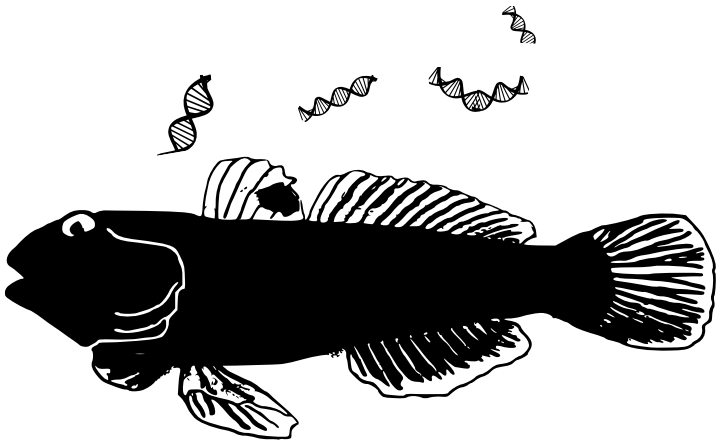
Investigating the Potential Introduction of Round Goby *Neogobius melanostomus* in Norwegian Ecosystems by Implementing Species Specific eDNA-sampling

Lars Martin Myhre



Supervisors:

Vivian Husa (The Institute of Marine Research)

Thomas Dahlgren (NORCE Norwegian Research Centre)

Rudolf Svensen (Museum Stavanger)

Henrik Glenner (University of Bergen)

Master of Science in Biology – Marine Biology

Department of Biology, University of Bergen

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Abstract

Over the last 150 years, various human activities have altered or weakened ecological barriers. In marine environments the most damaging activity is the ever-increasing, global commercial fleet. By passively transporting species through ballast water or biofouling, the commercial fleet has created pathways across ecological barriers. A species that has dispersed far from its natural range in the Black and Caspian seas through the commercial fleet, is the round goby (*Neogobius melanostomus* Pallas, 1814). The round goby was spread to the Great Lakes and to the Baltic Sea during the nineties. From there the invasive species has further dispersed both naturally, and through passive transportation. As it has been present Gothenburg, Sweden since 2010, it is expected to arrive in Norwegian ecosystems soon. Although the ecological impact from round gobies is difficult to predict, there are species native to Norway with overlapping niches, and benthic invertebrate taxa that potentially will be negatively impacted by the increased predation pressure. It is therefore of interest to detect the round goby early to limit its dispersal, and to early on map out ecological impacts. This study aimed to investigate a potential introduction of the round goby in the Oslo Fjord. Through fishing campaigns in September 2021 and August 2022, we attempted to verify its presence by catching round gobies. As newly introduced species tend to be of low abundance and patchy distributed, we also implemented eDNA-sampling to further build upon the notion that the method is an asset for detection and monitoring of invasive species. As a positive control both methods were tested in Gothenburg, where populations of round goby are well-established.

As expected from other literature we proved that both methods can be implemented to detect round gobies. We found however that eDNA-sampling was more efficient at detecting round gobies than fishing methods, as we only caught the fish at three out of five stations in Gothenburg. More importantly our eDNA results showed weak positive signals in four out of eight stations in the Oslo Fjord. This suggests that the round goby is introduced to the Oslo Fjord, but with significantly lower abundance than in Gothenburg. We did not manage to visually detect the invasive species, even during our second fishing campaign where fishing efforts around two of the positive stations were tremendously increased.

We hope that our findings can be applied to other investigative attempts in the Oslo Fjord to focus more fishing efforts at the positive tested stations. More eDNA-sampling should however be collected to help locating the alleged round goby populations.

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1. Introduction

1.1. Ecological Barriers and Vectors for Alien Species

Ecological barriers can be defined as physical, physiological, behavioural, or other ecological features that separate two or more ecosystems, taxa, or populations. Maintaining these barriers are crucial for biodiversity, especially for β-diversity (diversity in species between localities) and γ-diversity (overall species richness across all localities) (Seehausen et al. 2008). Over the past 150 years, human activities have increasingly weakened and altered one ecological barrier in particular, dispersal barriers (Vermeij 1978, Shucksmith and Shelmerdine 2015). Besides direct removal and damage to natural barriers by creating massive passages and steppingstones for marine traffic (e.g., the Panama- and Suez-Canal), barriers have also been weakened by passive and active transport of species from their natural habitats (Vermeij 1978, Shucksmith and Shelmerdine 2015).

Historically, humans have altered dispersal barriers by actively transporting species from their habitat for aquaculture. A well-recognised example of this is the pacific oyster (*Magallana gigas* Thunberg, 1793). The high economic value as a delicacy stimulated shellfish production in areas outside its natural habitat such as in Western Europe (Wrange et al. 2010). The pacific oyster was introduced to Norwegian aquaculture already in the 1970s with a well-documented argument that it would be unable to reproduce and naturally disperse due to low temperatures (Flagella and Abdulla 2005, Wrange et al. 2010). As time passed, warmer summers and milder winters weakened this limitation and the oyster is now widespread and reproduces along the west coast of Sweden and south coast of Norway (Wrange et al. 2010). While the pacific oyster itself can be a problem for ecosystems and recreational services, its introduction might also bring companion species (Maggs and Stegenga 1998, Reise et al. 1998, Sjøtun et al. 2008). Examples of species that are associated with pacific oyster are: *Dasya baillouviana* (Montagne, 1841) and *Dasysiphonia japonica* (H.-S.Kim, 2012) (Den Hartog 1964, Sjøtun et al. 2008). Though it is difficult to prove that oyster-introductions have been the main vector for introducing companion species, the distribution of companion species show clear patterns around oyster-farms (Maggs and Stegenga 1998, Sjøtun et al. 2008).

The most impactful vector for biological introduction is currently the ever-growing global commercial fleet (Carlton 1979, Shucksmith and Shelmerdine 2015, Letschert et al. 2021). Marine traffic creates pathways for marine species by passively transporting organisms or their propagules through ballast water and hull fouling, weakening the dispersal barriers (Godwin 2003, Ruiz et al. 2015). Notable examples of this are the zebra mussel (*Dreissena polymorpha* Pallas, 1771), the toxic dinoflagellate *Gymnodinium catenatum* (H.W.Graham, 1943), and the tunicate *Didemnum vexillum* (Graham 1943, Flagella and Abdulla 2005, McKenzie et al. 2017).

Ballast water is generally used by vessels to ensure stability and manoeuvrability when transporting light cargo or no cargo at all. The volume of ballast water is adjusted corresponding to cargo-load, fuel load etc. As a result of adjusting ballast to cargo-load, it normally implies that discharging/filling of ballast water happens while being anchored or moored. This conduces water from one port to be transported to another port. In many cases this water exchange happens across dispersal barriers such as distance, salinity levels, temperature, etc. When taking on ballast water there is a possibility for organisms to be caught in the ballast tank. Most species that can be caught in ballast tanks are microorganisms, but macroorganisms have also been found in ballast during their larvae and juvenile stages (Hayden and Miner 2009). Given that conditions in the ballast tank are tolerable, species caught in the tank can survive during transportation and be released in new areas. In 2017, it was estimated that 10 billion tonnes of ballast water were transported worldwide (IMO 2017).

Due to its vast volumes, ballast water has until recently been considered one of the vectors with highest risk for introducing nonindigenous marine species (David et al. 2015, Ruiz et al. 2015). This is anticipated to change over the coming years due to the recent ballast water convention implemented in 2017 (Husa et al. 2022). The ballast water convention inaugurated two standards for ballast water management, D1 and D2. D1 imposes ships to replace ballast water in open seas rather than at the ports, and the D2 standard imposes ships to install mechanical-, physio-chemical-, or electro-chemical ballast water management instruments (IMO 2017, Lakshmi et al. 2021). Combinations of different treatments, such as mechanical filtration followed by chemical hydroxyl treatment has been proven “to inactivate 100% of organisms in ballast tanks” (Lakshmi et al. 2021).

Although the IMO convention will reduce ballast water to a negligible vector, the commercial fleet continues to be a substantial vector of marine biological introductions (Husa et al. 2022). As the hull of ships is in direct contact with water, it will always be exposed to marine life growing on it. This growth is commonly known as biofouling. Biofouling is especially

pronounced while ships are stationary e.g., anchored, or moored. Hulls are primarily exposed to growth of species that typically inhibit hard substrate, such as algae, barnacles, tunicates, molluscs, and bryozoans (Coutts et al. 2010). Hard substrate biofouling can however facilitate habitat and shelter for other species such as fish and crustaceans (Coutts et al. 2010). With ballast water losing some of its importance as a vector for marine biological introductions, it can be argued that biofouling now has become the new main vector (Husa et al. 2022). With commercial vessels as the main vector, one would expect areas of introduction to create a pattern correlated to cargo harbours. This pattern is for instance documented for the round goby (*Neogobius melanostomus* Pallas, 1814*)*(Wiesner 2005).

1.2. Round Goby

The round goby originates from the Caspian and Black seas, and their tributaries. The species has therefore adapted to inhabit fresh-, brackish-, and, to some extent, marine waters (Ellis and MacIsaac 2009). This reflects on its current global distribution, being abundant in e.g., the Baltic Sea, the Great Lakes, and Eurasian river-systems (Kornis et al. 2012, Kotta et al. 2016). Although being mainly benthic, the round goby is reported to display nocturnal, pelagic activity (Jůza et al. 2016). This activity is especially apparent for foraging juvenile fish (Hayden and Miner 2009, Jůza et al. 2016). The round goby is associated with both rocks/rough sand and with sandy/muddy substrate (Skóra and Stolarski 1993, Charlebois et al. 1997). Though a tolerance for salinity as high as 40.5‰ is reported (Moskal'kova 1996), no populations have been reported to fully live in oceanic habitats (Charlebois et al. 1997). Laboratory experiments have in fact demonstrated that the round goby cannot survive more than 48 hours in 30‰ salinity (Ellis and MacIsaac 2009). In its native range the round goby spawns every 3rd-4th week from April to September (Charlebois et al. 1997). While the number of eggs laid per spawning seems to correlate with female body mass, one can roughly estimate the round goby to lay between 150-10 000 eggs per spawning (Charlebois et al. 1997, MacInnis and Corkum 2000, Wandzel 2000). The round goby has a thermal tolerance of -1 – 30°C but prefers temperatures close to its energetic optimum around 26°C (Moskal'kova 1996, Lee and Johnson 2005).

