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Master

Designing a species-specific method, based on analysis of eDNA, for detection of lesser weever fish, and testing it at chosen locations along the coastline of Agder, Southern Norway

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Research is to see what everybody else has seen, and to think what nobody else has thought
Albert Szent-Györgyi

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Abstract

Temperature is largely influencing species dispersion, and when the temperature conditions within an area are changing, species populations often respond by moving to other, more suitable areas. Such behavior has often been observed in marine species, because of increased water temperatures in the ocean. Throughout the Northeastern Atlantic Ocean, including the North Sea and Skagerrak, increased water temperatures have been registered over the last three decades, which are likely to have affected the availability of suitable habitats for various fish species, and therefore their local presence. This could explain how the warm water fish species lesser weever, *Echiichthys vipera,* over the past few years have been registered in Norwegian waters for the first time. The species is mostly known for its painful and venomous stings, while relatively little is known about its life cycle or ecological importance. Studying marine species within their natural environment is generally considered challenging since they are “less visible” than terrestrial species. However, utilization of molecular methods for faster and more precise species detection can ease future research.

In this study a species-specific method, based on analysis of environmental DNA (eDNA), has been designed for detection of lesser weever fish. The eDNA was sampled at chosen locations along the Agder coast, and was analysed using real-time PCR with primers and a TaqMan probe designed to amplify only DNA from this species. The analysis results show that lesser weever DNA was successfully detected in four locations despite very low concentrations. This proves that the designed method is good, and that the sensitivity is high. The high sensitivity was also shown using serial dilutions of genomic DNA, while the specificity was ensured through an in-silico PCR, and by testing the primers and probe on the closely related species greater weever fish. Generally, for the aim of detecting the potential presence of a species in a chosen location, independent on abundance, the impression of this method has a high utility and efficiency.

Norsk oppsummering

Temperatur har stor innvirkning på artsfordeling, og når temperaturforholdene i et område endres vil artspopulasjoner ofte respondere med å flytte til andre, mer passende områder. Denne atferden har ofte blitt observert hos marine arter, på grunn av økte vanntemperaturer i havet. I løpet av de tre siste tiårene har økte vanntemperaturer blitt registrert over hele det nordøstlige Atlanterhavet, inkludert Nordsjøen og Skagerrak, noe som trolig har påvirket tilgangen på passende habitater for ulike fiskearter, og dermed deres lokale forekomst. Dette kan forklare hvordan varmtvannsfisken dvergfjesing, *Echiichthys vipera*, har blitt registrert i norske farvann i løpet av de siste årene, for første gang. Denne arten er først og fremst kjent for sine smertefulle og giftige stikk, mens man vet relativt lite om livssyklusen eller den økologiske betydningen. Generelt sett er det å studere marine arter i deres naturlige miljø utfordrende, siden de er «mindre synlige» enn terrestriske arter. Ved å benytte molekylære metoder kan imidlertid arter oppdages raskere og mer presist, noe som kan gjøre det enklere å studere disse i fremtiden.

I dette studiet har en artsspesifikk metode, basert på analyse av miljø-DNA (eDNA), blitt designet for påvisning av dvergfjesing. Miljø-DNA ble samlet inn fra utvalgte stasjoner langs kysten av Agder, og ble analysert ved hjelp av real-time PCR med primere og en TaqMan-probe som var designet til å bare kopiere DNA fra denne arten. Analyseresultatene viser at dvergfjesing-DNA ble oppdaget ved fire stasjoner til tross for veldig lave konsentrasjoner. Dette beviser at en god metode har blitt designet, og at sensitiviteten er høy. Den høye sensitiviteten ble også vist gjennom en seriefortynning som ble gjort på genomisk DNA, mens spesifisiteten ble sikret ved hjelp av en in-silico PCR, og ved å prøve primerne og proben på den nært beslektede arten fjesing. Generelt oppleves denne metoden som veldig nyttig og effektiv dersom målet er å påvise forekomsten til en art i et valgt område, uavhengig av artsoverflod.

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Preface

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Kristiansand, Thursday May 4th

Jimmi Sjøstrøm

1 Introduction

Temperature has a large influence on species dispersion (Martínez et al., 2018). It is a key determinant to various biological processes like growth, metabolism, and reproduction, and for this reason all species have an optimal temperature range for normal functioning (Sen Gupta et al., 2015). If the temperature conditions within an area is changed, some indirect effects may occur, like the appearance of new predators, disappearance of prey, and alterations to various physical and biochemical processes (Sen Gupta et al., 2015). Consequently, species populations typically respond by moving to more suitable areas (Sen Gupta et al., 2015). This behavior has often been observed from marine species, both in shallow- and deep-water environments (Chaikin et al., 2022).

1.1 Warming of the ocean

It is a well-established fact that the ocean has been experiencing rising water temperatures over the last decades (Chaikin et al., 2022). This realization is based on various measurements including duration of marine heatwaves, rates of ocean warming, and mean sea surface temperature (SST) (Chaikin et al., 2022). It is presumably one of many impacts from climate change, something that anthropogenic emission of greenhouse gases is a major contributor to (Paul J. Durack et al., 2018). The ocean is known to possess a very high heat capacity, and during recent decades about 90% of excess heat has been absorbed by it (Paul J. Durack et al., 2018). Although the increased temperatures often are associated with surface level, results of new analyses indicate that warming sometimes goes all the way down to the abyss (Paul J. Durack et al., 2018).

Throughout the Northeast Atlantic shelf increased water temperature has been registered over the last three decades, likely to have affected the availability of suitable habitats for various fish species, and therefore affecting their local presence (Bauon et al., 2020). For instance, tropical species entering Atlantic-European waters has become a common phenomenon (Awaluddin et al., 2016), while cold-water species have shifted to colder areas (Kleisner et al., 2017). It has been noted that movements typically are towards the poles, and that the global ranges can be both extended and limited (Hammerschlag et al., 2022). Due to the influence of various abiotic and biotic factors, tolerance differences between species, and varying quality of natural ecosystems, the local responses can be very different (Hammerschlag et al., 2022).

Since it also depends on the season and the region, the local rearrangement of species may differ from the global trends (Awaluddin et al., 2016). The species that manage to survive and form viable populations in new regions are considered a potential threat to native species, communities, and even whole ecosystems (Awaluddin et al., 2016).

1.2 Altered temperature conditions in the Skagerrak

The composition of marine species within high latitudes waters is traditionally driven by big variations to environmental conditions, e.g., temperature, during a year (Gran-Stadniczeñko et al., 2018; Freitas et al., 2021). Within the Skagerrak the sea surface temperatures typically vary from 0 during the winters to above 20°C during the summers (Freitas et al., 2021). Fish species have different temperature preferences, and thus their local presence and activity in such regions largely depends on the season (Freitas et al., 2021). As an example, cold water species like Cod, *Gadus morhua*, are often less abundant at shallow depths during summer since the temperature often is above 15°C, while warm water species like the ballan wrasse, *Labrus bergylta*, are most abundant and active at during this season (Freitas et al., 2021).

The North Sea and Skagerrak are among the regions that are claimed to have experienced increased temperatures during the last decades (Trannum et al., 2018). Long-established dynamics of species abundance in this region can potentially be altered if the warmer conditions become more permanent, and for the future it has been discussed whether cold-water species may hide at larger and colder depths for longer periods of the year, while high abundance of warm-water species may become a more common trend a at shallower depths (Chaikin et al., 2022). The Institute of Marine Research have observed a stable increase to water temperatures within Skagerrak since the 1980s, and the appearance of "new species" from southern areas is a highly expected consequence ("Økosystemet i Nordsjøen og Skagerrak," 2018).

1.3 A new warm-water fish species registered in Mandal, Southern Norway

The fish species lesser weever, *Echiichthys vipera,* attracted considerable attention by local media, inhabitants, and marine scientists when numerous specimens were found at beaches in Mandal, Southern Norway, during the late summer of 2021 (Lepperød, 2021; Mjaaland,

2021). Until a few specimens were discovered by chance by local fishers in 2018, this species had never been registered within Norwegian waters (Lepperød, 2021; Mjaaland, 2021). The traditional prevalence of lesser weever is within the Mediterranean Sea and the Eastern Atlantic Ocean, from Morocco in the south to the Shetlands Isles and Danish coast of Skagerrak in the north (Vasconcelos et al., 2004). In the North Sea, Lesser weever is traditionally reported to be abundant in the southernmost parts due to its preference for warmer water (Scott & Henderson, 2016). However, over the last decades the temperature of the North Sea waters has increased, affecting the arrangement of various marine species (Perry et al., 2005). Warm-water species have extended their distribution northwards as temperatures have become more sufficient than before (Perry et al., 2005). The newly detection of the lesser weever in Southern Norway indicates that the temperature conditions in that area have become more suitable for this species during late summer.

This tiny (150 mm SL (Standard length)) demersal fish belongs to the family Trachinidae, which is mainly known for its painful and venomous stings (Vasconcelos et al., 2004). For beach tourists it is considered a big annoyance, because it is often found in the shallowest parts of the waters during summer (Gorman et al., 2021). It is very hard to see, because it buries itself in the sand during daytime, with only the head and venomous spikes above (Scott & Henderson, 2016). Although its venom is not considered deathly to humans it is described as extremely painful, and to possibly causing infection if not treated immediately (Emerson, 2012). Other than its venom, lesser weever has received limited research compared to other fish species (Scott & Henderson, 2016), and therefore little is known about its life cycle or ecological importance. Studying marine species within their natural environment is generally considered challenging since they are “less visible” than terrestrial species (Karenyi et al., 2018). This applies particularly to marine species that are rare or have low local abundance (Karenyi et al., 2018). If their presence can be detected more effectively in the future, without the need to catch them first, further research may be carried out more easily.

1.4 Species detection using environmental DNA

Since scientists started to develop and utilize molecular techniques, the process of detecting species has become faster and more precise (Kim et al., 2018; Marín et al., 2017; Liu et al., 2020). Today, collecting environmental DNA (eDNA), and analyzing it using real-time

polymerase chain reaction (qPCR or real-time PCR) has proven to be especially effective, and is frequently used on most species (Fukuta et al., 2013; Kim et al., 2018). Shortly explained, the principle is that the presence of eDNA from a species can be detected using large scale amplification (Freeland, 2020). Additionally, the amount of DNA in a sample can be quantified (Freeland, 2020, p. 19-22).

Environmental DNA (eDNA) is defined as remnant DNA which can be extracted from any cellular material that is shed by organisms (like skin, blood, mucus, excrements, etc.) into aquatic or terrestrial environments, and which can be sampled using molecular methods (Taberlet et al., 2012; Balasingham et al, 2016; Buxton et al., 2017, Freeland, 2020, p. 87). The use of eDNA has many advantages. Firstly, samples can be obtained without the need to catch, disturb, or harm organisms (Thomsen & Willerslev, 2015; Balasingham, 2016; Freeland, 2020, p. 87). Secondly, organisms can be detected even though they cannot be seen, since any form of organismal material will most likely have been left behind if they recently have been present in the environment (Freeland, 2020, p. 87). Finally, the detection ability is independent on developmental stage and population size (Kim et al., 2018).

