . Retrieved from

Tsuji, S., & Shibata, N. (2021). Identifying spawning events in fish by observing a spike in environmental DNA concentration after spawning. *Environmental DNA, 3*(1), 190-199.

Tsukamoto, K., & Arai, T. (2001). Facultative catadromy of the eel Anguilla japonica between freshwater and seawater habitats. *Marine Ecology Progress Series, 220*, 265-276.
Tzeng, W.-N., & Tsai, Y. (1994). Changes in otolith microchemistry of the Japanese eel, Anguitta japonica, during its migration from the ocean to the rivers of Taiwan. *Journal of fish biology, 45*(4), 671-683.
Van Kessel, N., Dorenbosch, M., De Boer, M. R. M., Leuven, R., & Van der Velde, G. (2011). Competition for shelter between four invasive gobiids and two native benthic fish species. *Current Zoology, 57*(6), 844-851. Retrieved from <Go to ISI>://WOS:000298293800021
Vøllestad, A., Skurdal, J., & Qvenild, T. (1986). Habitat use, growth, and feeding of pike(Esox lucius L.) in four Norwegian lakes. *Archiv fur Hydrobiologie. Stuttgart, 108*(1), 107-117.
Vøllestad, L. A., & Jonsson, B. (1986). Life-History Characteristics of the European Eel Anguilla anguilla in the Imsa River, Norway. *Transactions of the American Fisheries Society, 115*(6), 864-871. doi:https://doi.org/10.1577/1548-8659(1986)115<864:LCOTEE>2.0.CO;2
Westin, L., & Limburg, K. E. (2002). Newly discovered reproductive isolation reveals sympatric populations of Esox Indus in the Baltic. *Journal of fish biology, 61*(6), 1647-1652.
Wetzel, R. G., Likens, G. E., Wetzel, R. G., & Likens, G. E. (1991). Decomposition: relative bacterial heterotrophic activity on soluble organic matter. *Limnological Analyses*, 271-279.
White, R. A., & Zhang, C. (2023). csmaps: Preformatted Maps of Norway that Don't Need Geolibraries.
White, R. A. Z., C. . (2024). csdata: Structural Data for Norway. R package version 2024.4.21.
Winter, M., Schweiger, O., Klotz, S., Nentwig, W., Andriopoulos, P., Arianoutsou, M., . . . Hejda, M. (2009). Plant extinctions and introductions lead to phylogenetic and taxonomic homogenization of the European flora. *Proceedings of the National Academy of Sciences, 106*(51), 21721-21725.
Wonham, M. J., Carlton, J. T., Ruiz, G. M., & Smith, L. D. (2000). Fish and ships: relating dispersal frequency to success in biological invasions. *Marine Biology, 136*, 1111-1121.
Wood, S. (2017). Generalized Additive Models: An Introduction with R, 2 edition. .
Woodford, D. J., Barber-James, H. M., Bellingan, T. A., Day, J. A., de Moor, F. C., Gouws, J., & Weyl, O. L. (2013). Immediate impact of piscicide operations on a Cape Floristic Region aquatic insect assemblage: a lesser of two evils? *Journal of Insect Conservation, 17*, 959-973.
Wootton, R. J. (2009). The Darwinian stickleback Gasterosteus aculeatus: a history of evolutionary studies*. *Journal of Fish Biology, 75*(8), 1919-1942. doi:https://doi.org/10.1111/j.1095-8649.2009.02412.x
Östergren, J., Lundqvist, H., & Nilsson, J. (2011). High variability in spawning migration of sea trout, Salmo trutta, in two northern Swedish rivers. *Fisheries Management and Ecology, 18*(1), 72-82.
Aarestrup, K., & Jepsen, N. (1998). *Spawning migration of sea trout (Salmo trutta (L)) in a Danish river.* Paper presented at the Advances in Invertebrates and Fish Telemetry: Proceedings of the Second Conference on Fish Telemetry in Europe, held in La Rochelle, France, 5–9 April 1997.

Appendix A

Overview of eDNA- samples missing in the dataset

Sampling locations	Date	Species	Type of error
1	22.12.21	European eel Brown trout Northern pike Three-spined stickleback	Ice coverage on the lake
11	22.12.21	Northern pike Three-spined stickleback	Sample empty or lost
10	09.05.22	European eel	Sample empty or lost
1	04.03.22	Brown trout	Sample empty or lost
1	23.01.23	Brown trout Three-spined stickleback	Sample empty or lost

Appendix B

Primers and probe details showing sequences, target species, gene and fragment size