Both horizontal and vertical, seasonal migration occurs in round goby populations (Christoffersen et al. 2019, Behrens et al. 2022). In lakes it is typical for the round goby to migrate to deeper waters during winter, and have been found as deep as 155 meters in Lake Ontario (Pennuto et al. 2021). Meanwhile, studies from Sjælland, Denmark has shown that

round gobies in estuaries have a variation of winter strategies within a population (Christoffersen et al. 2019). Christoffersen et al. (2019) showed that out of 50 tagged fishes, 18 winter-migrated seawards (and thereby deeper), 11 resided inside the estuary throughout the whole winter, and 3 winter-migrated upstream into the river. The water temperature in the estuary during Christoffersen et al. study reached below 2°C on its coldest, showing that, although capable of migrating 1-2 km per day, the round goby is flexible and opportunistic (Christoffersen et al. 2019).

Despite the round goby's migration capabilities evidence strongly suggests that its near-global distribution (Figure 1.1) has relied on passive transport through marine traffic (Kornis et al. 2012). According to studies exploring genetic similarities between introduced round goby populations, "local" marine traffic has also accelerated the dispersal within areas of introduction (LaRue et al. 2011). Juvenile round goby is proven to undergo a diel vertical migration, foraging on plankton during night (Hayden and Miner 2009). Hayden and Miner (2009) illustrated that juvenile round goby would vertically migrate from depths ≥10 meters to as high as 2 meters, with higher densities from 5-8 meters. As many freighters take in ballast water from 6-9 meter below the surface, replacing ballast water during night could result in transportation of juvenile round gobies (Hensler and Jude 2007, Hayden and Miner 2009). Some researchers also suggested that the round goby might be able to attach its eggs to ships (Tsepkin et al. 1992, Moskal'kova 1996, Hirsch et al. 2016). However, as Adrian-Kalchhauser et al. (2017) put it "the invasive round goby might attach its eggs to ships or boats – but there is no evidence".

The round goby was first observed in the Baltic Sea in June 1990 in the Gulf of Gdansk (Skóra and Stolarski 1993). Through both marine traffic and natural dispersal, the round goby was introduced to other areas of the Baltic Sea in the following years (AquaNIS 2019). For instance, it was introduced to Lithuania in 2002, likely through shipping, and later to Latvia in 2004 through natural dispersal from Lithuania (AquaNIS 2019). In 2008 the round goby was found along the east coast of Sweden, and two years later in Gothenburg (AquaNIS 2019, Artportalen 2021). As of 2022, round gobies are still not observed in Norwegian waters. The current perception in the Norwegian research community for alien species is that the round goby is expected to be introduced in Norwegian ecosystems. One can therefore classify the round goby as a "door-knocker" in Norwegian ecosystems (Forsgren and Hanssen 2022).

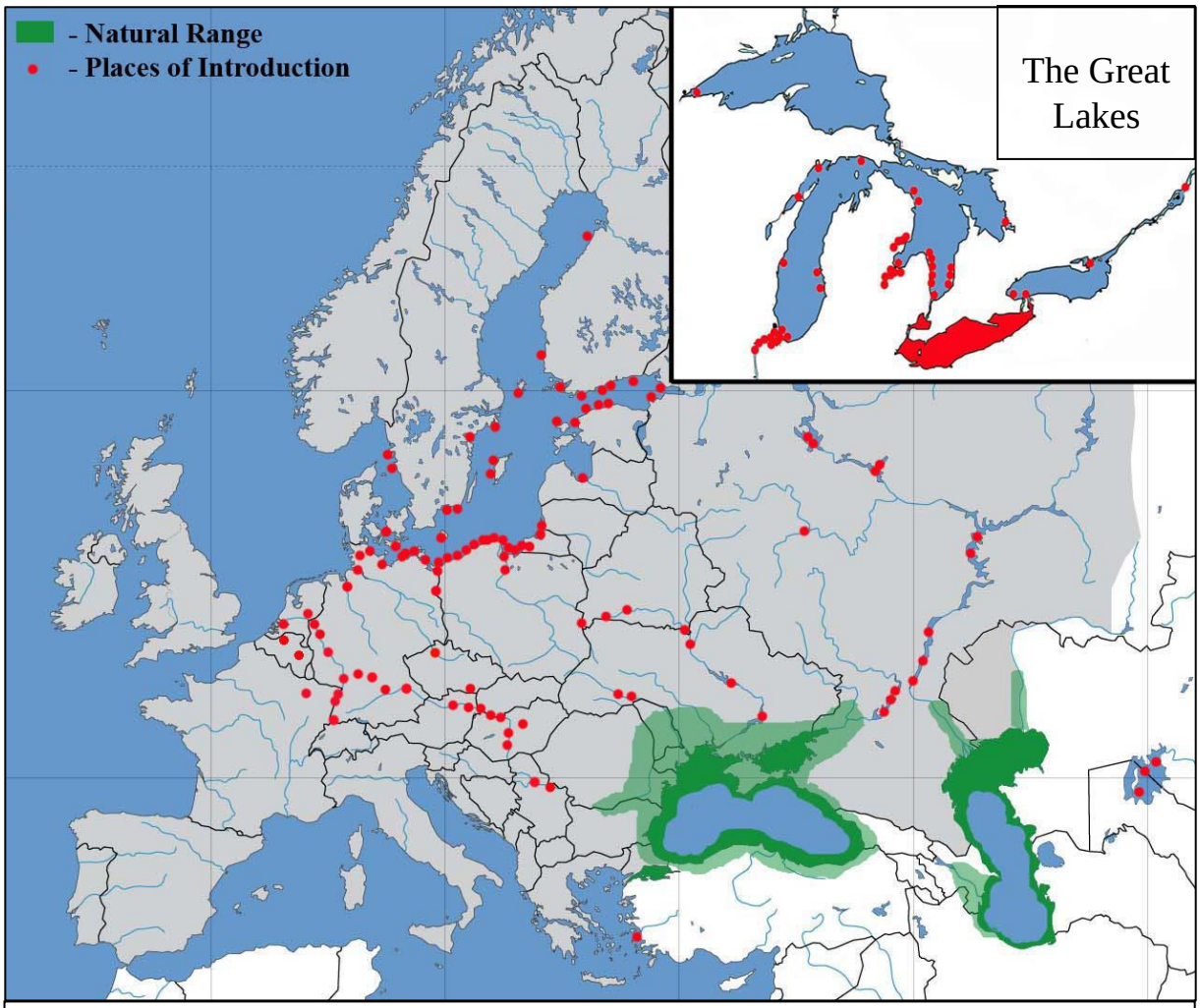

Figure 1.1: Global documented distribution of round goby. Red shows places of introduction and green illustrate its natural range. Retrieved from WikiMedia Commons (CC BY-SA 4.0), created by Yuriy Kvach (2014), last updated 1st June 2019.

Although the round goby is ranked amongst the top 100 most invasive species in Europe, the impact it inflicts on native fish assemblages is evidently case-specific (Hirsch et al. 2016, Janáč et al. 2019). Studies conducted in River Meuse suggest that round gobies have played a role in the rapid decline of the river bullhead (*Cottus perifretum*, Freyhof, Kottelat and Nolte, 2005) population (Kessel et al. 2016). Meanwhile, a long-term study on the river bullhead's close relative, the European bullhead (*Cottus gobio*, Linnaeus, 1758), in Austrian Donau showed that round gobies inflicted no significant impact (Janáč et al. 2018). Studies have also illustrated that similar fish assemblages within the same area can vastly differ in how they are affected by introduced round gobies (Janáč et al. 2019). This becomes clear when investigating studies from various locations in Lake Michigan (Janssen and Jude 2001, Kornis et al. 2013). The study conducted by Janssen and Jude in 2001 along the southern coast of Lake Michigan found that

round gobies had a significant impact on the recruitment success in native fish. Kornis et al. (2013) however, claimed that the round goby had no significant impact on native fish assemblage in the central coast of Lake Michigan.

Lastly, round gobies are proven to cause negative, positive, and neutral effects concurrently with a fish assemblage (Morissette et al. 2018). Morissette et al. (2018) suggested the round goby to negatively impact the abundance of tessellated darter (*Etheostoma olmstedi* Storer, 1842) through competitive exclusion. The same study claimed that other species, like brook silverside (*Labidesthes sicculus* Cope, 1865) and emerald shiner (*Notropis atherinoides* Rafinesque, 1818), increased in abundance (Morissette et al. 2018).

While studies referred to so far illustrate unpredictable and complex impacts, the overall trend is that round gobies tend to negatively affect small benthic fish populations in one way or another (Hirsch et al. 2016, Janáč et al. 2019). Numerous studies have also proven that round gobies negatively affect other benthic fauna through predation (Kuhns and Berg 1999, Lederer et al. 2008, Bradshaw-Wilson et al. 2019). A cage experiment that was conducted on round goby found that its predation pressure significantly decreased the abundance, biomass, and taxon richness of invertebrates native to the Baltic Sea (Henseler et al. 2021). Most studies regarding round gobies predation on invertebrates have been focusing on dreissenid mussels, but newer research has illustrated that the round goby also predates on other taxa native to Norwegian ecosystems like: unionid-, mytilid-, and cardiid mussels (Bradshaw-Wilson et al. 2019, Henseler et al. 2021)

While it is impossible to accurately predict how a potential introduction of round goby would affect Norwegian ecosystems, there are fish species native to Norwegian waters that have overlapping diet and habitat with the round goby: e.g., turbot, European flounder, European plaice, and European perch (Skora and Rzeznik 2001, Karlson et al. 2007, Hirsch et al. 2016, Ustups et al. 2016). There are also mytilid, cardiid, and unionid mussels in Norwegian ecosystems that could stand at risk of increased predation pressure such as: blue mussel, cockle, and freshwater pearl mussel. With the unpredictable and complex impacts from round goby, one could argue that detecting it early is important.

1.3. Detection and Monitoring of the Round Goby and eDNA-sampling

Detection and mapping of the round goby using conventional sampling methods can be challenging as many methods are both resource and time demanding. This is increasingly challenging when investigating an introduced species over a large area. Other observational methods such as aquascope, snorkelling, SCUBA, and video are all good alternatives but are for one, also time consuming, and secondly, limited to certain habitats. Observational methods are for instance hard to apply in estuaries as visibility usually is remarkably worse in brackish water than fresh- or saltwater.

Another challenge with applying observational methods to investigative campaigns is that newly introduced species, in most cases, still exist with low and/or patch densities. This makes it difficult for the observer to be at the right place at the right time. As the round goby is classified as a "door-knocker" in Norway, it calls for methods that are effective at detecting the species during preliminary stages of establishment. To cover a larger area, it is preferable to fish with passive equipment such as small, easily transportable traps (which accumulate catch over time) or using environmental DNA-sampling.