When analysing eDNA the main steps are to collect samples from the relevant environment (water, soil, etc.), to extract eDNA from the samples by isolating it from all other content, and finally to analyse the extracted eDNA either with a species-specific assay, or with metabarcoding (figure 1) (Freeland, 2020, p. 87-88). Although these steps are used for all types of samples, there are some differences to the procedures (Freeland, 2020, p. 87-88). For example, water samples are filtered before eDNA can be extracted from a filter paper (Freeland, 2020, p. 87-88). When analysing the eDNA through a species-specific assay, primers are designed to hybridize only to DNA of a chosen target-species, and (Freeland, 2020, 87-88). When analysing through metabarcoding, primers are designed to hybridize to DNA of many different species (universal primers), and each species is then identified using high-throughput DNA sequencing (Freeland, 2020, p. 88). Sequencing can be used on the amplified product of a standard PCR, or alternatively, directly on the extracted eDNA (Freeland, 2020, p. 88).

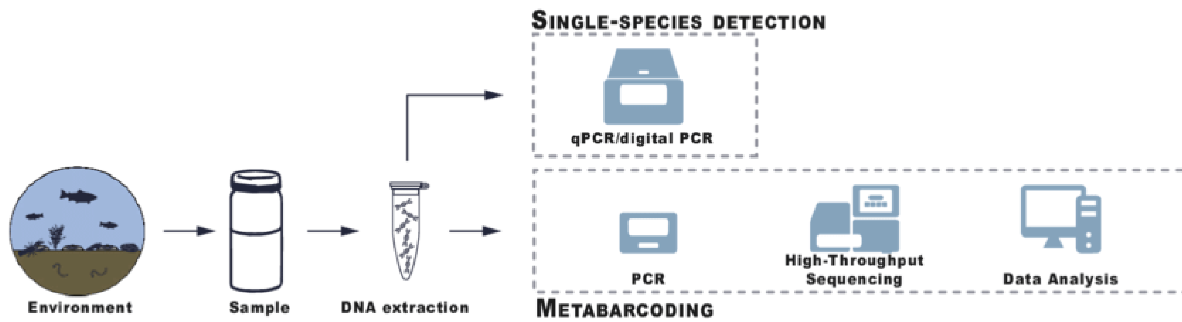

Figure 1: The main steps in eDNA analysis. The extracted eDNA is either analysed with species-specific primers (single-species detection) through real-time PCR (qPCR), or universal primers (metabarcoding) through conventional PCR, followed by high-throughput sequencing and data analysis (Pawlowski *et al*., 2020)

By analyzing eDNA using real time PCR (quantitative PCR/qPCR) or modern sequencing techniques (Thomsen & Willerslev, 2015) it is now possible to distinguish between species even when analyzing very short DNA-fragments (Takahara et al., 2012). It has become an important method for early detection of invasive species (Kim et al., 2018), and detecting and studying rare species and cryptic species has become less challenging (Buxton et al., 2017). However, despite that the big potential of eDNA has received increasing attention from researchers, there are still some regions of the world, like the tropics, where it remains under-utilized (Huerlimann et al., 2020). This is very unfortunate because of the relatively low cost-requirements for using the method, which could benefit countries with low budgets (Huerlimann et al., 2020).

1.5 eDNA analysis using real-time PCR with species-specific primers and TaqMan™ probe

Among the known PCR variants, real time PCR is claimed to be the most time-effective, and to have the highest sensitivity and specificity (Wang et al., 2023). The high specificity is due to, in several of the real-time PCR techniques, the inclusion of a hydrolysis probe, better known as a TaqMan™ probe (Chen et al., 2022). It is described as a fluorogenic single stranded oligonucleotide that will only hybridize to a DNA sequence between two PCR primers (Chen et al., 2022). Like the primers, the TaqMan probe is designed to be complementary to a unique DNA-sequence (Chen et al., 2022).

The mechanism of the TaqMan probe is illustrated in figure 2. When it has hybridized to the target-sequence, the heat-stable enzyme Taq-DNA polymerase will cleave the sequence using a 5'-3' exonuclease activity (Geiger et al., 2019). The probe has a unique fluorescent reporter dye attached at the 5'-end, and a quencher at the 3'-end (Geiger et al., 2019). The fluorescent signal is made by the reporter upon amplification of the target-sequence, when it is separated from the quencher (Geiger et al., 2019). Shortly explained, the probe is degraded by the Taq polymerase during the amplification. It will send out fluorescent signals only when the target sequence is present, and thus is getting amplified during real-time PCR (Chen et al., 2022). The signal strength increases as more amplifications of the target sequence are produced, and the product can be quantified after each cycle (Chen et al., 2022).

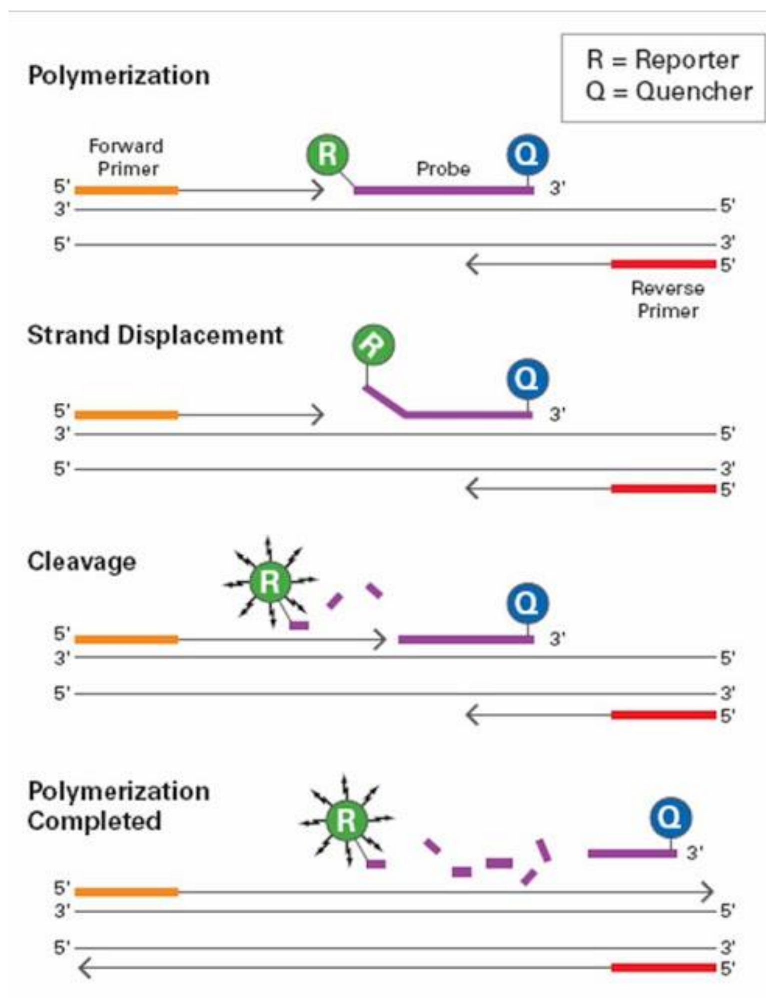

Figure 2: Mechanism of a TaqMan probe (UPODMG 1516 irivbrro, 2015).

Today species-specific primers and TaqMan probes are usually designed to hybridize to sequences within the CO1 (Cytochrome Oxidase subunit 1) gene (Freeland, 2020, p. 87). It is one of the most used genes for the detection of animal species (Wang et al., 2021; Shi et al., 2022; Kim et al., 2018). One reason is that the sequences of this gene, which contains about 600 base pairs, are highly conserved intraspecifically, meaning that there are little to no sequence differences within the same species (Muhammad Fahmi Zuhdi & Hawis Madduppa., 2020; Geller et al., 2013). At the same time there are enough differences in this sequence when comparing it among different species, and in this way, it is possible to detect the species-specific sequence, and consequently which species' DNA is present in a sample (Muhammad Fahmi Zuhdi & Hawis Madduppa., 2020; Geller et al., 2013). Real-time PCR-amplification based on the COI-gene has also become a popular tool for the verification of fish species, even the ones that are rare and difficult to detect otherwise (Nantón et al., 2015). The process of designing species-specific primers and TaqMan probes can be assisted using biomedical databases and software tools from the National Center for Biotechnology Information (NCBI) or similar sources.

1.6 Ensuring sufficient quality of eDNA samples

Negative results despite high DNA concentrations, so called false negatives, is a common issue due to the presence of proteins, salts, or other substances that may hinder the amplification process (Buxton et al., 2017). When extracting eDNA, it is therefore crucial that the concentration and purity are quantified, to ensure sufficient quality of all samples, and thus avoid such false negatives (Buxton et al., 2017). Quantification can be done spectrophotometrically, where the purity is indicated by the 260/280 and 260/230 absorbance ratio. A good sample should have a 260/280 ratio of 1,7-2,0, and a 260/230 ration of 2.0-2.2 (Claassen et al., 2013). Environmental samples often have a very low 260/230 ratio due to the presence of humic acid (HA) (Sutlovic et al., 2008). One way to solve this issue is to choose a PCR master mix with ingredients that can counteract PCR inhibitors (Schrader et al., 2012). If only negative results are obtained for a target-species, the sample quality can be tested by analysing with primers and probes designed for a control-species, which is commonly found within the sampling areas (Burian et al., 2021). Potential positive results for the control-species will prove that the samples have sufficient quality, even though the target species has not been detected (Burian et al., 2021). If for example the aim is to detect DNA of lesser

weever, a good choice of a control-species is sand goby, since it is a common and abundant species within Norwegian waters (Pethon, 2005, p. 394).

1.7 Ensuring the specificity of designed primers and probe.

When a new test is designed, the specificity of the new primers and probe can be ensured by testing them on many different species that are expected to be present in the sampling areas (Peixoto et al., 2021). Another way to test the specificity is to use DNA sequencing on products obtained while conducting a conventional PCR analysis on samples of unknown content (Freeland, 2020). Since the exact order of the base pairs (bp) is seen directly, this is a verification that the correct species is recorded (Freeland, 2020, p. 16-19). Consequently, any result from a real-time PCR analysis is considered trustworthy (Freeland, 2020, p. 19-21).

1.8 Aims of the study.

The aim of this study is to design a species-specific method, based on analysis of environmental DNA (eDNA), for detection of lesser weever fish, *Echiichthys vipera*. The eDNA is sampled at chosen locations along the Agder coastline and analysed using real-time PCR with primers and a TaqMan probe that are designed to amplify DNA only belonging to this species.

2 Methods

2.1 Sampling locations

Seawater samples were collected from 15 chosen locations distributed among three main areas along the Agder coast, in Southern Norway (figure 3). Coordinates for these locations are shown in table 1. Nine of the locations were in Mandal (figure 4) since lesser weever previously had been detected at two of the locations. Three additional locations in Søgne (figure 5), and three more in Kristiansand (figure 6) were sampled to detect possible spreading.