Target species	Primer F and R, and probe sequence	Gene	Fragment size	Primer and probe design
A. anguilla	F- 5′-CACCCATACTTCTCCTACAAAGACCTA-3′ R- 5′-TCTGGGTCTCCAAGCAGGTT-3’ P- 5-FAM-TTCATTATCATGCTCACC-MGBEQ-3’	cytb	101 bp	Halvorsen et al. (2020)
S. trutta	F-5′-CCACCCCTACTTCTCATA-3′ R- 5′-GGAGGTTGGGTGCGAATAGA-3’ P-5′- FAM-CTTGGATTCGTAGCTAT-MGBEQ -3’	cytb	88 bp	Halvorsen et al. (2020)
E. lucius	F-5′CCTTCCCCCGCATAAATAATATAA3′ R-5′GTGTTGAAGCTGGTGCTGGTAC3′ P-5′-FAM-CTTCTGACTTCTCCCC-MGB-NFQ-3′	COI	94 bp	Olsen et al. (2015)
G. aculeatus	5’-ACGCCACCTTAACACGTTTCTT-3’ R-5’-AGAGCCTGTCTGGTGAAGGAAA-3’ P-6FAM-5’-GGTGCCACACTTGTT-3’-MBG-NFQ	cytb	101 bp	Thomsen et al. (2012)

Appendix C

Model outputs for generalized additive mixed models for all four fish species studied, describing the proportion of positive eDNA samples over time. Parametric coefficients represent the overall intercept of each habitat and smooth terms shows the complexity of the non-linear additive terms and the random intercept for ach sampling station. The sample size (no of stations x no of sampling occasions x no of triplicates) is shown below each model, and deviations here are caused by occasional depletion of sample DNA. Consistently not positive eDNA samples in both upstream stations caused a lack of conversion and unreliable estimates for this habitat in the pike model.

	Three-spined stickleback				
Component	**Term**	**Estimate**	**Std Error**	**t-value**	**p-value**
A. parametric coefficients	Intercept (Lake)	1.976	1.541	1.282	0.200
	Habitat: Sea	0.493	2.198	0.224	0.822
	Habitat: Inlet	-4.747	2.104	-2.257	0.024
Component	**Term**	**edf**	**Ref. df**	**F-value**	**p-value**
B. smooth terms	Time (Lake)	4.766	5.215	69.913	<0.001
	Time (Sea)	3.579	4.245	7.536	0.096
	Time (Inlet)	2.640	3.259	9.603	0.031
	Random intercept (Station)	6.406	7.000	35.382	<0.001
	Adjusted R-squared: 0.594, Deviance explained 0.557, N=531				
	European pike				
Component	**Term**	**Estimate**	**Std Error**	**t-value**	**p-value**
A. parametric coefficients	Intercept (Lake)	-3.469	0.783	-4.433	<0.001
	Habitat: Sea	-7.427	14.204	-0.523	0.6011
	Habitat: Inlet				
Component	**Term**	**edf**	**Ref. df**	**F-value**	**p-value**
B. smooth terms	Time (Lake)	4.761	5.343	64.234	<0.001
	Time (Sea)	2.103	2.381	2.512	0.2377
	Time (Inlet)				
	Random intercept (Station)	0.001	6	0.001	0.5001
	Adjusted R-squared: 0.617, Deviance explained 0.639, N=534				
	Brown trout				
Component	**Term**	**Estimate**	**Std Error**	**t-value**	**p-value**
A. parametric coefficients	Intercept (Lake)	-0.077	0.185	-0.415	0.6778
	Habitat: Sea	1.831	0.381	4.805	<0.001
	Habitat: Inlet	3.977	0.847	4.694	<0.001
Component	**Term**	**edf**	**Ref. df**	**F-value**	**p-value**
B. smooth terms	Time (Lake)	5.77	5.977	67.073	<0.001

	Time (Sea)	1	1	0.187	0.6654
	Time (Inlet)	1	1	1.234	0.2667
	Random intercept (Station)	2.399	7	5.009	0.0471
	Adjusted R-squared: 0.429, Deviance explained 0.381, N=531				
	European eel				
Component	**Term**	**Estimate**	**Std Error**	**t-value**	**p-value**
A. parametric coefficients	Intercept (Lake)	-1.022	0.233	-4.382	0
	Habitat: Sea	1.098	0.424	2.59	0.0096
	Habitat: Inlet	1.022	0.424	2.409	0.016
Component	**Term**	**edf**	**Ref. df**	**F-value**	**p-value**
B. smooth terms	Time (Lake)	5.59	5.923	47.558	0
	Time (Sea)	1.001	1.001	6.027	0.0141
	Time (Inlet)	2.207	2.738	3.851	0.2849
	Random intercept (Station)	4.305	7	11.425	0.0097
	Adjusted R-squared: 0.196, Deviance explained 0.174, N=507				