Since the late 2000s an accelerating number of studies have used environmental DNA (eDNA) for detection and monitoring (Sepulveda et al. 2020). eDNA includes intra- and extra-cellular forms of DNA from an organism found in the environment (Cristescu and Hebert 2018). Mediums that typically used for DNA analysis are soil, water, sediment and even air (Ruppert et al. 2019, Sepulveda et al. 2020). Animal DNA can be released to the environment from skin, mucous, gametes, faeces, urine, blood and rotting bodies (Bohmann et al. 2014). eDNA can be detected from environmental samples through PCR by using either universal primers (typically used for mapping and biodiversity research), or species-specific primers (Ruppert et al. 2019). Species-specific primers can be used for monitoring species with low density or low abundance like species on the verge of extinction, or invasive species in early stages of establishment (Ruppert et al. 2019). This was for instance demonstrated when Jerde (2021) detected eDNA from the non-indigenous bighead carp (*Hypophthalmichthys nobilis* Richardson, 1845) close to Lake Michigan prior to visual detections.

As with most methods there are considerations and challenges to address with eDNA-sampling. In the field of aquatic- and marine- biology, where much of eDNA-sampling is done by sampling water, currents and streams disperse eDNA from its source (Forsström and Vasemägi 2016). This is especially the case for marine sampling where, contrary to most aquatic sampling,

there are massive volumes of medium and currents that makes it hard to find the source of DNA (Forsström and Vasemägi 2016). Another challenge with eDNA-sampling is to account for degradation over time. DNA-degradation is caused by several biotic and abiotic factors that are habitat-dependent such as bacterial activity, salinity, temperature, pH and UV (Strickler et al. 2015). In addition to degradation rate, initial DNA amounts shed from the source are significant when looking at turnover time. A study on degradation and turnover illustrated that, while degradation is higher in marine sediment compared to the water column, the turnover time in water is much shorter due to less eDNA content (Dell'Anno and Corinaldesi 2004). This demonstrates that research on the degradation and dispersal distances of eDNA can be difficult to implement into other studies. Overall, it is difficult to determine how much time after release from its source, and therefore how far away, one could expect to detect eDNA. While there are studies claiming a half-life (time for half of the eDNA to degrade) of 0.7 hours, there are other studies showing half-life over 300 hours (Strickler et al. 2015, Seymour et al. 2018). In water with natural biochemical conditions studies span from 0.7 hours to 71 hours (Cowart et al. 2018, Seymour et al. 2018).

Application of eDNA-sampling in marine and aquatic research is a relatively novel approach. Even though there are uncertainties regarding degradation rates, the method has proven itself as an asset in marine and aquatic research (Thomsen et al. 2012, Sundberg et al. 2018, Kutti et al. 2020). Throughout the development of eDNA-sampling, the method has proven to be more suitable for some groups of species than others. Research has for instance showed that eDNA-sampling is difficult to implement when sampling for crustacean DNA. The probable cause for this has been argued to be low shedding rates from exoskeletal species (Forsström and Vasemägi 2016, Sundberg et al. 2018). Several species groups with higher DNA shedding rates, such as filter-feeders and fish, have been proven detectable and possible to monitor with eDNA-sampling. There have been successful attempts on mapping and monitoring cold-water coral reefs by approaching eDNA as a passive particle (Kutti et al. 2020). Kutti et al. (2020) demonstrated that eDNA-sampling could be indicative for a predicative distribution model of the cold-water coral *Desmophyllum pertusum* (Linnaeus, 1758) with a 1-2.5 km precision (Kutti et al. 2020). Research using eDNA meta barcoding for marine fish diversity found that the method performed as good (and sometimes better) as conventional methods when identifying fish diversity (Thomsen et al. 2012). Additionally, there are numerous studies that have applied species specific eDNA in monitoring of alien and invasive fish species, including the round goby (Jerde et al. 2011, Nevers et al. 2018, Sundberg et al. 2018).

1.4. Digital Droplet Polymerase Chain Reaction (ddPCR)

Natural quantities of eDNA from the organism of interest are small and mixed in a pool of DNA from other organisms. eDNA methods must therefore include PCR techniques to amplify wanted sequences to detectable and measurable quantities. PCR enables researchers to target one or several specific DNA-sequences and to amplify it to quantities that can be used for analyses. To further enhance the readability, it is common to use probes labelled with a fluorescent reporter (a fluorophore) or DNA-binding dye during the PCR (qPCR). By exposing the sequences to a certain wavelength of light, the fluorophore will be excited and thereby emit fluorescent light. The amplitude of emitted light can be analysed to see if the targeted sequence of DNA is present or not, and consequently to estimate the amount of DNA sequences in the sample.

ddPCR is a development to PCR that has enhanced the quantifiability of eDNA-samples. This technique involves dividing the PCR-mastermix (PCR reagents and eDNA-sample) in up to 20 000 droplets prior to PCR. This enables PCR amplification to perform separately in each droplet. Droplets containing the targeted sequence will then react with the fluorescent reporter and emit fluorescence. During droplet reading, droplets emitting fluorescence will be registered as positive or negative based on the intensity of emitted fluorescence. What makes the results from ddPCR more quantifiable than qPCR, is that from a total of 15 000-20 000 droplets, there will be quantifiable portions of negative droplets and positive droplets. This will more accurately correlate with the concentration of the targeted sequence in the mastermix which can be used to calculate the proportion of the sequence in the sampled habitat. It is important to mention that this technique is well developed for detecting low concentrations of target DNA, such as the case with many environmental samples (Kokkoris et al. 2021).

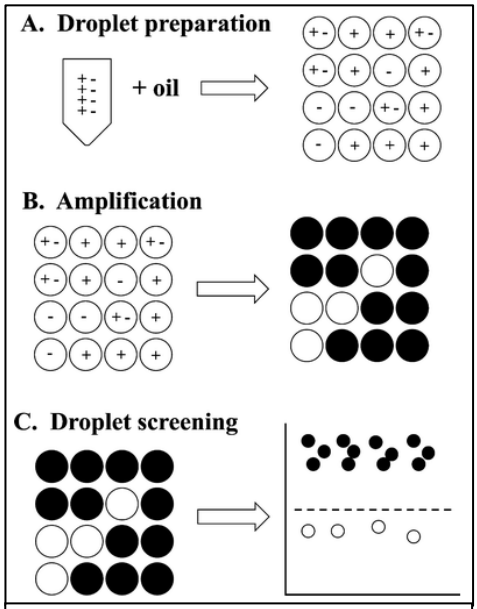

Figure 1.2: ddPCR technique (Nathan et al. 2014)

A: Sample with targeted and non- targeted DNA is separated into ~20 000 droplets. Each droplet has targeted (+) and/or non-targeted (-) DNA.

B: Droplets are amplified through PCR amplification, resulting in positive (black) or negative (white)

C: Droplets is read by an instrument that gives a distribution of positive and negative droplets. Droplets containing both positive and negative strains are read as positive

1.5. Aim

The round goby is ranked as one of the top 100 most invasive fish species in Europe (Hirsch et al. 2016). As of the launch of this project there were no registered sightings of round gobies in Norway. Detecting the species early can be beneficial as its impacts are unpredictable for ecosystems important for Norwegian conservational and commercial interests. The main aim of this project was to investigate whether the round goby was introduced in the Oslo Fjord. As there are challenges with sampling in habitats where one would expect to find the round goby and time consuming to study a large area as the Oslo Fjord, this project included eDNA-sampling. Sampling with eDNA has successfully been applied to monitoring and investigative work on round goby earlier in both the Great Lakes and the Baltic Sea (Nathan et al. 2014, Sundberg et al. 2018, George et al. 2021). This study aimed to further build on the notion that eDNA can be useful for both monitoring and detection of the species.

2. Material and Methods

2.1. Stations and Sampling Methods

Three separate field campaigns were executed in Eastern Norway and/or Western Sweden during this project. The first one was executed 13th-19th of September 2021, where both eDNA-sampling and fishing were performed in Norway and Sweden. Due to eDNA-samples from Norway being contaminated in the first campaign, a second eDNA-sampling campaign was executed from 30th-31st of March 2022 to recollect samples. Lastly, a third campaign was executed between 12th-14th of August 2022, to further increase the fishing efforts at the Norwegian stations (see section 2.8.).

All data was collected from 15 stations in total: 5 in Gothenburg, 5 along eastern Oslo Fjord, and 5 along western Oslo Fjord. Prior to field work the stations were tentatively placed based on available information. In Gothenburg there were sufficient previous observations registered in artportalen.se, which was the framework when planning for station locations. In Norway the station plan was made regarding ideal habitats, especially focusing on salinity and sediment type. As such factors are difficult to know for sure beforehand, some stations were moved to a different location *in situ*. Different methods were used to investigate the possible presence of round goby: fishing (angling and minnow traps) and water sampling for eDNA-testing. Additional data collected at each site included temperature, substrate type, and depth. A full overview over stations and station locality from the first field campaign is found in Table 2.2 and Figure 2.2 respectively. One of the 15 stations (station 10) was not introduced until the last campaign and is not listed in Table 2.2 nor Figure 2.2.