Table 1: Coordinates for all sampling locations

Location	Coordinates
1. Sjøsanden (Mandal)	58.020020, 7.448964
2. Lordens (Mandal)	58.016137, 7.441083
3. Stumpestrendene (Mandal)	58.014656, 7.440582
4. Kanelstranda (Mandal)	58.014771, 7.437361
5. Spidsbo (Mandal)	58.014799, 7.434286
6. Trappestranda (Mandal)	58.014693, 7.433467
7. Banken (Mandal)	58.017238, 7.430284
8. Torkelshola (Mandal)	58.018404, 7.428363
9. Lillebanken (Mandal)	58.018691, 7.430341
10. Høllensanden (Søgne)	58.074345, 7.808543
11. Høllensanden/Årossanden (Søgne)	58.072734, 7.813242
12. Årossanden (Søgne)	58.073063, 7.818392
13. Bystranda (Kristiansand)	58.145863, 8.007623
14. Nodeviga (Kristiansand)	58.142058, 7.997688
15. Bendiksbukta (Kristiansand)	58.138593, 8.003354

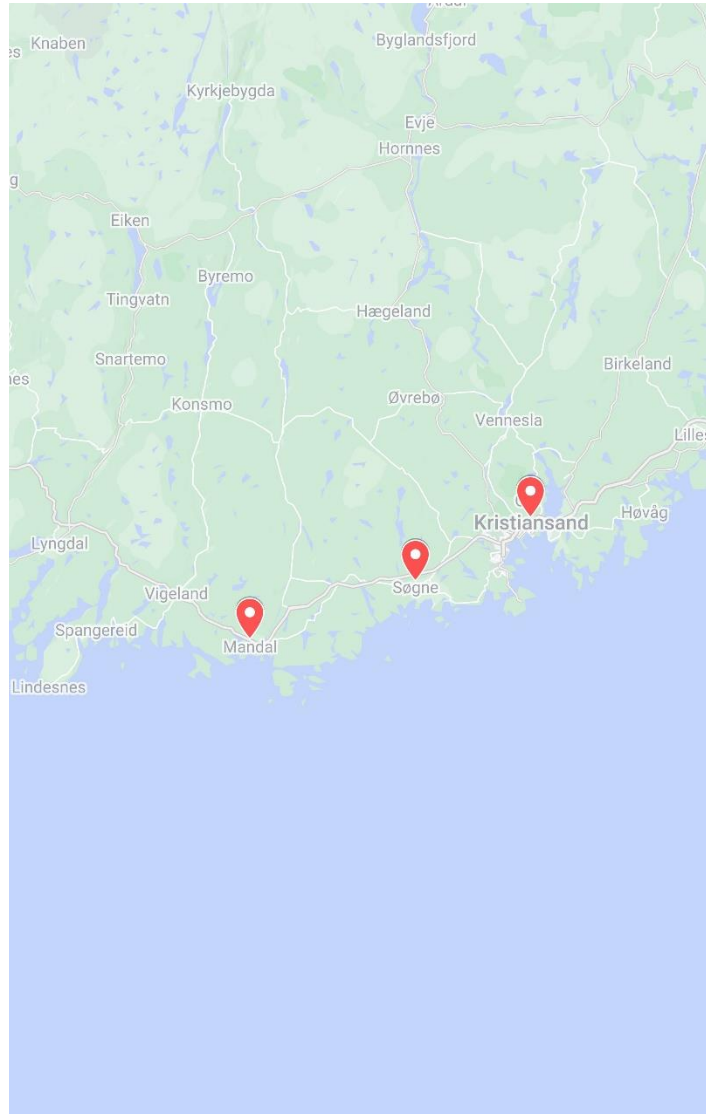

Figure 3: All sampling locations were distributed among three main areas, Mandal, Søgne, and Kristiansand, located along the Agder coastline in Southern Norway. Modified map extracted from Google.com (2022).

Figure 4: Sampling location 1-9 were in Mandal. Modified map extracted from Google.com (2022).

Figure 5: Sampling location 10-12 were in Søgne. Modified map extracted from Google.com (2022).

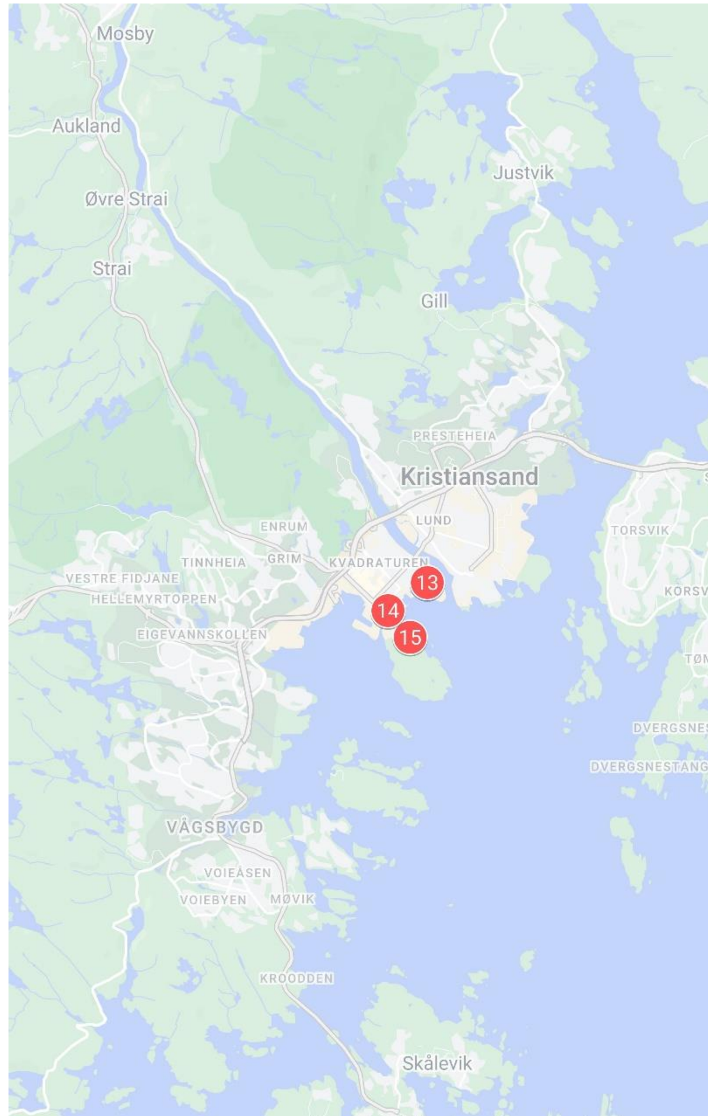

Figure 6: Sampling location 13-15 were in Kristiansand.
Modified map extracted from Google.com (2022).

2.2 Sampling technique and equipment

One litre of seawater was sampled from each of the 15 locations during August-September, when the water temperature was expected to be sufficient for the presence of lesser weever. The nine locations within Mandal were sampled once more during October, to register the expected absence of the species due to colder water temperatures. All sampling locations were sandy beaches, and all samples were collected using the same equipment and technique. Before sampling, all sample bottles were rinsed with 10% chlorine to degrade any DNA present. They were then flushed with seawater right before sampling to avoid damage to the DNA in the collected sample. At each location, a 50 ml conical centrifuge tube attached to a broom handle was lowered to about 1 meter of depth and used to fill a 1 L polypropylene sample bottle. Evenly spread at each location 50 ml samples were collected and combined in the sampling bottle. Sampling date, water temperature, location type, and sampling depth were registered before sampling. All samples were carried to the lab in a thermal bag, and the seawater was filtered using a vacuum filtration system. The type of filters used were Thermo Scientific™ Nalgene™ Single Use Analytical Filter Funnels (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), with a pore size of 0,45 µm. When all seawater (~1 liter from each location) was filtered through the system, each of the filters (1 from each location) were put in separate 1,5 ml microcentrifuge tubes, which were labelled with location number and date of sampling, and finally stored in the freezer (-20ºC) until the extraction process.

2.3 DNA extraction and quantification

DNA was extracted from the filters using a QIAGEN DNeasy© blood & tissue kit (Qiagen GmbH, Hilden, Germany). All steps were conducted following the instructions from the manufacturer with some changes to dosage of the ingredients. All filters were cut into pieces and put in separate microtubes (2 mL) containing silica magnetic beads, before adding 720 µL buffer ATL to promote lysis. All microtubes were shaken for 45 seconds, incubated for 30 minutes (56ºC), and shaken for 45 seconds again. 80 µL proteinase K. was added to promote digestion of contaminating proteins, before the microtubes were incubated (56 ºC) again, but for about 2 hours. The microtubes were further vortexed for a few seconds, centrifuged with 8000 rpm (revolutions per minute) for one minute, before about 500 µL supernatant was transferred to new microtubes (1,5 µL). The same volume of buffer AL was added to promote lysis, the microtubes were vortexed for a few seconds. Then the same volume of ethanol (96%) was added, and the microtubes were vortexed again. 600 µL of the content was then

transferred to spin columns, which were centrifuged (8000 rpm/1 minute). This step was continued until all content was centrifuged through the same column. Contaminants were washed away from the DNA through two steps. First 500 µL buffer AW1 was added, the spin columns were spun (8000 rpm/1 minute), and the eluate was thrown away. Then 500 µL buffer AW2 was added, the spin columns were spun (13400 rpm/1 minute), and the eluate was thrown away again. 150 µL buffer AE was added to elute DNA from the spin columns into new microtubes (1,5 mL), and after being incubated in air temperature for 1 minute, the microtubes containing the eluted DNA were centrifuged (8000 rpm/1 minute), before being stored in the freezer (-30ºC).

The concentration and purity of the extracted product was quantified with a Thermo Scientific™ NanoDrop™ One Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachuset, USA). First, the upper and lower pedestals of the instrument were wiped with a lab wipe before the it was calibrated by pipetting 1 µL Buffer AE (blanking solution) onto the lower pedestal. Then, after 1 µL sample solution was pipetted onto the lower pedestal, the sample measurement was automatically started when the arm was lowered. The wiping of the pedestals was repeated each time a new sample was quantified. DNA concentration (ng/µL) and ratio of absorbance (260/280 and 260/230) were registered for all eDNA samples. The extracted product was stored in the freezer (-30ºC) prior to the amplification process.

2.4 Designing species-specific primers and a TaqMan™ probe for lesser weever

Species-specific primers and a TaqMan™ probe were designed based on sequences within the mitochondrial COI gene, using the nucleotide database and the "Pick Primers" tool on the website of The National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/, Bethesda, Maryland, USA). Melting temperatures (Tm), product size, and other preferences were chosen, and the tool was set to suggest ten sets of primer pairs and probes after it had compared the target-sequence among all species expected to be present in the sampling locations. To induce the most effective binding of the primer pair and probe, a length between 18-30 base pairs, GC-content between 40-60%, and a 3' end with either G or C were aimed for. The size of the chosen probe was further modified using the software PrimerExpress v.3.0 from Thermo Fisher, since it was going to be used with a MGB protein attached to the 3' end, in addition to the quencher. The MGB protein would increase the Tm of the probe, so that a shorter and more specific version could be designed

while keeping the Tm of the original version. A 6-FAM fluorescent dye was attached to the 5' end of the probe, while an MGB-NFQ nonfluorescent quencher was attached to the 3' end. The primers and probe were ordered from Thermo Fisher and were obtained in a lyophilized state. They were dissolved in buffer AE from the extraction kit to a concentration of 100 µM, before 10 µM working stocks were made by diluting it with nuclease free water.