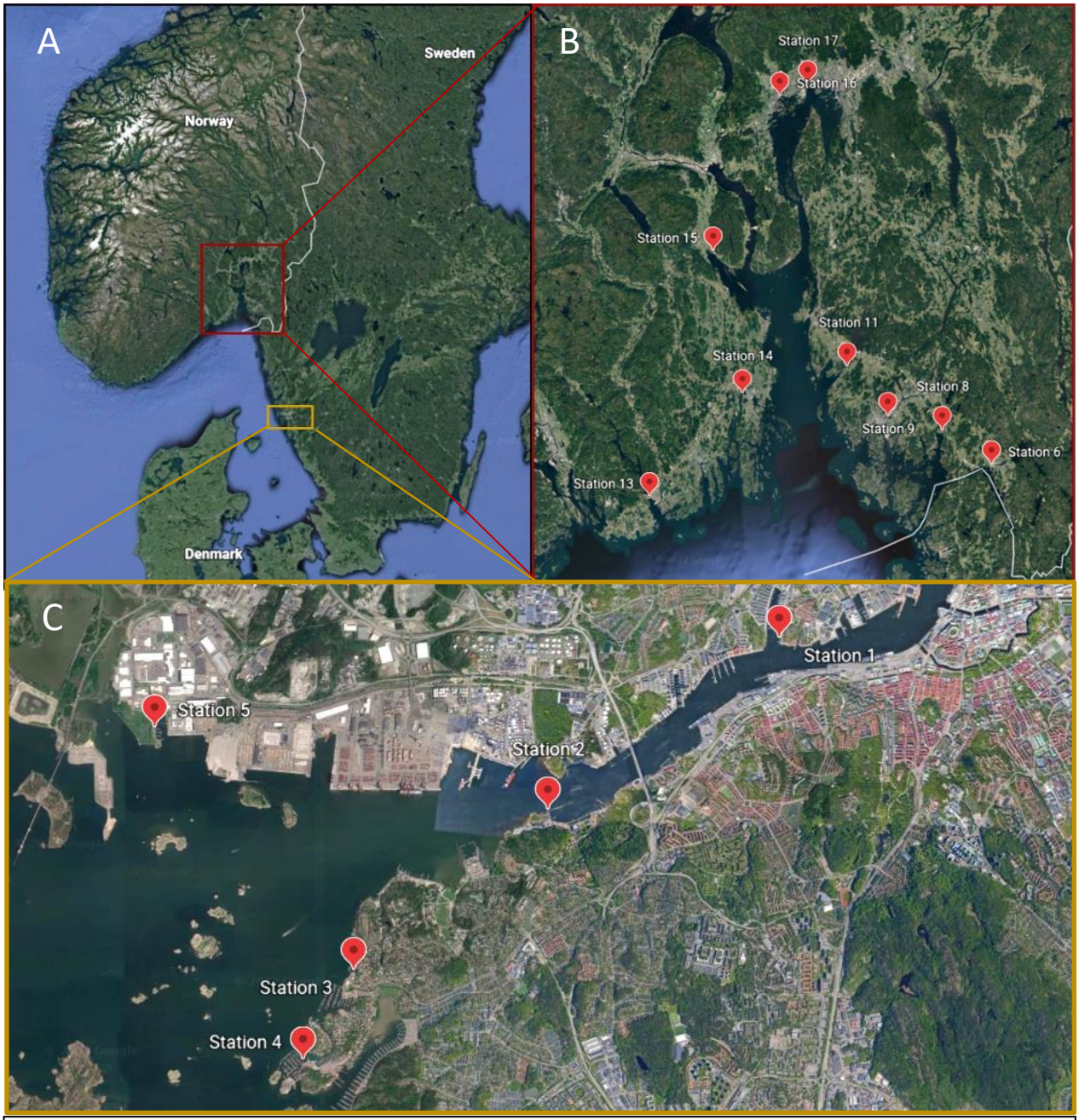

Figure 2.1:

- **A.** Map of Southern Scandinavia illustrating the distance between Gothenburg (yellow square) and the Oslo Fjord (red square).
- **B.** Overview of stations along the Oslo Fjord (St. 6-17).
- **C.** Overview of stations in Gothenburg (St. 1-5).

Note: Station 7 and 12 are not to be found, as these stations were removed during field, re-labelling the stations after removal were not done to reduce the risk of miss-labelling.

Table 2.1: Overview and description of each station from the first field campaign

Station	Description
St. 1 – Slottsberget	Furthest up-stream of all stations in Göta älv. The station was located at a ferry terminal in the outermost part of a wide channel in the river. Clear constructional changes, with a semi-natural rip-rap zone along the terminal. Poor visibility. Pots placed at 1 meter deep, all other sampling done at 1-4 meters depth.
St. 2 – Nya varvet	Constructed riprap zone with poor visibility. Covered from waves due to a constructed marina, but still with good water exchange from the river stream, as there were openings in both ends of the marina. All sampling performed from 1-2 meters depth.
St. 3 – Långedrag	Good coverage from both waves and river-stream, with seemingly low water exchange. Station was in a marina at the innermost part of the Göta älv estuary. The bottom was sandy/muddy and showed signs of physical alterations from the marina. All sampling performed from 1-1.5 meters depth. Good visibility.
St. 4 – Saltholmens brygga	Station located at a marina + ferry terminal on the southern part of the Göta älv estuary. Medium visibility. With exception to currents from ferries, the station was protected from streams and currents by natural and non-natural constructions. All sampling was taken from 1-1.5 meters depth.
St. 5 – Måsholmen	Station located at a small marina between two constructed rip-rap zones. The station was farthest away from Göta älv and was located at the northern side of the Estuary. The sampling was done between 2-3 meters, mostly on sandy bottom, but some fishing was done at the edge between sandy and riprap.
St. 6 – Halden	Station located along a constructed riprap zone near heavy industrialised harbours at the estuary of Tista River. All sampling was done at 0.5-3.5 meters. The bottom was a mosaic of sandy and rocky. Slightly exposed to currents and stream with a seemingly medium-low water exchange.
St. 8 – Høysand	Station located at a marina in the innermost part of an inlet/fjord, with run-off from agriculture through small streams nearby. The whole area is characterised by recreational boat traffic. All sampling was done at 2-3 meters depth along a riprap breakwater built on a sandy bottom. Seemingly low amounts of water exchange. Good visibility.
St. 9 – Fredrikstad	Station located along Glomma River by a small marina, with seemingly good water exchange. All samples were taken from 1.5-2 meters depth. Rocky bottom. Poor visibility (unable to see bottom clearly at 1.5 meters).
St. 11 – Råde	Station located in the marina at the innermost part of a small fjord with run-off from agriculture through small streams. Water exchange was medium poor. All sampling was done along the marina at 1-2 meters depth. Visibility was too poor to see the sandy bottom.

St. 13 – Larvik	Station located along the end of Farris River. Along both sides of the river, there were constructed riprap, while the middle of the river was rocky/gravely. The river provided good water exchange. The visibility was good enough to see the deepest part of the river (~4 meters deep). All sampling was done at 1.5-3 meters depth.
St. 14 – Tønsberg	Station located on an island facing the estuary of Auli River. Samples were taken along a small swimming pier at 1.5-2 meters depth. Visibility was barely good enough to see the muddy bottom. While the bottom was muddy, the pier was built on massive boulders with a lot of gaps in between them.
St. 15 – Sande	Station located in the estuary of Selvik River. Samples taken along land at 1-2 meters depth. Visibility was too poor to see the muddy/sandy bottom clearly. The slow running river gave a good water exchange. Station was located close to the constructed riprap.
St. 16 – Sandvika	Station located at a promenade along the end of Sandvik River. Samples were taken along the promenade at ~2 meters depth. Visibility was too poor to clearly see the bottom. Good water exchange from the river. The bottom was concrete and with little to none rocks or other substrate to hide in. Yet the promenade was built on boulder with cracks in between.
St. 17 – Lysaker	Station located on a breakwater along the estuary of the Lysaker River. Samples from the inner side of the breakwater. All samples were collected from 1-2 meters depth. The water exchange is low due to the breakwater. Visibility is barely good enough to see the bottom.

Angling was done through a simple rod-and-hook setup. Shrimp were consistently used as bait on the hooks, and a light sinker was attached to the line to keep the hook from floating to the surface. During the first fishing campaign we fished for a total of 1 hour at each station. At some stations the total time was divided between multiple rods to save time e.g., 30 min. divided between two rods, or 20 min. between three rods. This allowed us to cover a larger area and save time on fishing. All catches were counted and logged to document fishing effort and fishing success.

In addition to angling, five minnow traps were placed at each station. The minnow traps measures were 44 cm long, 23 cm in diameter, and had a grid size of 0,6 cm. At both ends of the trap there was a 2,5 cm opening. Each trap was equipped with a small bottle (250 mL) as a bait-container to ensure that the bait was not eaten before the fishing period was over. The bottles were each baited with 3-4 shrimps. Prior to the field campaign a piece of rope was attached to the bottle-cap to easily hang the bottles inside the traps with cable ties. This was

done to slightly elevate the bottles from the bottom to hinder endobenthic fauna from consuming all the bait. Multiple holes were drilled in the bottles to ensure that scents from the bait were released to the surrounding environment. The traps fished for a minimum of 12 hours at each station. Once the traps were retrieved, all catches were counted before release. When possible, we preserved 10 black gobies (*Gobius niger* Linnaeus, 1758) and round gobies from each station in 70% ethanol to use for further research. The pelvic fin of all round gobies was clipped and preserved in 97% ethanol for potential future DNA analysis. The preservation of black gobies was also done with potential future research in mind.

To avoid cleaning the minnow traps between stations but still ensuring a minimum risk of cross contamination of eDNA, water samples were collected prior to placing the minnow traps. Three water samples were collected at each station by using a Ruttner water sampler. The Ruttner sampler can be shut by pushing the top-part of the apparatus. Thereby, using a brass attached to the line, the apparatus can be lowered to the correct depth before dropping the brass and shutting the apparatus. By lowering the sampler to just above the sea floor before shutting, it collected water from where higher concentrations of round goby eDNA was expected. The sampler was equipped with a thermometer which was used to retrieve temperature data simultaneously as water samples. The attached line was marked with tape every meter, which was utilised to gain information about the depth where the samples were collected. One water sample corresponded to roughly 1.8 litres which each were transferred to clean buckets for transportation. Between every sampling the Ruttner sampler and the buckets was thoroughly cleaned by using a sponge and chlorine. During the first field, the water samples were transported back to base for filtration within 3 hours after collection.

2.2. Water Filtration:

The filtration set-up used in September can be divided into three main functional parts: A vacuum pump, filter-stands, and a collection tank (Figure 2.2). The stand had four pedestals for filters with detachable 3 dl cups held in place with magnets. Each pedestal had a lock that could be closed when not in use. This made it possible to do four replicates simultaneously, but also allowed for different replicates to flow with different pace. When one replicate was complete the corresponding pedestal was shut to avoid depressurising the system. All four pedestals drained off to a hose that was connected to the collection tank. Also connected to the tank was an additional hose leading to a vacuum pump. As both hoses are connected to the lid, water falls into the tank without being sucked into the electric pump.

Firstly, one "Whatman nitrocellulose membrane filter" (from here referred to as "filter paper"), with a pore-size = 0.45 µM, and diameter 4,7 cm, was placed on each pedestal before the cups were attached. While being were locked, all four cups were filled up with 3dl of water before opening the pedestals, three with water samples and one with a negative control (tap water). The vacuum pump was turned on to decrease the pressure in the collection tank before all locks were opened. As it was desirable to filtrate one litre, the cups were continuously filled up. To make filling up easier, one-litre-containers were used to transfer water from the "field buckets" to the cups. All filtrations were decided to do at least one litre of water per sample. However, as shore water can be muddy, filtrating one litre is not always possible on a time-schedule. A one-hour filtration-time limit therefore was implemented to the method as well.