2.5 Real-time PCR with species-specific primers and TaqMan probe

The default real-time PCR mix consisted of 12,5 µL TaqMan™ Environmental Master Mix 2.0 (Thermo Fisher), 4,3 µL PCR grade water, 2 µL forward primer (10 µM stock), 2 µL reverse primer (10 µM stock), and 1,2 µL TaqMan probe (10 µM stock). The volume of each ingredient was multiplied with the number of reactions (tubes) planned for the real-time PCR analysis, with 10 % extra being added to compensate for possible pipetting errors. The ingredients were mixed in a microcentrifuge tube by carefully pipetting up and down several times, before 23 µL was distributed in real-time PCR tubes. 3 µL DNA from the samples was added to separate tubes, and all samples were analysed in triplicates. The tubes were centrifuged for about ten seconds before being placed in a 96-well StepOnePlus™ Real-Time PCR system. The plate setup and run method was set using the attached StepOne software. The holding stage had a 10-minute step with a temperature of 95ºC, and the cycling stage had a 2-minute step with 60ºC. The total number of cycles was set to 60, with a fluorescence signal detection conducted after each cycle. When all cycles were finished, the cycle threshold value (Ct-value), which showed how many cycles that was needed for acquiring a positive result, was registered for all positive reactions.

An additional real-time PCR analysis was conducted on the environmental samples, using identical procedure, ingredients, and settings, but with primers and a TaqMan probe designed for the species sand goby, *Pomatoschistus minutus*. The concentration used was 549 µM for the forward primer, 451 µM for the reverse primer, and 60 µM for the probe. Since this species was expected to be common within the sampling areas, potential positive results would ensure sample quality.

2.6 Positive and negative controls

Throughout the study various positive and negative controls were included to avoid false negative and false positive results. During the sampling process extra bottles filled with 1 L distilled water (dH_2O) were brought along to the three main sampling areas. They were not filled with seawater samples, but the filtration, extraction and quantification processes were identical. During the real-time PCR analysis, the eluate of the extraction step (dH_2O) was included as a negative control to make sure that there was no contamination present in the reagents and consumables that were used during the extraction and amplification process. It was also included to detect possible inter-sample contamination, namely that the content of each sample unintentionally was mixed during the sampling and extraction process. In addition to DH_2O, template DNA was replaced by the same volume of PCR grade water as an extra negative control (termed blank). As a positive control lesser weever DNA was extracted from a frozen tissue sample using the DNeasy Blood & Tissue kit but following the instructions for tissue samples. After being diluted to 10%, it was included during the real-time PCR analysis, to control that the extraction and amplification processes were conducted properly.

2.7 Sensitivity and specificity estimation

Sensitivity measures of the analysis method was based on the lowest amount of lesser weever DNA that could be detected when using the real-time PCR assay. First, a tenfold serial dilution was conducted on genomic DNA. The dilution step consisted of mixing 10 µL sample in 90 µL PCR grade water using a vortex mixer for about one minute. The original sample was diluted nine times using this dilution step. 3 µL of each dilution was then put in separate real-time PCR tubes which contained the exact same type and amount of PCR-mix which was used on the seawater samples. Additionally, the real-time PCR amplification was conducted using identical settings. A second serial dilution test was conducted on each of the positive eDNA samples, using the same dilution step (1:10) but with fewer (5) dilutions due to the starting DNA concentration being much lower than the genomic DNA. A third, two-fold, serial dilution test was conducted to test if the system could detect a value between the lowest positive dilution and highest negative dilution. Here the dilution step consisted of mixing 50 µL sample with 50 µL PCR grade water. The concentration value of the last positive dilutions was further used to calculate the sensitivity mathematically. Since each PCR tube contained 3 µL DNA and 22 µL PCR mix (25 µL in total), this value was multiplied with

3, then divided by 25 to determine the lowest concentration (ng/µL) at which the DNA could be detected (detection limit).

An in-silico PCR analysis was conducted to ensure primer and probe specificity. Using the database, and the multiple sequence alignment tool Clustal Omega on the website of the European Molecular Biology Laboratory (EMBL, Heidelberg, Germany, https://www.embl.org/), the sequences of the primers and probe designed for lesser weever were aligned and compared among sixteen lesser weever individuals, nineteen other fish species, and one crab species.

The specificity was also tested by analysing DNA of the closely related species greater weever, *Trachinus draco*, with the primers and probe designed to be unique for lesser weever. The DNA was extracted from a frozen tissue sample, diluted using a tenfold serial dilution test.

2.8 Final verification of the species

The eDNA samples that were positive for lesser weever were analysed again but using conventional PCR with the same primers, and a gel electrophoresis. Purification and DNA sequencing was intended on the amplified product as a final verification of the species.

Four reactions were planned for the conventional PCR run. Three tubes contained the eDNA samples that were positive during the real-time PCR analysis, while the last tube contained a negative control. 50 µL 2x Phusion Green Hot Start II high-fidelity PCR-master mix, 25 µL PCR grade water, 5µL forward primer (10 µM stock), and 5 µL reverse primer (10 µM stock) was mixed for about 1 minute using a vortex mixer. 17 µL of the solution was further put in each of the PCR tubes, before adding 3 µL DNA in the three tubes containing samples.

The PCR analysis was conducted on an Applied Biosystems™ Veriti™ Thermal Cycler (60-well) and consisted of 40 cycles. The temperature was increased to 98°C during the denaturing stage (0:30), lowered to 60°C during the annealing stage (0:10), and finally increased to 72°C during the extending stage (0:05).

The agarose gel electrophoresis was conducted to separate the DNA fragments. A 1,8 % agarose gel was made using a standard recipe (see Appendix A). The gel was poured into a tray, a well comb was placed to form wells, and the gel was hardened at room temperature for about 45 to 60 minutes. The hardened gel was further immersed in 1xTAE buffer (charged buffer solution) within the electrophoresis unit (gel box). A 1Kb+ DNA ladder (Thermo Fisher) was loaded into the first lane (well) of the gel, and the samples were carefully loaded into the additional wells. A loading dye buffer (Thermo Fisher) was added to all samples (see appendix B for recipe). The gel was run at 85 V due to the high concentration (1,8 %) for about 1 hour. When the run was completed, before the gel was carefully removed from the gel box and placed in a Syngene™ NuGenius-machine (Syngene, Cambridge, UK) to visualize the DNA-fragments (bands/lines). The gel was exposed to long-wavelength UV for as short a time as possible to limit damage to the DNA.

In case of a negative result (no visible bands) the remaining content of the four tubes was intended to be used as a template for a new attempt using a different master mix (TaqMan ™ Environmental Master Mix 2.0) for the PCR.

3 Results

3.1 Water temperatures registered at the sampling areas

The measured water temperature was 17-18℃ in all three sampling areas during August-September (table 2). It had decreased to 12℃ when new samples were collected in Mandal during October.

Table 2: Sampling dates and measured water temperatures for the three sampling areas.

Sampling area	Sampling date	Water temp. (℃)
Mandal	15.08.2022	17
Søgne	04.09.2022	18
Kristiansand	06.09.2022	17
Mandal	13.10.2022	12

3.2 Quantification of DNA extracted from the environmental samples

Based on the quantification using a NanoDrop spectrophotometer the environmental samples had a DNA concentration ranging from 9 to 82,2 ng/µL, a 260/280 absorbance ratio ranging from 1,61 to 2,11, and a 260/230 absorbance ratio ranging from 0,25 to 1,48 (table 3). The negative controls had a DNA concentration ranging from 1,5 to 3 ng/µL, a 260/280 absorbance ratio ranging from 2,43 to 8,19, and a 260/230 absorbance ratio ranging from 0,07 to 0,27.

Table 3: DNA concentration (ng/µL), 260/280 absorbance rate, and 260/230 absorbance rate for the extracted product of all environmental samples, plus the negative controls, quantified with a NanoDrop spectrophotometer.

Locat+A1:D29ion	Concentration (ng/µL)	A260/280	A260/A230
1. Sjøsanden (Aug.)	16,4	2,11	0,45
2. Lordens (Aug.)	9	1,76	0,25
3. Stumpestrendene (Aug.)	30,6	2	0,86
4. Kanelstranda (Aug.)	36,5	1,89	0,79
5. Spidsbo (Aug.)	6,5	2,07	0,34
6. Trappestranda (Aug.)	14,8	1,84	0,45
7. Banken (Aug.)	8,3	2,08	0,39
8. Torkelshola (Aug.)	15,2	1,93	0,47
9. Lillebanken (Aug.)	47,2	1,61	0,9
Neg01	2,6	2,58	0,12
10. Høllensanden	51,5	1,96	1,22
11. Høllensanden/Årossanden	47,8	1,98	0,99
12. Årossanden	48,2	1,95	1,1
Neg02	3	8,19	0,1
13. Bystranda	82,2	1,9	1,48
14. Nodeviga	79,4	1,94	1,44
15. Bendiksbukta	36,2	1,83	1,01
Neg03	1,5	2,43	0,27
1. Sjøsanden (Oct.)	18,8	1,67	0,42
2. Lordens (Oct)	18,1	1,69	0,41
3. Stumpestrendene (Oct.)	11,9	1,76	0,43
4. Kanelstranda (Oct.)	14,7	1,73	0,45
5. Spidsbo (Oct.)	28,8	1,89	0,36
6. Trappestranda (Oct.)	17,3	1,78	0,51
7. Banken (Oct.)	12,6	1,64	0,37
8. Torkelshola (Oct.)	19,9	1,72	0,75
9. Lillebanken (Oct.)	24,9	1,82	0,7
Neg04	1,7	2,96	0,07

3.3 Details about the primers and TaqMan probe designed for lesser weever

The chosen forward primer had the sequence 5’ CGGATGGACTGTTTACCCCC 3’, with a length of 20 base pairs, and a 60% GC-content. The chosen reverse primer had the sequence 5’ GAAGAAATCCCGGCTAAGTGG 3’, with a length of 21 base pairs, and a 52% GC-content. The chosen TaqMan probe had the sequence 5’ CTCGCACACGCAGGA 3’, with a length of 15 base pairs, and a 67% GC-content. The probe also had a 6-FAM reporter dye at attached to the 5’ end, and a MGB-NFQ quencher attached to the 3’ end. Details about the chosen primers and probe can be seen in table 4.

Table 4: The sequence (5’ to 3’ direction), length (number of base pairs), and GC-content (%) of the primers and probe designed for lesser weever. Also, the type of reporter (5’end) and quencher (3’ end) attached to the probe.

Oligonucleotide	Sequence (5' -> 3')	Length (bp)	GC-content (%)	Reporter (5' end)	Quencher (3' end)
Forward primer	CGGATGGACTGTTTACCCCC	20	60		
Reverse primer	GAAGAAATCCCGGCTAAGTGG	21	52		
TaqMan probe	CTCGCACACGCAGGA	15	67	6-FAM	MGB-NFQ

3.4 Real-time PCR analysis of environmental samples with primers and probe designed for lesser weever

Based on real-time PCR analysis of the environmental samples, Lesser weever DNA was detected in 4 of 15 locations, namely location 1 (Sjøsanden), location 2 (Lordens), location 4 (Kanelstranda), and location 5 (Spidsbo) (table 5). An example of a positive amplification plot is illustrated in figure 7. All positive samples were collected within Mandal, during August. Three reactions for location 1, two from location 2, one from location 4, and one from location 5 were positive. The mean Ct-values ranged from 40 to 41,3 on the positive samples, and 20-27 on the positive controls. All samples from location 6-15 were negative, equal to the extra samples collected from location 1-9 in October. All negative controls and blank controls were negative.

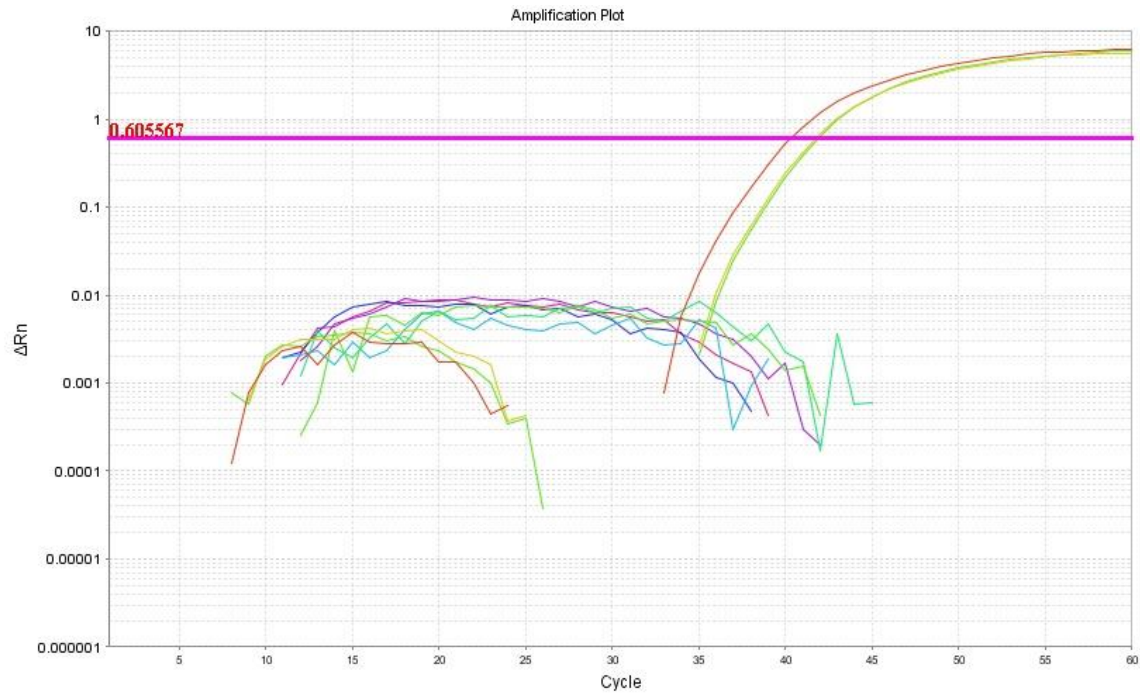

Figure 7: An example of a positive result from the real-time PCR analysis conducted on the environmental samples. This amplification plot is based on the sample from location 1 (Sjøsanden), where lesser weever DNA was detected in all three reactions (Red, yellow, and green curves). The purple threshold line separates relevant amplification signal from the background.

Table 5: Results from the real-time PCR analysis conducted on the environmental samples with the primer pair and a TaqMan probe designed for lesser weever, *Echiichthys vipera*. For each sample location (1-15) a positive (+) or negative (-) result was registered for all three reactions (triplicates), and the mean Ct-value was calculated for the positive reactions.

Real-time PCR analysis of environmental samples (lesser weever)				
Sample location	Results (triplicates)			Mean Ct-value
1. Sjøsanden (Aug.)	+	+	+	41.3
2. Lordens (Aug.)	+	+	-	40
3. Stumpestrendene (Aug.)	-	-	-	-
4. Kanelstranda (Aug.)	+	-	-	40.7
5. Spidsbo (Aug.)	+	-	-	40
6. Trappestranda (Aug.)	-	-	-	-
7. Banken (Aug.)	-	-	-	-
8. Torkelshola (Aug.)	-	-	-	-
9. Lillebanken (Aug.)	-	-	-	-
10. Høllensanden	-	-	-	-
11. Høllensanden/Årossanden	-	-	-	-
12. Årossanden	-	-	-	-
13. Bystranda	-	-	-	-
14. Nodeviga	-	-	-	-
15. Bendiksbukta	-	-	-	-
1. Sjøsanden (Oct.)	-	-	-	-
2. Lordens (Oct.)	-	-	-	-
3. Stumpestrendene (Oct.)	-	-	-	-
4. Kanelstranda (Oct.)	-	-	-	-
5. Spidsbo (Oct.)	-	-	-	-
6. Trappestranda (Oct.)	-	-	-	-
7. Banken (Oct.)	-	-	-	-
8. Torkelshola (Oct.)	-	-	-	-
9. Lillebanken (Oct.)	-	-	-	-

3.5 Real-time PCR analysis of environmental samples with primers and probe designed for sand goby

Based on the real-time PCR analysis of the environmental samples, with a primer pair and a TaqMan probe designed for sand goby, DNA was detected in 10 of 15 locations. One reaction for location 2 (Lordens), one for location 4 (Kanelstranda), one for location 5 (Spidsbo), three for location 6 (Trappestranda), one for location 7 (Banken), three for location 8 (Torkelshola), two for location 10 (Høllensanden), three for location 11 (Høllensanden/Årossanden), three for location 12 (Årossanden), and two for location 13 (Bystranda) were positive (table 6). The mean Ct-value ranged from 38,1 to 44,6.

Table 6: Results from the real-time PCR analysis conducted on the environmental samples with the primer pair and a TaqMan probe designed for sand goby, *Pomatoschistus minutus*. For each sample location (1-15) a positive (+) or negative (-) result was registered for all three reactions (triplicates), and the mean Ct-value was calculated for the positive reactions.

Real-time PCR analysis of environmental samples (sand goby)				
Sample location	Results (triplicates)			Mean Ct-value
1. Sjøsanden	-	-	-	-
2. Lordens	-	-	+	44.4
3. Stumpestrendene	-	-	-	-
4. Kanelstranda	-	+	-	43.4
5. Spidsbo	-	+	-	45
6. Trappestranda	+	+	+	44.6
7. Banken	-	-	+	42.3
8. Torkelshola	+	+	+	44.1
9. Lillebanken	-	-	-	-
10. Høllensanden	-	+	+	41.1
11. Høllensanden/Årossanden	+	+	+	39.7
12. Årossanden	+	+	+	38.1
13. Bystranda	+	+	-	41.7
14. Nodeviga	-	-	-	-
15. Bendiksbukta	-	-	-	-

3.6 Measuring the sensitivity of the real-time PCR analysis for lesser weever

Based on the real-time PCR analysis conducted on tenfold dilutions of the genomic DNA, positive results were obtained for the undiluted DNA and the five first dilutions. The mean Ct-value ranged from 21.5 on the original sample, to 39.4 on the last positive dilution (table 7). The DNA concentration value of the last positive dilution was 2,65 x 10^{-4} ng/µL, and the lowest concentration at which the DNA could be detected (detection limit) was calculated to 3,18 x 10^{-5} ng/µL. Based on the twofold serial dilution test, no value was detected between the fifth and sixth dilution. All dilutions were negative when the positive environmental samples were analysed (table 8). For location 1 (Sjøsanden) the DNA concentration value of the undiluted positive sample was 16,4 ng/µL, and the lowest concentration at which the DNA could be detected (detection limit) was calculated to 1,97 ng/µL. For location 2 (Lordens), the concentration value was 9 ng/µL, and the detection limit was calculated to 1,08 ng/µL. For location 4 (Kanelstranda) the concentration value was 36,5 ng/µL, and the detection limit was calculated to 4,38 ng/µL. For location 5 (Spidsbo) the concentration value was 6,5 ng/µL, and the detection limit was calculated to 0,78 ng/µL.

Table 7: Results from the real-time PCR analysis conducted on the original and diluted (1:10) genomic DNA (lesser weever). For each dilution a positive (+) or negative (-) result was registered for all three reactions (triplicates), and the mean Ct-value was calculated for the positive reactions.

Real-time PCR analysis of diluted genomic DNA (lesser weever)				
Sample	Results (triplicates)			Mean Ct-value)
Original	+	+	+	21.5
Dilution 1	+	+	+	25.9
Dilution 2	+	+	+	30.6
Dilution 3	+	+	+	33.9
Dilution 4	+	+	+	36.9
Dilution 5	+	+	+	39.4
Dilution 6	-	-	-	-
Dilution 7	-	-	-	-
Dilution 8	-	-	-	-
Dilution 9	-	-	-	-
Dilution 10	-	-	-	-

Table 8: Results from the real-time PCR analysis conducted on original and diluted (1:10) positive eDNA samples (lesser weever). For each dilution a positive (+) or negative (-) result was registered for all three reactions (triplicates, and the mean Ct-value was calculated for the positive reactions.

Real-time PCR analysis of diluted eDNA samples				
Sample	Results (Triplicates)			Mean Ct-value
Sjøsanden, undiluted	+	+	+	41
Sjøsanden, dilution 1	-	-	-	-
Sjøsanden, dilution 2	-	-	-	-
Sjøsanden, dilution 3	-	-	-	-
Sjøsanden, dilution 4	-	-	-	-
Sjøsanden, dilution 5	-	-	-	-
Lordens, undiluted	+	+	-	40
Lordens, dilution 1	-	-	-	-
Lordens, dilution 2	-	-	-	-
Lordens, dilution 3	-	-	-	-
Lordens, dilution 4	-	-	-	-
Lordens, dilution 5	-	-	-	-
Kanelstranda, undiluted	+	-	-	41
Kanelstranda, dilution 1	-	-	-	-
Kanelstranda, dilution 2	-	-	-	-
Kanelstranda, dilution 3	-	-	-	-
Kanelstranda, dilution 4	-	-	-	-
Kanelstranda, dilution 5	-	-	-	-
Spidsbo, undiluted	+	-	-	40
Spidsbo, dilution 1	-	-	-	-
Spidsbo, dilution 2	-	-	-	-
Spidsbo, dilution 3	-	-	-	-
Spidsbo, dilution 4	-	-	-	-
Spidsbo, dilution 5	-	-	-	-

3.7 Testing the specificity of the primers and TaqMan probe designed for lesser weever

Based on the in-silico PCR analysis conducted on the sequence of the primers and TaqMan probe there was a 100% match for all 16 lesser weever individuals. Compared with the 20 other species the forward primer had three to six sequence differences, the probe had two to five differences, and the reverse primer had three to seven differences (table 9).

Table 9: Results of the in-silico PCR analysis where the sequences of the primers and TaqMan probe designed for lesser weever (*Echiichthys vipera*) were compared among 16 lesser weever individuals, and among 20 other species to ensure primer and probe specificity. Green colour indicates matching bases, while red colour indicates sequence differences.