When one sample was done the corresponding pedestal was closed off to maintain the pressure within the system. The paper filters were removed from the pedestal, folded, and placed in 2ml sample tubes using a clean tweezer. Before storing the tubes in a freezer, 1.6ml of buffer ATL was added in each tube to preserve the DNA contained in the filters. When all samples were filtrated everything in contact with the water-samples prior to filtration, or the filters after filtration, were cleaned with chlorine and rinsed with water. The outgoing tubes and collection tank were not cleaned in between samples, as these were in no risk of contaminating the samples.

Figure 2.2: Filtration set-up during field campaign September 2021. From the left: a vacuum pump was connected to the collection tank via the outgoing orange hose. The ingoing white hose was connected to the filter stands. The four white containers to the right for the filter stand were used to transfer water from "field buckets" to the 3 dl cups.

A simpler filtration was used during the field campaign in March. Instead of collecting, transporting, and then filtrating water, a peristaltic pump was used in combination with Sterivex filters (Bürcle, Vampire Sampler). This made it possible to filtrate in the field immediately after sampling with less handling. The water samples were collected using the same Ruttner sampler as used during the first campaign. After tapping the water into a bucket, it was pumped through the hose and further through a Sterivex filter (45 µm pore-size). After filtration, the Sterivex capsules was filled with ATL-buffer using a sterile syringe. Like the September samples, three replicates and one control from bottled/tap water were performed at each station. After filtrating one replicate, the hose and syringe was disposed, and the bucket plus the Luer lock were washed in chlorine and rinsed with water. Each filter-capsule was labelled and stored in individual zip-lock bags. The zip-lock bags were stored in a storage freezer at -24°C until extraction.

2.3. DNA-extraction – DNeasy Blood and Tissue Kit

All DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen).

Positive DNA-controls for PCR were prepared 19th of November 2021. DNA was extracted from tissue provided by a round goby specimen that was caught in Gothenburg. Using 25 mg tissue, the extraction was done according to the Qiagen "Purification of total DNA from animal tissues (spin-column protocol)". The three positive controls were made simultaneously (P1, P2 and P3). All positive controls were tested for optimum dilution and PCR conditions. A 1:100 dilution of P1 was used as positive control for all PCR assays.

DNA from the Gothenburg samples were extracted from 12th-13th October and from 14th-15th October, while DNA from the Oslo Fjord samples were extracted from 2nd-4th of May 2022.

The samples were always thawed at room temperature before any lab treatments. After thawing, 100µl Proteinase K was added to both the filter papers and Sterivex filters and incubated/lysed at 56°C overnight. DNA was extracted from the samples the following day according to the spin-column protocol. One main alteration was implemented to the protocol: instead of a starting sample volume of 100µl, it was changed to 1 ml. Any other alterations to the protocol were done to fit the new starting volume. Using 1 ml starting volume left ~0.6ml from the filter paper samples, and ~1ml from the Sterivex filter samples, as back up. When the protocol was completed, the final elution volume of 100µl was equally divided into two Eppendorf tubes, one for long storage at -20 °C and one for short storage at 4°C.

2.4. Qubit

Before performing PCR, the overall quantity of DNA (target and non-target) was measured using Quibit™. This was done according to the "DNA quantification using Qubit™ dsDNA HS Assay Kit" protocol (hereby referred to as the dsDNA protocol). Every sample was diluted according to the quantity of DNA in the samples. In this experiment, all samples with more than 5.00 nanograms per millilitre (ng/mL) were diluted 1:10. All samples from 2022 were diluted 1:2. Extraction controls were not diluted as these were not expected to contain target DNA. Dilutions was later considered when calculating copies/L in the field (see section 2.7.).

2.5. Primers and DNA-binding Dye

The primers used to detect the round goby were developed by Adrian-Kalchhauser & Burkhardt-Holm (2016). The primers were designed from the mitochondrial cytochrome B sequence which was retrieved from the NCBI database (Adrian-Kalchhauser and Burkhardt-Holm 2016). The primers were each 19 nucleotides long and were designed to amplify a fragment of 85 base pairs. In addition to designing the primers, Adrian-Kalchhauser & Burkhardt-Holm (2016) also tested primer-specificity against other Ponto-Caspian gobies, other goby species, and fish present in Switzerland. Mentionable examples of species which the primer-specificity was tested against is; *Pomatoschistus pictus* (Malm, 1865), *Aphia minuta* (Risso, 1810), *Pomatoschistus microps* (Krøyer, 1838), *Pomatoschistus minutus* (Pallas, 1770), *Gasterosteus aculeatus* Linnaeus, 1758, *Perca fluviatilis* Linnaeus, 1758, *Salmo trutta* Linnaeus, 1758 (Adrian-Kalchhauser and Burkhardt-Holm 2016).

Table 2.2: Primers used for PCR assay

Primer name	Primer sequence (5'-3')
SL_eDNA_NM_F1 (F1-primer)	TATGTGATGATCGGACAGC
SL_eDNA_NM_R1 (R1-primer)	GTTCTCTAGTCAGCTCGCT

The DNA-binding dye is one of the components in QX200™ ddPCR™ EvaGreen Supermix (Bio-Rad). The supermix is a universal mix that contains all components required for an EvaGreen-based ddPCR assay except for primers and of course the DNA template (Bio-Rad 2022). As the DNA-binding dye was included in the mix, no internal probe was used in this assay.

2.6. ddPCR

The master mix used for PCR was made according to standard procedure and consisted of: 11µl EvaGreen Supermix, 0.55µl F1-primer, 0.55µl R1-primer, 4.4µl ultrapure water, and 5.5µl of template. The samples were divided into around 15.000 droplets each using the BioRad QX200 droplet generator, which combined 20µl mastermix with 70µl BioRad droplet oil in a cartridge, creating 40µl of droplets.

Before the samples were analysed with ddPCR, different PCR conditions were tested to optimise the results. After testing multiple PCR-programs, the following program was used to amplify all samples:

Table 2.3: PCR-program used for amplification of all samples throughout the experiment.

Program consisted of 45 amplification cycles.		
Step	**Time (min:sec)**	**Temperature**
Denaturation	5:30	95°C
Annealing	1:00	58°C

After PCR, the droplets were analysed by a BioRad QX200 droplet reader. The droplet reader analysed the fluorescent light amplitude of each individual droplet. After reading, the distribution of droplets along and gradient of amplitude was possible to examine using either "QuantaSoft™" or "QX manager™. Environmental samples can in some cases have an unclear distribution of positive and negative droplets. This was the case for this experiment and a signal-threshold (purple line in Figure 2.3) were therefore put just above the values from the extraction control, to distinguish positive and negative droplets. Droplet distributions from all lab-samples are listed in Appendix A1-A7.

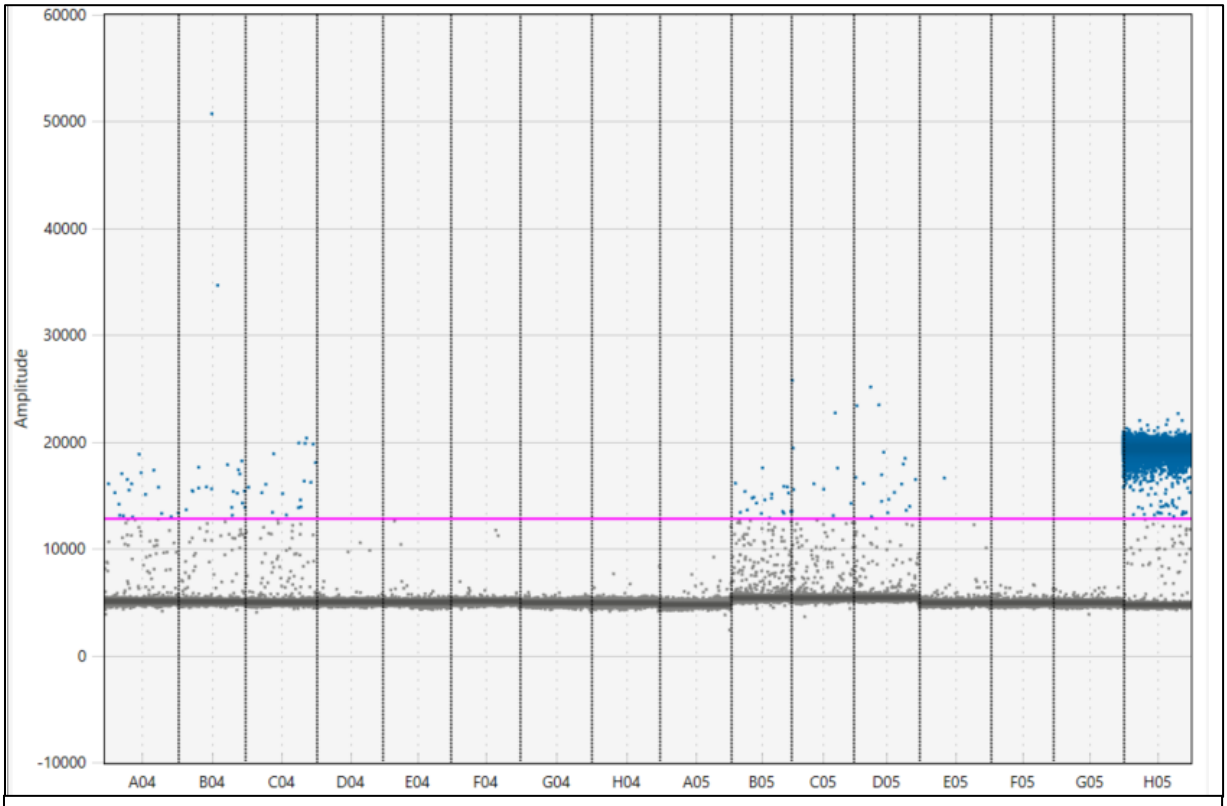

Figure 2.3: A screen capture from QXmanager™ illustrating how a signal threshold was placed. Y-axis measures amplitude and X-axis separates PCR-wells from each other. In this example the extraction control is G04-A05 and H05 is the positive PCR-control

2.7. eDNA Data Management

ddPCR gave an output on positive copies per µL of the master mix. This number was used to calculate copies per litre of water.