Species	Forward primer (5' -> 3')	Probe (5' -> 3')	Reverse primer (5' -> 3')
Echiichthys vipera	CGGATGGACTGTTTACCCCC	CTCGCACACGCAGGA	GAAGAAATCCCGGCTAAGTGG
Trachinus draco	TGGTTGAACAGTTTACCCTC	TTCGACCCGGCAGGA	GAGGAAATACCGGCTAGATGT
Pomatoschistus minutus	GGGGTGAACGGTTTACCCCC	CTAGCACATGCTGGA	GAAGAAATGCCTGCTAGGTGA
Pollachius virens	AGGCTGAACTGTTTATCCCC	CTAGCACACGCCGGG	GATGAAATCCCTGCTAAATGA
Platichthys flesus	AGGGTGAACTGTATATCCCC	CTAGCACACGCCGGG	GATGAAATTCCGGCAAGGTGA
Pollachius pollachius	AGGGTGAACTGTTTATCCGC	CTCGCTCACGCAGGG	GATGAAATCCCTGCTAAGTGA
Labrus bergylta	CGGATGAACAGTATACCCCC	CTGGCCCACGCGGGA	GATGAAATCCCTGCTAAGTGA
Ctenolabrus rupestris	TGGGTGGACAGTTTACCCCC	CTGGCCCACGCGGGG	GAAGAAATCCCCGCCAGGTGG
Myoxocephalus scorpius	CGGGTGAACAGTCTACCCTC	CTGGCCCACGCGGGA	GAAGAGATTCCTGCTAGATGT
Taurulus bubalis	CGGTTGAACGGTCTACCCTC	CTAGCCCACGCAGGC	GAGGAGATCCCTGCTAAGTGC
Pleuronectes platessa	AGGGTGAACCGTATACCCCC	CTAGCACACGCTGGG	GATGAAATTCCAGCAAGGTGG
Limanda limanda	TGGGTGAACTGTATACCCTC	CTAGCACACGCTGGA	GATGAAATCCCGGCAAGGTGG
Carcinus maenas	AGGATGAACAGTCTATCCTC	ATCGCCCATGCAGGA	GAAGAAACCCCGGCTAAATGT
Eutrigla gurnardus	AGGGTGAACTGTCTACCCTC	CTCGAACATGCCGGA	GAGGAAATCCCGGCCAGATGA
Gadus morhua	AGGCTGAACTGTCTATCCAC	CTCGCTCATGCTGGG	GATGAAATCCCTGCTAGATGA
Scomber scombrus	TGGCTGAACAGTCTACCCCC	CTAGCGCATGCCGGG	GAGGAAACACCTGCTAGGTGA
Clupea harengus	CGGGTGAACGGTATATCCTC	CTGGCCCATGCAGGA	GAGGAAATACCTGCTAGATGA
Merlangius merlangus	AGGTTGAACTGTCTATCCCC	CTCGCTCATGCTGGG	GATGAAATTCCTGCCAGATGA
Symphodus melops	CGGCTGAACAGTATACCCGC	CTTGCCCACGCAGGA	GAGGAGATTCCTGCCAGGTGG
Labrus mixtus	CGGGTGAACAGTGTATCCCC	CTAGCCCACGCAGGA	GAAGAAATACCAGCTAAGTGT
Gobius niger	CGGATGAACGGTCTACCCCC	CTAGCACATGCTGGA	GACGAAATCCCGGCCAGGTGA

Based on the real-time PCR analysis conducted on tenfold dilutions of genomic DNA from greater weever, a positive result was obtained for the undiluted DNA and the first dilution (table 10), with the average Ct-values being relatively high, 36.2 and 41 respectively. The DNA concentration value of the last positive dilution was 3,99 ng/µL, and the lowest concentration at which the DNA could be detected (detection limit) was calculated to 0,48 ng/µL. The positive results were only obtained with highly concentrated greater weever DNA present, while continuous negative results were obtained once the concentration was diluted a little.

Table 10: Results from the real-time PCR analysis conducted on the original and diluted (1:10) genomic DNA (greater weever). For each dilution a positive (+) or negative (-) result was registered for all three reactions (triplicates), and the mean Ct-value was calculated for the positive reactions.

Real-time PCR analysis of diluted genomic DNA (greater weever)				
Sample	Results (triplicates)			Mean Ct-value
Original	+	-	-	36.2
Dilution 1	-	+	-	41
Dilution 2	-	-	-	-
Dilution 3	-	-	-	-
Dilution 4	-	-	-	-
Dilution 5	-	-	-	-
Dilution 6	-	-	-	-
Dilution 7	-	-	-	-
Diltuion 8	-	-	-	-
Dilution 9	-	-	-	-
Dilution 10	-	-	-	-

4. Discussion

4.1 The efficiency and utility of the method

In this study a species-specific detection method, based on eDNA analysis, was designed for lesser weever fish. Environmental samples were collected in chosen locations along the Agder coastline, and the extracted eDNA was analysed using real-time PCR with primers and a TaqMan probe designed to hybridize only to lesser weever DNA. In earlier studies lesser weever has successfully been detected by analysing eDNA using PCR with universal primers, and subsequent DNA sequencing (Collins et al., 2019; Judith et al., 2020), but no sources have been found about the species being detected through a TaqMan real-time PCR assay. However, the method has been frequently used to detect other fish species (McCarthy et al., 2022). The analysis results show that lesser weever DNA was detected in four of fifteen sampling locations, confirming that a good method has been developed for detection of this species, without the need for catching or disturbing it in any way. Successful detection of lesser weever DNA despite very low concentrations in the positive samples, as indicated by the high Ct-values (table 5), shows the high sensitivity of real-time PCR which also has been pointed out in other studies (Nga et al., 2010; Da Costa Lima et al., 2013). High sensitivity was also shown by the calculated detection limit, which was based on analysis of diluted genomic DNA, and found to be as low as 3,18 x 10^{-5} ng/μL. In comparison, all attempts at achieving visible bands on the agarose gel following a conventional PCR on the positive samples, with the same primers, failed. Execution of the different procedures had relatively low requirements for time, equipment, and skill level. This applies in particular to the sampling procedure. It took only a few hours to sample at all fifteen locations, and very simple sampling technique and equipment could be used, especially since the target species, lesser weever, is commonly found on clean sandy bottoms at the shallowest depths (Vasconcelos et al., 2004). The general impression is that the method is especially useful when the goal is to simply determine whether a species has been present or not, independent on abundance, something which also have been expressed in studies where it has been used on other species (Fukuta et al., 2013; Buxton et al., 2017; Kim et al., 2018).

4.2 Reliability of the real-time PCR results

4.2.1 Sample quality

While conducting all steps prior to the real-time PCR analysis, the aim has been to ensure sufficient quality of the samples, to obtain reliable results. Based on the quantification of the extracted DNA all samples had a low 260/230 absorbance ratio, but as mentioned this is a common issue when analysing environmental samples (Sutlovic et al., 2008). If it was not for the fact that the chosen PCR master mix is resistant to humic acid and other possible amplification inhibitors, lesser weever DNA could potentially have been present in all samples. With a 260/280 ratio within the approved range of 1,7-2,0 (Claassen et al., 2013) for all samples, and with positive results obtained in ten of the fifteen sampling locations when testing on sand goby, which is a common and abundant species within Norwegian waters (Pethon, 2005, p. 392), there are good indications that the sample quality is sufficient. meaning that there is nothing that should hinder the amplification. Therefore, possible DNA detection is only a matter of the concentration, and all results for lesser weever seems highly reliable. It should be noted that there was no control for possible false negatives in the five negative samples when testing on sand goby. Optimally, as was done for lesser weever, an additional commonly present species could have been tested for. A more advanced variant would be to use multiplex PCR, where multiple targets are detected in a single reaction (Malik et al., 2019). It would however require having access to DNA of all species, and to design multiple specific primers and probes (Malik et al., 2019).

4.2.2 The specificity of the primers and TaqMan probe designed for lesser weever

The in-silico PCR analysis shows that the primers and TaqMan probe designed for lesser weever in theory have enough specificity to avoid hybridization to the DNA of other species. It is based on the sequences always having a 100% match when conducting an intraspecific comparison, while having several mismatches when compared interspecifically, at least among the 19 chosen species (table 8). One way in which the specificity is shown in practice is that the positive control (tissue sample), was detected every time an eDNA sample was analysed, and was always having a low Ct-value (<28). One thing that is questioning the specificity to some extent is that a positive result was obtained when testing on the closely related species greater weever, by analysing tenfold dilutions of the tissue sample. According to Ye *et al*., (2012) a so-called non-specific target amplification, meaning that primers that in

theory only match to a single species also hybridize to other species, sometimes can happen, because "different parts of chromosomes or transcripts may share some nucleotide similarity due to either homologous regions or fortuitous matches". Several studies have investigated the effects of mismatches between targets and primers, and they have shown that a target can be amplified even if it has a few mismatches to the primers. Even the inclusion of a TaqMan probe has not always been a guarantee against such occurrence, as seen in a study conducted by Yao et al., (2006), where positive results were obtained even though the TaqMan probe contained up to five mismatches. Over the last decades it has been discussed whether the specificity is somewhat dependent on the location of potential mismatches between primers and template (Ledeker & De Long, 2013). For example, if a mismatch is located at the 3' end it has been suggested in some studies that there is a higher possibility of a negative effect on the result (Ledeker & De Long, 2013). Additionally, the effect of single mismatches compared to multiple mismatches has been highly debated, with some researchers going so far as to claim that single mismatches tend to have no effect at all (Ledeker & De Long, 2013). Though the latter claim may have been disapproved by newer studies, and the relationship between mismatches and amplification is difficult to establish, there is a general agreement that amplification is possible with a few mismatches, even at the 3' end (Ledeker & De Long, 2013).

Taking all this into account, a way to demonstrate the specificity of the primers and TaqMan probe even further, would probably have been to include them while conducting a real-time PCR analysis on the DNA of all the 19 chosen species, or maybe even more species. However, it would require much more time and work, and having access to the DNA of all those species. When comparing the number of dilutions with a detectable fluorescence signal, and the mean Ct-values of the positive dilutions, the results speak more for lesser weever, than for greater weever (table 6 and 7). Additionally, the calculated detection limit, based on the concentration value of the last positive dilution, was much lower for lesser weever (see section 3.6 and 3.7). Shortly said, relatively high concentrations of greater weever DNA were required for detection. Thus, without diminishing that a non-specific target amplification is possible, the positive result for lesser weever seems highly convincing. In a potential continuation of this study, different variants of the designed primers and probe could have been tested, to check for false positive results. Alternatively different temperature could have been tested during the PCR.