Copies/L ($C/_L$) were calculate using the following formula:

$$\frac{\frac{X_{dd} * V_{dd}}{V_{templ}} * V_{elu} * D_f}{V_{samp}} * \frac{V_{ATL}}{V_{ext}} * 1000 = C/_L$$

Where:

X_{dd}	Number of copies per µl in the ddPCR mix
V_{dd}	Volume of ddPCR mix (20µl for all samples)
V_{templ}	Volume of template in the ddPCR mix (5µl for all samples)
V_{elu}	Elution volume after extraction (100µl for all samples)
D_f	Dilution factor
V_{ATL}	Total volume of ATL-buffer
V_{ext}	Extraction volume (1ml for all samples)
V_{samp}	Volume of filtrated water

As V_{dd}, V_{templ}, and V_{elu} was unchanged for all samples. By multiplying V_{dd} and V_{elu} together and dividing by V_{templ} the formula can be shortened down to:

$$\frac{400 * X_{dd} * D_f}{V_{samp}} * \frac{V_{ATL}}{V_{ext}} * 1000 = C/_L$$

For the stations in Gothenburg V_{samp} was measured while doing filtrations. For the stations along the Oslo Fjord, V_{samp} was rounded off to 500ml or 1000ml due to lack of measuring equipment. 500ml was used for stations that had too muddy water to filtrate 1000 ml.

2.8. Revisiting Stations for Further Investigation.

After not catching the round goby during the first fishing campaign but still reading weak positive eDNA signals in Norway, a new fishing campaign was planned. Between 12th-14th of August 2022, further investigations and detection attempts were made around station 9, 16 and 17, in addition to a new station (Hvaler, station 10). Station 9 and 16 were chosen due to positive eDNA signals. Station 17 and 10 were chosen due to their proximity to 9 and 16. Notably, Station 10 is in close proximity to the border between Norway and Sweden, and there were hearsay rumours about sightings of the round goby here. As this sampling was no longer intended to be comparable between other stations, but a high intensity investigation, the fishing effort differed between the stations. This was due to some areas being of more interest than others.

Table 2.4: Overview of fishing effort at the different stations from campaign in August 2022.

Location	Station	No. Pots	No. Rods	Duration pots (h)	Duration rods (h)	Total time pots (h)	Total time rods (h)
Fredrikstad							
	9	3	4	21	0,5	63	2
	9.1	0	4	--	0,5	--	2
	9.2	2	4	40	0,5	80	2
	9.3	2	4	18	0,5	36	2
	9.4	0	4	--	0,5	--	2
	9.5	3	4	18	0,5	54	2
Hvaler							
	10	0	3	--	0,5	--	1,5
	10.1	0	1	--	0,5	--	0,5
	10.2	0	4	--	0,5	--	2
Sandvika							
	16	3	3	52	0,5	156	1,5
	16.1	0	3	--	0,5	--	1,5
Lysaker							
	17	0	2	--	0,5	--	1
	17.1	0	2	--	0,5	--	1

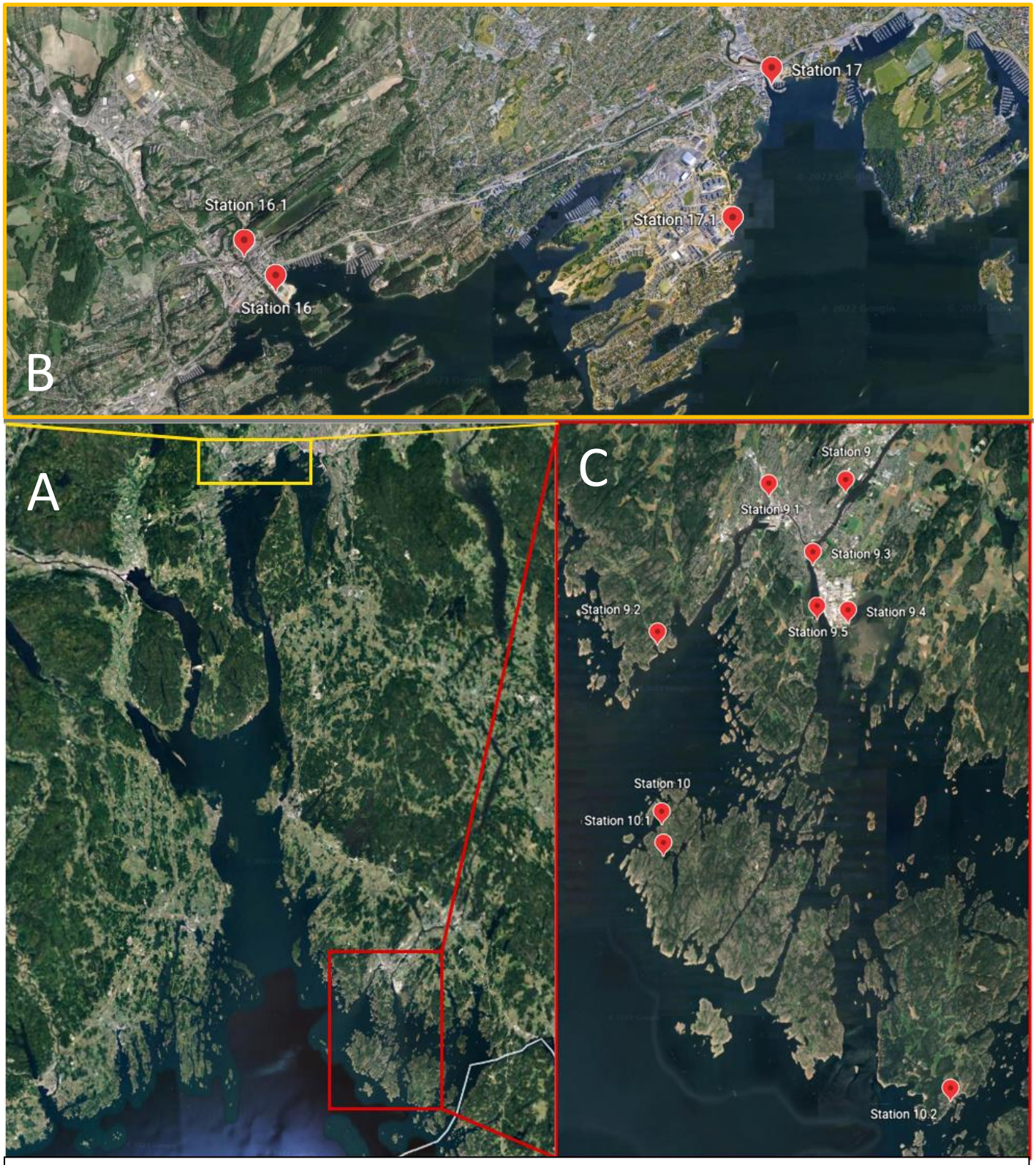

Figure 2.4:

A: Map of the Oslo Fjord. Off-white line in bottom right corner illustrates Norway-Sweden border. **B:** Map of Sandvika (16) and Lysaker (17). **C:** Map of Fredrikstad (9) and Hvaler (10)

3. Results:

3.1. Catch Data from September 2021 Field Campaign

We caught a total of ten round gobies in Gothenburg from three different stations: 1, 2, and 4 (see table 3.1). Seven of the round gobies were caught through angling, while the remaining three were caught with minnow traps. We did not manage to catch any round gobies with any of the fishing methods in the Oslo Fjord.

Table 3.1: Species caught during field campaign in September 2021

Station	*N. melanostomus*	*G. niger*	*Labidae*	*C. maenas*	*A. anguilla*	*G. aculeatus*	*P. adspersus*	Other
1	3	0	0	2	0	1	0	3
2	6	2	0	0	2	0	0	0
3	0	21	0	5	0	0	1	2
4	1	34	1	5	0	0	0	2
5	0	14	10	1	0	0	1	2
6	0	1	0	0	13	68	1	2
8	0	43	0	14	0	0	0	1
9	0	0	0	0	0	0	0	0
11	0	10	0	1	0	0	68	1
13	0	1	9	0	4	0	0	10
14	0	30	0	60	0	0	61	4
15	0	30	6	5	1	0	0	0
16	0	79	0	16	0	0	23	352*
17	0	60	12	2	0	0	0	0

* Catch was dominated by *Tritia reticulata* (Linnaeus, 1758).

3.2. eDNA Results and Statistical Significance

From eDNA-sampling, the amount of copies/L after amplification was calculated at each station (Figure 3.1). Figure 3.1 illustrates a clear difference in eDNA signal (Copies/L) between Sweden and Norway, where the five stations with strongest signals are all from Gothenburg. Måsholmen measured both the strongest single signal overall (≈49500 copies/litre), and the strongest mean signal over all (≈35500 copies/litre). The weakest mean signal overall was in Halden (≈570 copies/litre), while the strongest mean signal in the Oslo Fjord was in Sandvika (≈4370 copies/litre). This illustrates that there was a varying weak positive signal between the stations in Norway.

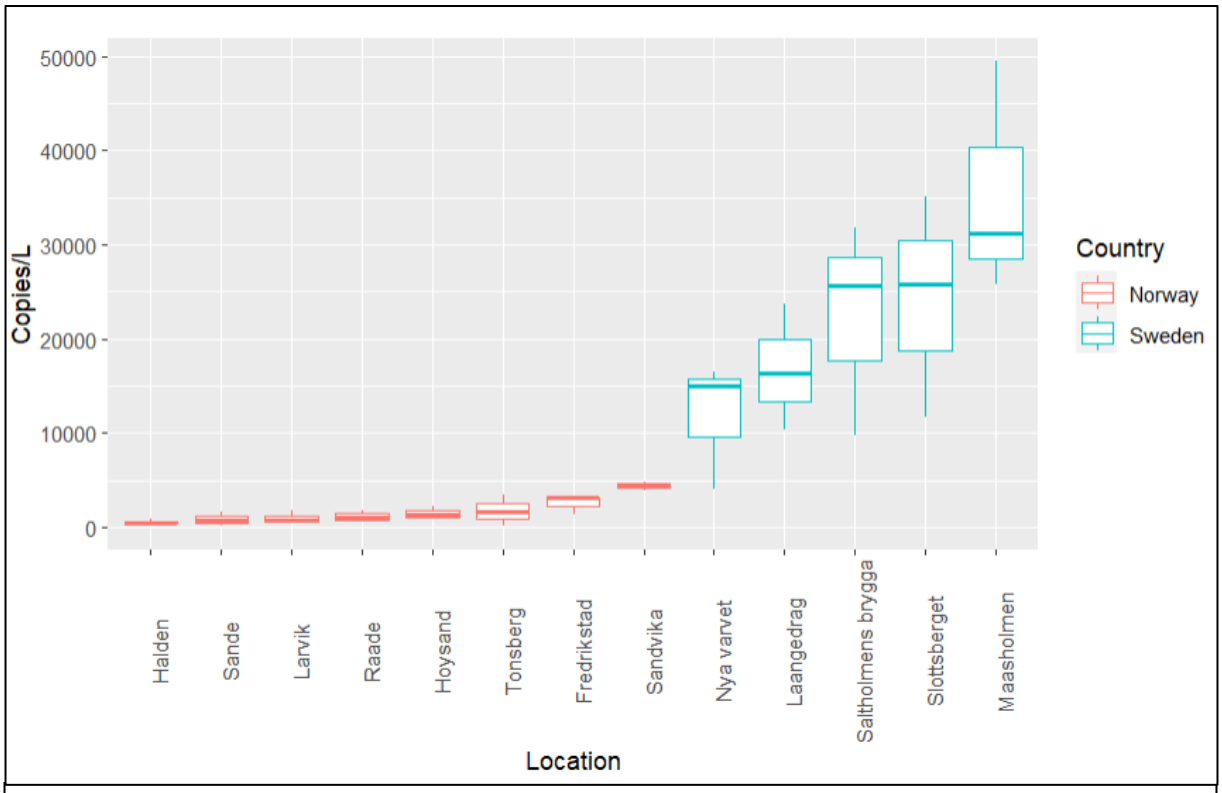

Figure 3.1: Boxplot showing distributions of mean eDNA signal per sample at each station. The Y-axis describes calculated number of copies per litre of habitat-water after amplification. The X-axis describes the stations and are sorted in increasing signal from left to right. The colours represent countries.