4.2.3 Release and degradation of eDNA

The efficiency and utility of analysing eDNA using a TaqMan real-time PCR assay has been acknowledged many times by this and similar studies. Additionally, the sample quality is expected to be sufficient, and the most efficient primer pair and TaqMan probe probably has been chosen. Still there is one more thing that potentially is affecting whether the analysis results truly reflect the real situation, namely if the lesser weever has been present or not. It is related to the rate of which DNA is released and degraded in the environment, which in addition to the already mentioned amplification inhibitors may lead to false negative results (Freeland, 2020, p. 89). During the last decades, while still praising the method for its advantages, research has increasingly focused this and other challenges of analysing eDNA (Pilliod et al., 2014). The degradation rate of DNA shed in the environment is probably dependent on various abiotic factors like water temperature, pH, salinity, and UV radiation (Freeland, 2020, p. 89). There have also been discussions about how biotic factors like biomass, development stage, and behaviour possibly affects how much DNA as organism leave behind (Freeland, 2020, p. 89). In that case, even if the target species truly has been present in the chosen sampling locations, there is no guarantee that its DNA is obtained in the samples. Luckily the obtained results of this study have demonstrated that very low DNA concentrations are needed for it to be detected using the species-specific real-time PCR, which is the only purpose of this study. At the same time, it is unknown how many individuals that are needed to for a positive result, or as Sigsgaard et al., (2020) express it, how many individuals that are contributing to the eDNA pool. Whether they are positive or negative the analysis results tell nothing about the number of individuals present at each sampling location, or within a larger area (Mandal, Søgne, or Kristiansand). If the sampling locations are close to each other, the eDNA that has been detected in two different locations may have been shed by the same individual. If a total of ten individuals were present in the Mandal area, where there was a short distance between the nine sampling locations, and only one was present at location 1 (Sjøsanden) during the sampling procedure, it is possible that the amount of eDNA in this location was not enough to be detected during the analysis. While praising eDNA analysis for its many utilities, Sigsgaard et al., (2020) has pointed this issue as one of the limitations. Hopefully, the number of individuals required for a positive result is very low, so that a negative result is a good indication that the species has not been present, or at least that its presence is scarce. But as mentioned, the focus of this study was to show that the designed method is useful for detecting the presence of lesser weever, and the results show that it indeed is.

4.3 The presence of lesser weever along the Agder coast as indicated by this study

The results of this study indicate that the lesser weever has been present in four locations within Mandal during the late summer 2022. Mandal is presumably the only area in Norway where the species has previously been detected, and in that case, these results also indicate that a population has been established in this area, at least during late summer/early autumn. It is also possible that individual fish has entered the Norwegian side of Skagerrak by chance ("Dvergfjesing funnet på Sørlandet", 2021). Since apparently no similar studies have been conducted on lesser weever in Norway, it is difficult to confirm this, or to make any further interpretations. A study conducted on the species within the southern parts of the North Sea has found that its abundance in shallow waters is seasonal, with several maximums and minimums (Scott & Henderson, 2016). The lowest abundance was found between November and March, followed by a maximum in May, then a decline during June-July, and finally a new but more varying top in August-September (Scott & Henderson, 2016). Future research will be required to confirm whether this trend is followed by the lesser weever in Mandal or not, but some additional samples could possibly have been collected during May. Bearing in mind that eDNA analysis, which have been praised for its many utilities, has a limitation regarding estimates of the number of fish within an area (abundance), it would be difficult to measure seasonal changes in this species abundance using this method. At least the results of the samples collected during October, which were all negative, support the theory that the abundance is declining prior to the winter months. The choice of collecting all other samples during late summer/early autumn was based on articles published by the Institute of Marine Research (IMR) ("Dvergfjesing funnet på Sørlandet", 2021) and various newspapers, which so far is the only official statements that has been found about the presence of lesser weever along the Agder coast. The samples collected within Søgne and Kristiansand, which also were all negative, were attempts at detecting possible spreading of lesser weever to other areas of the Agder coast, something which so far has not been found any statements about. Due to its infamous stings a potential discovery of the species outside Mandal would most likely receive great attention.

5. Conclusion

5.1 Concluding remarks

The aim of this study was to design a species-specific method, based on eDNA analysis, for detection of lesser weever fish, and to test it in fifteen chosen locations along the Agder coast, in Southern Norway. To do so eDNA samples were collected at these locations were analysed using a real-time PCR assay with primers and a TaqMan probe designed to amplify DNA only from this species. The analysis results show that lesser weever DNA was successfully detected in four locations despite very low concentrations. This proves that the designed method is good, and that the sensitivity is high. The high sensitivity was also shown using serial dilutions of genomic DNA, while the specificity was ensured through an in-silico PCR, and by testing the primers and probe on the closely related species greater weever. Generally, for the aim of detecting the potential presence of a species in a chosen location, independent on abundance, the impression of this method has a high utility and efficiency.

5.2 Limitations and further research

This study has shown that the developed method indeed can be used to detect the presence of lesser weever, but to determine whether this species has established in Mandal, or have spread to other areas of the Agder coast, or Norwegian water in general, more comprehensive studies will be required in the future. It will most likely require collecting a considerably higher number of samples, from a higher number of location, which are more evenly distributed, over a wider time span.

Bibliography

Bauon, A. R., Brunel, T., Blanchet, M.-A., Hidalgo, M., Chust, G., Brown, E. J., Kleisner, K. M., Millar, C., Mackenzie, B. R., Nikolioudakis, N., Fernandes, J. A., & Fernandes, P. G. (2020). Changing fish distributions challenge the effective management of European fisheries. *Ecography (Copenhagen), 43*(4), 494–505. https://doi.org/10.1111/ecog.04864

Balasingham, K. D., Walter, R. P., & Heath, D. D. (2017). Residual eDNA detection sensitivity assessed by quantitative real-time PCR in a river ecosystem. *Molecular Ecology Resources*, 17(3), 523–532. https://doi.org/10.1111/1755-0998.12598

Burian, A., Mauvisseau, Q., Bulling, M., Domisch, S., Qian, S., & Sweet, M. (2021). Improving the reliability of eDNA data interpretation. Molecular Ecology Resources, 21(5), 1422–1433. https://doi.org/10.1111/1755-0998.13367

Buxton, A. S., Groombridge, J. J., & Griffiths, R. A. (2017). Is the detection of aquatic environmental DNA influenced by substrate type? *PloS One, 12*(8), e0183371–e0183371. https://doi.org/10.1371/journal.pone.0183371

Chaikin, S., Dubiner, S., Belmaker, J., & MacNeil, A. (2022). Cold-water species deepen to escape warm water temperatures. *Global Ecology and Biogeography, 31*(1), 75–88. https://doi.org/10.1111/geb.13414

Chen, Y., Long, H., Feng, T., Pei, Y., Sun, Y., & Zhang, X. (2022). Development of a Novel Primer–TaqMan Probe Set for Diagnosis and Quantification of Meloidogyne enterolobii in Soil Using qPCR and Droplet Digital PCR Assays. *International Journal of Molecular Sciences*, 23(19), 11185. https://doi.org/10.3390/ijms231911185

Claassen, S., du Toit, E., Kaba, M., Moodley, C., Zar, H. J., & Nicol, M. P. (2013). A comparison of the efficiency of five different commercial DNA extraction kits for extraction of DNA from faecal samples. *Journal of Microbiological Methods, 94*(2), 103–110. https://doi.org/10.1016/j.mimet.2013.05.008

Collins, R. A., Bakker, J., Wangensteen, O. S., Soto, A. Z., Corrigan, L., Sims, D. W., Genner, M. J., Mariani, S., & Yu, D. (2019). Non-specific amplification compromises environmental DNA metabarcoding with COI. Methods in Ecology and Evolution, 10(11), 1985–2001. https://doi.org/10.1111/2041-210X.13276

Da Costa Lima, M. S., Zorzenon, D. C. R., Dorval, M. E. C., Pontes, E. R. J. C., Oshiro, E. T., Cunha, R., Andreotti, R., & de Fatima Cepa Matos, M. (2013). Sensitivity of PCR and real-time PCR for the diagnosis of human visceral leishmaniasis using peripheral blood. Asian Pacific Journal of Tropical Disease, 3(1), 10–15. https://doi.org/10.1016/S2222-1808(13)60003-1

Emerson, D. (2012). Treating envenomation caused by fish sting. Emergency Nurse, 20(3), 28–29. https://doi.org/10.7748/en2012.06.20.3.28.c9153

Freeland, J. R. (2020). *Molecular Ecology*. Wiley-Blackwell.

Freitas, C., Villegas-Ríos, D., Moland, E., & Olsen, E. M. (2021). Sea temperature effects on depth use and habitat selection in a marine fish community. *The Journal of Animal Ecology, 90*(7), 1787–1800. https://doi.org/10.1111/1365-2656.13497

Fukuta, Takahashi, R., Kuroyanagi, S., Miyake, N., Nagai, H., Suzuki, H., Hashizume, F., Tsuji, T., Taguchi, H., Watanabe, H., & Kageyama, K. (2013). Detection of Pythium

aphanidermatum in tomato using loop-mediated isothermal amplification (LAMP) with species-specific primers. *European Journal of Plant Pathology, 136*(4), 689–701. https://doi.org/10.1007/s10658-013-0198-3

Geiger, K., Leiherer, A., Brandtner, E.-M., Fraunberger, P., Drexel, H., & Muendlein, A. (2019). Direct blood PCR: TaqMan-probe based detection of the venous thromboembolism associated mutations factor V Leiden and prothrombin c.20210G>A without DNA extraction. *Clinica Chimica Acta, 488*, 221–225. https://doi.org/10.1016/j.cca.2018.11.016

Geller, J., Meyer, C., Parker, M., & Hawk, H. (2013). Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. Molecular Ecology Resources, 13(5), 851–861. https://doi.org/10.1111/1755-0998.12138

Gorman, Judge, S. J., Harris, J. B., & Caldwell, G. S. (2021). Lesser weever fish (Echiichthys vipera Cuvier, 1829) venom is cardiotoxic but not haemorrhagic. *Toxicon (Oxford), 194*, 63–69. https://doi.org/10.1016/j.toxicon.2021.02.002

Gran-Stadniczeñko, S., Egge, E., Hostyeva, V., Logares, R., Eikrem, W., & Edvardsen, B. (2019). Protist Diversity and Seasonal Dynamics in Skagerrak Plankton Communities as Revealed by Metabarcoding and Microscopy. *The Journal of Eukaryotic Microbiology, 66*(3), 494–513. https://doi.org/10.1111/jeu.12700

Hammerschlag, N., McDonnell, L. H., Rider, M. J., Street, G. M., Hazen, E. L., Natanson, L. J., McCandless, C. T., Boudreau, M. R., Gallagher, A. J., Pinsky, M. L., & Kirtman, B. (2022). Ocean warming alters the distributional range, migratory timing, and spatial protections of an apex predator, the tiger shark (Galeocerdo cuvier). *Global Change Biology, 28*(6), 1990–2005. https://doi.org/10.1111/gcb.16045

Huerlimann, Cooper, M. K., Edmunds, R. C., Villacorta-Rath, C., Le Port, A., Robson, H. L. A., Strugnell, J. M., Burrows, D., & Jerry, D. R. (2020). Enhancing tropical conservation and ecology research with aquatic environmental DNA methods: an introduction for non-environmental DNA specialists. *Animal Conservation, 23*(6), 632–645. https://doi.org/10.1111/acv.12583

Judith D. L. vanBleijswijk, Julia C. Engelmann, Lise Klunder, Harry J. Witte, Johannes IJ. Witte, & Henk W. van derVeer. (2020). Analysis of a coastal North Sea fish community: Comparison of aquatic environmental DNA concentrations to fish catches. Environmental DNA (Hoboken, N.J.), 2(4), 429–445. https://doi.org/10.1002/edn3.67

Kaimuddin, A. H., Laë, R., & de Morais, L. T. (2016). Fish Species in a changing world: The route and timing of species migration between tropical and temperate ecosystems in Eastern Atlantic. *Frontiers in Marine Science, 3*, 162. https://doi.org/10.3389/fmars.2016.00162