To further investigate the significant differences in eDNA signals between stations and between countries, the results were tested with a statistical model. In R the fit of four different linear models were tested by calculating the Akaike information criterion (AIC). The AIC test showed mod5 to be the best fit (see Figure 3.2). mod5 only described differences between Norway and Sweden, and mod2 was therefore used for statistical testing instead.

```
mod5 <- lm(copies_per_L ~ contry, data = plot_data)
mod4 <- lm(copies_per_L ~ location, data = plot_data)
mod2 <- lm(copies_per_L ~ contry*location, data = plot_data)
mod6 <- lm(copies_per_L ~ contry+location, data = plot_data)

AIC(mod5, mod4, mod2, mod6)
```

```
##      df      AIC
## mod5  3 809.4425
## mod4 14 805.5179
## mod2 14 805.5179
## mod6 14 805.5179
```

Figure 3.2: Results from the AIC test on mod5, mod4, mod2 and mod6.

An ANOVA-test was used on mod2 to investigate for significance both, between countries, and between stations. The ANOVA-test showed P-value= $3{,}095*10^{-10}$ (Table 3.2) between countries, meaning that the eDNA signal in Sweden is significantly stronger than in Norway. The P-value between stations was 0,0453, showing a significant difference in eDNA signals between stations. This shows that some stations are significantly different and further testing was performed to investigate where the significance derived from.

Table 3.2: ANOVA-test output from R studios. Showing P-value between countries, and P-value between stations.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**P-Value**
Country	1	3855035259	3855035259	96.418723	$3.095*10^{-10}$
Station	11	981293875	89208534	2.231205	0.0453
Residuals	26	1039537895	39982227	NA	NA

To investigate which stations had significantly stronger eDNA signals than others, all stations were tested against each other with a post-hoc test. All P-values from the post hoc test are listed in Appendix B. In summary, the post-hoc test showed that Slottsberget, Måsholmen and Saltholmens brygga were significantly stronger than all stations in the Oslo Fjord. Additionally, it illustrated that Måsholmen was significantly stronger than Nya varvet. The post-hoc did not show any significant difference between Nya varvet and stations in the Oslo Fjord nor between Långedrag and the Oslo Fjord stations.

As illustrated in Figure 3.3, some of the field controls have a weak eDNA signal (e.g., Måsholmen, Nya varvet, and Fredrikstad). It is common for field controls to have a weak signal, but it is important to distinguish this signal from true positive signals. To examine whether the eDNA signals in Norway simply were a result of "background noise" and small contaminations, all stations in Norway were statistically tested against the field controls using a linear model and ANOVA-test (Table 3.3).

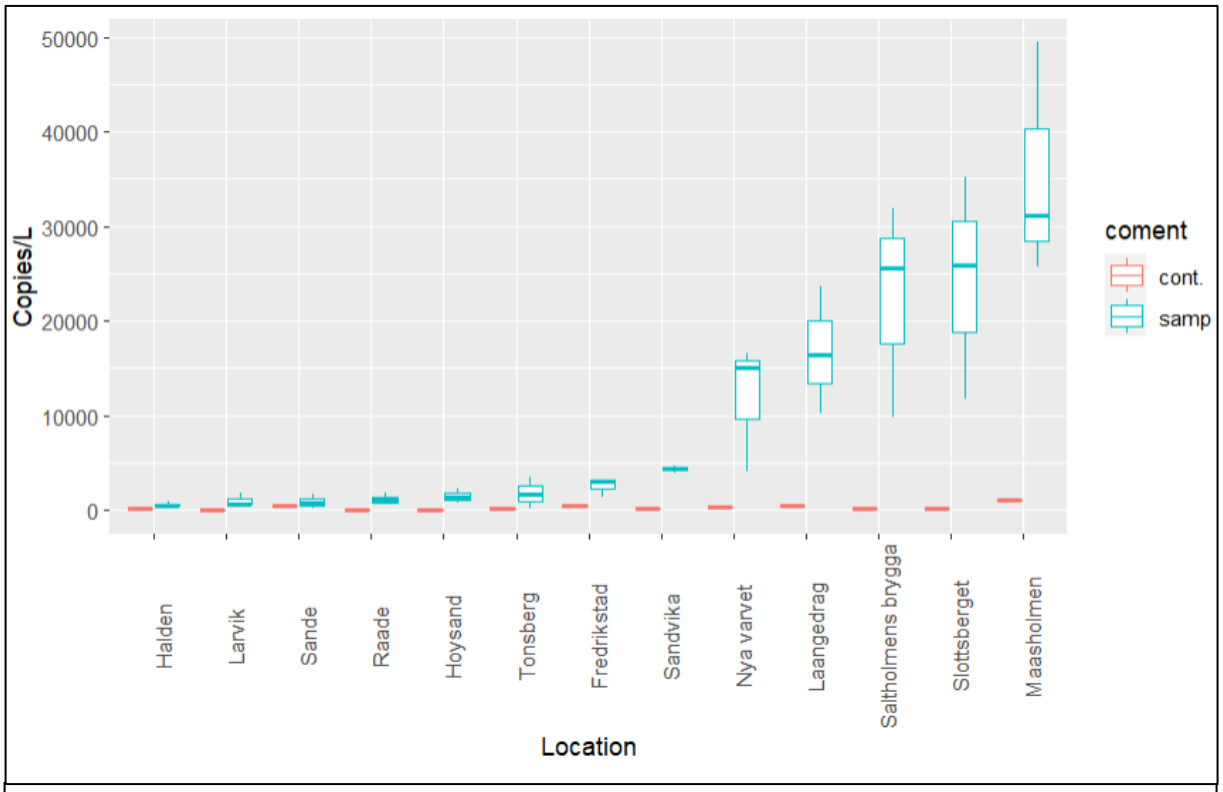

Figure 3.3: Illustrating differences between stations (samp), but also compared to with the negative field controls (cont.).

Table 3.3: ANOVA-test output from R studios. Showing P-value between field controls and stations. SampleID refers to where the sample was taken. All field controls had the same sampleID in this test.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
SampleID	8	46566387	5820798.4	10.76522	4e-06
Residuals	23	12436188	540703.8	NA	NA

The ANOVA-test showed that there was a significant difference between samples and field controls, meaning that the field controls were significantly weaker than the samples. To investigate between which stations the significance derived from, a summary in R studios was printed (Table 3.4).

Table 3.4: Summary output from R studios. Showing P-value between field controls and stations. "(Intercept)" describes P-value within field controls.

Predictors	*Estimates*	*CI*	*p*
(Intercept)	179.52	-358.28 – 717.33	0.497
Fredrikstad	2394.64	1364.83 – 3424.46	**<0.001**
Halden	391.69	-638.13 – 1421.50	0.439
Høysand	1319.47	289.66 – 2349.29	**0.014**
Larvik	808.60	-221.21 – 1838.42	0.118
Råde	972.23	-57.58 – 2002.05	0.063
Sande	675.27	-354.54 – 1705.09	0.188
Sandvika	4188.96	3159.14 – 5218.77	**<0.001**
Tønsberg	1634.89	605.08 – 2664.71	**0.003**
Observations	32		
R^2 / R^2 adjusted	0.789 / 0.716		

The summary shows that Fredrikstad, Sandvika, Tønsberg, and Høysand have significantly stronger eDNA signals than the field controls (intercept). Amongst these stations, Fredrikstad and Sandvika are the most significant stations ($P<0.001$). Both Fredrikstad and Sandvika were therefore revisited for further investigation.