Karenyi, Sink, K., Nel, R., Clark, A. E., & Altwegg, R. (2018). Imperfect detection distorts depth-related trends in marine macrofaunal species richness. *Ecography (Copenhagen), 41*(10), 1698–1706. https://doi.org/10.1111/ecog.03439

Kim, P., Kim, D., Yoon, T. J., & Shin, S. (2018). Early detection of marine invasive species, Bugula neritina (Bryozoa: Cheilostomatida), using species-specific primers and environmental DNA analysis in Korea. *Marine Environmental Research, 139*, 1–10. https://doi.org/10.1016/j.marenvres.2018.04.015

Kleisner, K. M., Fogarty, M. J., McGee, S., Hare, J. A., Moret, S., Perretti, C. T., & Saba, V. S. (2017). Marine species distribution shifts on the U.S. Northeast Continental Shelf under

continued ocean warming. *Progress in Oceanography, 153*, 24–36. https://doi.org/10.1016/j.pocean.2017.04.001

Ledeker, B. M., & De Long, S. K. (2013). The effect of multiple primer–template mismatches on quantitative PCR accuracy and development of a multi-primer set assay for accurate quantification of pcrA gene sequence variants. Journal of Microbiological Methods, 94(3), 224–231. https://doi.org/10.1016/j.mimet.2013.06.013

Lepperød, T. (2021, September 27th). Ny art funnet på strender I Mandal. *Nettavisen*. https://www.nettavisen.no/nyheter/giftig-dvergfjesing-for-forste-gang-pavist-pa-norske-strender/s/12-95-3424183934

Liu, K., Zhao, S., Yu, Z., Zhou, Y., Yang, J., Zhao, R., Yang, C., Ma, W., Wang, X., Feng, M., Tang, Y., Li, K., & Zhou, C. (2020). Application of DNA barcoding in fish identification of supermarkets in Henan province, China: More and longer COI gene sequences were obtained by designing new primers. Food Research International, 136, 109516–109516. https://doi.org/10.1016/j.foodres.2020.109516

Malik, S., Sharma, K., Vaiphei, K., Dhaka, N., Berry, N., Gupta, P., Sharma, M., Mallick, B., Kochhar, R., & Sinha, S. K. (2019). Multiplex Polymerase Chain Reaction for diagnosis of gastrointestinal tuberculosis. JGH Open, 3(1), 32–37. https://doi.org/10.1002/jgh3.12100

Marín, Villegas-Llerena, C., Fujimoto, T., & Arai, K. (2017). Novel decaplex PCR assay for simultaneous detection of scallop species with species-specific primers targeting highly variable 5′ end of the 16S rRNA gene. *Aquaculture Research, 48*(3), 920–930. https://doi.org/10.1111/are.12935

Martínez, B., Radford, B., Thomsen, M. S., Connell, S. D., Carreño, F., Bradshaw, C. J. A., Fordham, D. A., Russell, B. D., Gurgel, C. F. D., & Wernberg, T. (2018). Distribution models predict large contractions of habitat-forming seaweeds in response to ocean warming. *Diversity & Distributions, 24*(9/10), 1350–1366. https://doi.org/10.1111/ddi.12767

McCarthy, A., Rajabi, H., McClenaghan, B., Fahner, N. A., Porter, E., Singer, G. A. C., & Hajibabaei, M. (2023). Comparative analysis of fish environmental DNA reveals higher sensitivity achieved through targeted sequence-based metabarcoding. *Molecular Ecology Resources*, *23*(3), 581–591. https://doi.org/10.1111/1755-0998.13732

Mjaaland, A. (2021, September 27th). Svært giftig fisk oppdaget langs Sørlandskysten. *Aftenposten*. https://www.aftenposten.no/norge/i/MLjo3K/svaert-giftig-fisk-oppdaget-langs-soerlandskysten

Muhammad Fahmi Zuhdi, & Hawis Madduppa. (2020). Identifikasi Caesio cuning berdasarkan Karakterisasi Morfometrik dan DNA Barcoding yang didaratkan di Pasar Ikan Muara Baru, Jakarta. Jurnal kelautan tropis (Online), 23(2), 199–206. https://doi.org/10.14710/jkt.v23i2.7036

Muri, C. (2021, September 28th). Dvergfjesing funnet på Sørlandet: Dette vet vi om arten. *Havforskningsinstituttet*. https://www.hi.no/hi/nyheter/2021/september/dvergfjesing-funnet-pa-sorlandet-dette-vet-vi-om-arten

Nantón, Freire, R., Arias-Pérez, A., Gaspar, M. B., & Méndez, J. (2015). Identification of four Donax species by PCR–RFLP analysis of cytochrome c oxidase subunit I (COI). *European Food Research & Technology, 240*(6), 1129–1133. https://doi.org/10.1007/s00217-015-2416-z

Nga, T. V. T., Karkey, A., Dongol, S., Thuy, H. N., Dunstan, S., Holt, K., Tu, L. T. P., Campbell, J. I., Chau, T. T., Chau, N. V. V., Arjyal, A., Koirala, S., Basnyat, B., Dolecek, C., Farrar, J., & Baker, S. (2010). The sensitivity of real-time PCR amplification targeting invasive Salmonellaserovars in biological specimens. BMC Infectious Diseases, 10(1). https://doi.org/10.1186/1471-2334-10-125

Paul J. Durack, Peter J. Gleckler, Sarah G. Purkey, Gregory C. Johnson, John M. Lyman, & Tim P. Boyer. (2018). Ocean Warming: From the Surface to the Deep in Observations and Models. *Oceanography (Washington, D.C.), 31*(2), 41–51. https://doi.org/10.5670/oceanog.2018.227

Pawlowski J., Apothéloz-Perret-Gentil L., Mächler E. & Altermatt F. 2020: Environmental DNA applications in biomonitoring and bioassessment of aquatic ecosystems. Guidelines. Federal Office for the Environment, Bern. Environmental Studies. no. 2010: 71 pp. https://doi.org/10.5167/uzh-187800

Sara Peixoto, Cátia Chaves, Guillermo Velo-Antón, Pedro Beja, & Bastian Egeter. (2021). Species detection from aquatic eDNA: Assessing the importance of capture methods. *Environmental DNA (Hoboken, N.J.)*, *3*(2), 435–448. https://doi.org/10.1002/edn3.130

Perry, Low, P. J., Ellis, J. R., & Reynolds, J. D. (2005). Climate Change and Distribution Shifts in Marine Fishes. *Science (American Association for the Advancement of Science), 308*(5730), 1912–1915. https://doi.org/10.1126/science.1111322

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

Pethon, P. (2005). *ASCHEHOUGS STORE FISKEBOK*. Aschehoug & Co.

Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors - occurrence, properties and removal. *Journal of Applied Microbiology, 113*(5), 1014–1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x

Scott, A. L., & Henderson, P. A. (2016). Seasonal and age-related migrations in the unexpectedly long-lived benthic fish the lesser weever, Echiichthys vipera (Cuvier, 1829). *Journal of the Marine Biological Association of the United Kingdom, 96*(6), 1287–1293. https://doi.org/10.1017/S0025315415001460

Sen Gupta, A., Brown, J. N., Jourdain, N. C., van Sebille, E., Ganachaud, A., & Vergés, A. (2015). Episodic and non-uniform shifts of thermal habitats in a warming ocean. *Deep-Sea Research. Part II, Topical Studies in Oceanography, 113*, 59–72. https://doi.org/10.1016/j.dsr2.2013.12.002

Shi, Ge, S., Hou, Z., Xu, Y., Tao, J., Wu, H., & Zong, S. (2022). Species-specific primers for rapid detection of Monochamus saltuarius, an effective vector of Bursaphelenchus xylophilus in China. *Journal of Applied Entomology (1986), 146*(5), 636–647. https://doi.org/10.1111/jen.12972

Sigsgaard, E. E., Jensen, M. R., Winkelmann, I. E., Møller, P. R., Hansen, M. M., & Thomsen, P. F. (2020). Population-level inferences from environmental DNA—Current status and future perspectives. Evolutionary Applications, 13(2), 245–262. https://doi.org/10.1111/eva.12882

Sutlovic, D., Gamulin, S., Definis-Gojanovic, M., Gugic, D., & Andjelinovic, S. (2008). Interaction of humic acids with human DNA: Proposed mechanisms and kinetics. *Electrophoresis, 29*(7), 1467–1472. https://doi.org/10.1002/elps.200700699

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

Takahara, Minamoto, T., Yamanaka, H., Doi, H., & Kawabata, Z. (2012). Estimation of fish biomass using environmental DNA. *PloS One, 7*(4), e35868–e35868. https://doi.org/10.1371/journal.pone.0035868

Thomsen, & 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

Trannum, H. C., Gundersen, H., Oug, E., Rygg, B., & Norderhaug, K. M. (2018). Soft bottom benthos and responses to climate variation and eutrophication in Skagerrak. *Journal of Sea Research, 141*, 83–98. https://doi.org/10.1016/j.seares.2018.08.007

Vasconcelos, Prista, N., Cabral, H., & Costa, M. J. (2004). Feeding ecology of the lesser weever, Echiichthys vipera (Cuvier, 1829), on the western coast of Portugal. *Journal of Applied Ichthyology, 20*(3), 211–216. https://doi.org/10.1111/j.1439-0426.2004.00547.x

Wang, Li, H., Zhan, X., Ma, M., Yuan, D., Hu, Q., & Gong, Y. (2021). Development and application of quantitative real-time PCR based on the mitochondrial cytochrome oxidase subunit I gene for early detection of the grazer Poterioochromonas malhamensis

contaminating Chlorella culture. *Algal Research (Amsterdam), 53*, 102133. https://doi.org/10.1016/j.algal.2020.102133

Wang, G., Guo, X., Huang, X., Wang, D., Chen, Y., Qin, J., Yang, G., Tang, K. F. J., Dong, X., & Huang, J. (2023). The quantitative characteristics of infection with infectious precocity virus (IPV) revealed with a new TaqMan probe-based real-time RT-PCR method. *Aquaculture, 566*, 739179. https://doi.org/10.1016/j.aquaculture.2022.739179

Yao, Y., Nellåker, C., & Karlsson, H. (2006). Evaluation of minor groove binding probe and Taqman probe PCR assays: Influence of mismatches and template complexity on quantification. *Molecular and Cellular Probes, 20*(5), 311–316. https://doi.org/10.1016/j.mcp.2006.03.003

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics, 13*(1), 134–134. https://doi.org/10.1186/1471-2105-13-134

Økosystemet i Nordsjøen og Skagerrak er endret. (2018, May 15). Retrieved from https://www.hi.no/hi/nyheter/2018/mai/okosystemet-i-nordsjoen-og-skagerrak-er-endret

Appendix

A. Agarose gel recipe

1,8 grams of agarose powder was mixed with 100 mL 1xTAE-buffer in an Erlenmeyer flask. The flask was microwaved up to boiling point, then taken out and swirled. This step was repeated until all the agarose was completely dissolved. The solution was then cooled down to about 60°C, before 10 µL Invitrogen SYBR™ safe DNA Gel Stain was added to make the DNA visible under UV light.

B. Loading dye buffer recipe

50% glycerol + 50% Tris-HCL, pH8 + bromophenol (blue)