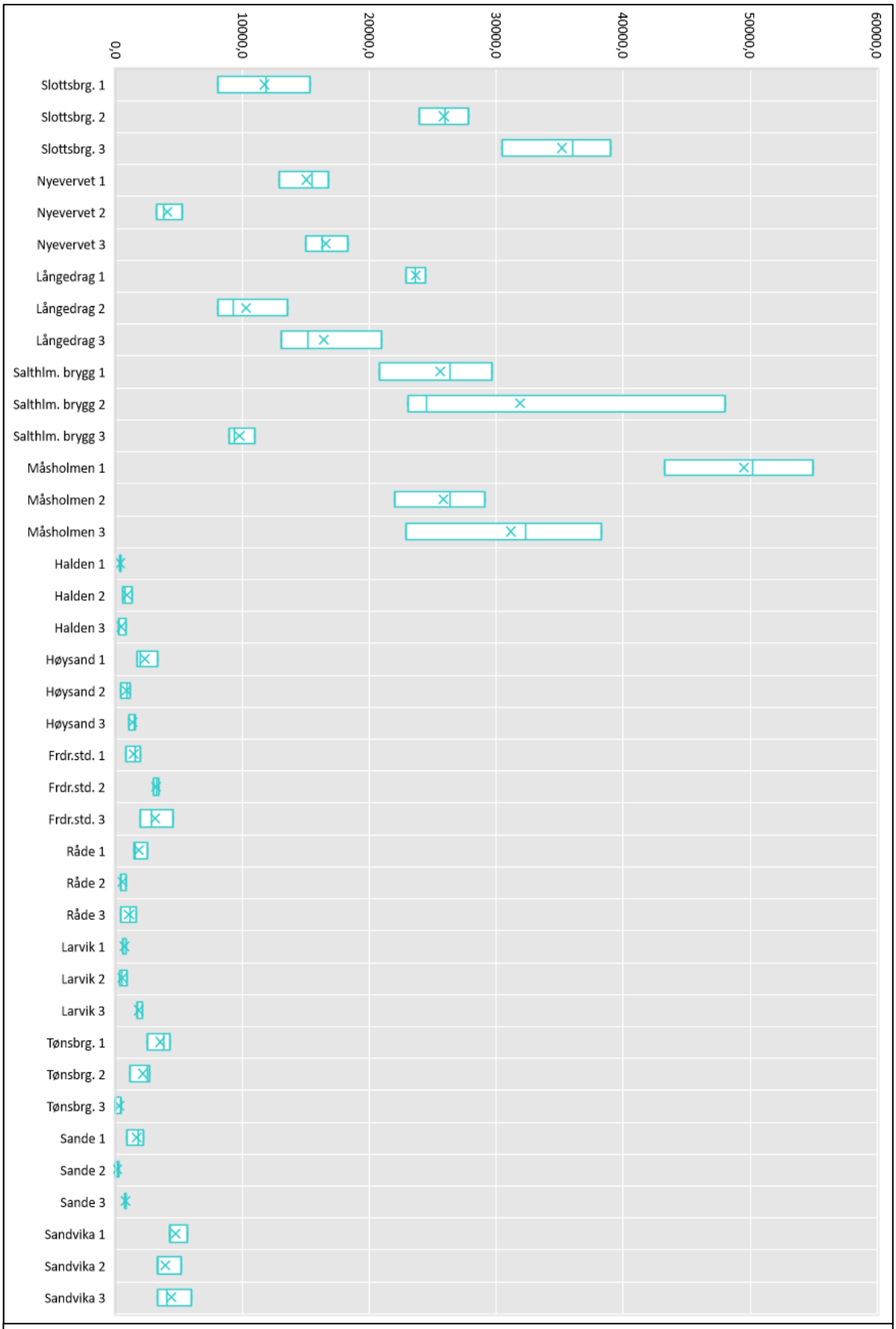

Figure 3.4: Box plot of all water samples before calculating mean of lab-triplicates. Illustrating the spread of signals within each sample.

Figure 3.4 shows variation between water samples taken on each station. Each box has a minimum (furthest to the left) and maximum value (furthest to the left), which shows the variation of signals in lab-triplicates from the same water sample. This figure shows that the samples from the Gothenburg stations varied significantly from sample to sample. Furthermore, lab triplicates from each sample did in some cases have a variation. The most extreme case of variation can be seen within the triplicates from "Saltholmens brygga 2" with a minimum value of 28 000 copies/L, and a maximum value of 48 000 copies/L. The variation both between samples and lab-triplicates is however more evident in the samples from Gothenburg than any of the other stations.

3.3. Catch Data from August 2022 Field Campaign

When revisiting stations of interest during the field campaign in August 2022, we still failed to detect the round goby using conventional methods. Notably, we caught the two first specimens of the alien *Hemigrapsus takanoi* (Asakura & Watanabe, 2005) on the eastern side of the Oslo Fjord at station 9.2 (See Table 3.5).

Table 3.5: Species caught during field campaign August 2022

Station	*G. niger*	*Labidae*	*C. meanas*	*A. anguilla*	*P. adsperus*	*H. takanoi*	Other
9	0	0	0	0	0	0	0
9.1	0	0	0	1	0	0	0
9.2	22	6	1	1	11	2	1
9.3	0	0	0	0	0	0	0
9.4	0	0	0	0	0	0	0
9.5	2	0	1	0	0	0	1
10	10	1	0	0	0	0	0
10.1	10	1	0	0	0	0	0
10.2	36	2	0	0	0	0	1
16	44	1	7	0	4	0	0
16.1	9	0	0	0	0	0	0
17	6	3	0	0	0	0	0
17.1	5	3	0	0	0	0	0

4. Discussion:

4.1. Findings

The main aim of this study was to investigate whether the round goby had been introduced in the Oslo Fjord. According to our eDNA results, some stations in the Oslo Fjord have significantly stronger signals than the negative controls from the field campaign. These results indicate that there is DNA from the round goby in the Oslo Fjord which implies that low densities of the round goby are introduced in Norway. The eDNA signals from the Oslo Fjord are significantly weaker than the signals from Gothenburg and should be interpreted as significantly lower abundance of round gobies. If the round goby is introduced to the Oslo Fjord this would not be the first instance where eDNA detects introduction of invasive fish prior to visual detection. Jerde et al. (2011) registered weak eDNA signals of the bighead carp (*Hypophthalmichthys nobilis* Richardson, 1845) in The Calumet River 8 months prior to visual detection.

The strongest mean eDNA signal in the Oslo Fjord was in Sandvika (≈4370 Copies/L) and Fredrikstad (≈2570 Copies/L). In a study similar to this project, comparing fishing methods and eDNA in Lake Michigan, eDNA signals in an area with documented round goby populations varied from approximately 3400-19500 copies/L (Nevers et al. 2018). The stations with weak positive signals in the Oslo Fjord should therefore be noted as areas of interest in future detection attempts. These areas of interest correspond with a study led by The Norwegian Institute for Nature Research (NINA), attempting to identify high-risk areas for introduction of the round goby (Forsgren and Hanssen 2022). The study modelled high-risk areas from factors provided from previous research such as: distance to nearest established population, distance to nearest international harbour, salinity, wave exposure, and sea temperature (Kotta et al. 2016, Florin et al. 2018). All our stations that gave weak positive eDNA signals in the Oslo Fjord aligns with their modelled high-risk areas.

eDNA-sampling with ddPCR is extremely sensitive to contamination as it is designed to detect eDNA at very low densities. Addressing the possibility of false positives are therefore in place. False positives can appear due to errors both in the field and in the lab. Factors that could cause false positives during this project are: contamination between stations or samples, sampling independent contamination (e.g., ballast water or faeces from predators), or vertical transport (Burian et al. 2021). Since there was used controls during the field campaign and during all lab

stages, contaminations between stations and samples are tested for and can be ruled out as a possible source of false positives. The nearest documented source of round goby eDNA that could vertically disperse to the Oslo Fjord, are in Sweden, more than 120 km away from Fredrikstad (Artportalen 2021). According to a study conducted using mesocosms, round goby eDNA half-life estimation are 15.85 hours (12°C) (Nevers et al. 2018). It would take well over 48 hours for eDNA to disperse to the Oslo Fjord (Appendix C), making this a highly unlikely scenario. Some of our stations were close to international harbours accommodating ships from areas with established round goby populations (Husa et al. 2022). Theoretically this could allow ballast water to contaminate an area with eDNA but considering the already mentioned half-life of round goby eDNA and ballast water treatment (i.e., UV-light) this would have to happen within a small window of time on several separate occasions.

We did not catch the round goby in the Oslo Fjord during our fishing campaigns, which can indicate no introduction. However, not finding something does not prove absence. A typical pattern for newly introduced species is a low initial abundance and a patchy distribution. Our results from the fishing campaigns might just illustrate that our efforts were not enough to detect the round goby if it follows this pattern. When comparing the catch results in Gothenburg with data found at www.artportalen.se, we caught fewer round gobies on all five stations totally than previous catch efforts in the same areas. For instance, only one day after we failed to catch any round gobies at station 3 (Långedrag), a different research group reported 48 specimens 40 meters downstream from our station (Artportalen 2021). Another example is at station 2 (Nya Varvet), where our efforts caught a total of 6 specimens and previous efforts (November 2019) have reported 27 specimens over the span of one day (Artportalen 2021). Prior to the project we investigated if minnow traps were a suitable catch method. We found previous work that successfully applied a similar fishing method to catch round gobies (Nevers et al. 2018). Nevers et al. (2018) used the same minnow traps, fished for 14-19 hours, and deployed the same number of traps at each station as in our project. In their campaign they caught between 0-7 round gobies per trap compared to our 0-2 per trap. It is worth mentioning that Nevers et al. (2018) study was constructed in Lake Michigan, where the species has been thriving since the 90s (Janssen and Jude 2001).

Our low catch success can also be explained by that our fishing efforts in Gothenburg were stationary, and that we only angled for 1 hour. There are many other fishing and detection methods that could have been implemented that are proven to increase detection success such as multi-mesh gill nets and hand-towed beach seines (Uspenskiy et al. 2021). These methods

are time consuming, demand more people and in some cases boats which, in this project, was not logistically possible. Since recently established populations tend to have low abundance and/or patchy distribution, we needed the ability to easily relocate stations and move around to cover larger areas. This was especially put in motion during the second fishing campaign, where fishing was focused on areas around the Oslo Fjord with stronger eDNA signal. Knowing that we missed populations that were approximately 40 meters away during our first campaign, a higher effort was put into moving along the stations to cover larger areas.

Another aim of this study was to further build upon the notion that eDNA is a useful tool when monitoring and detecting invasive marine species. According to our results, eDNA was a more successful detection method than the conventional methods that were used in this project. This is apparent in Gothenburg, where all stations were placed in accordance with earlier documented sightings. Out of five stations in Gothenburg, fishing methods only detected the round goby on three stations while eDNA detected the invader in all stations. This illustrates that even in areas where the round goby is well established, population patchiness does occur, and fishing methods might miss the patches and fail to detect the species. In cases like this, eDNA-sampling is a useful tool to investigate large areas of interest and can aid narrowing down areas. This aligns with previous research on eDNA-sampling of the round goby both connected to the Great Lakes and the Baltic Sea (Nevers et al. 2018, Sundberg et al. 2018, George et al. 2021). Our eDNA results cannot confidently draw any conclusion on abundance of round goby in neither Gothenburg nor the Oslo Fjord. While there is a clear pattern of stronger eDNA signals in Gothenburg, there is not enough catch data to check for correlation between eDNA signal and amount of caught fish. Furthermore, there are pronounced variations of eDNA signals from water-samples within the same station. This variation is less pronounced in samples from the Oslo Fjord, but there is no catch data to correlate the eDNA against.

4.2. Evaluating the Method

4.2.1. Different Filtration Methods

The filtration from September and the filtrations from March were performed differently, which makes the results from Gothenburg and the Oslo Fjord less comparable. Two different filters were used between the two localities; paper filters and Sterivex filters (see material and methods). When using varying material, it raises the question of to what degree this affects the results. Using paper filters is a so-called "open filtration" that requires more handling than using enclosed filtration (as with Sterivex filters). Using enclosed filtration has been argued to better preserve eDNA, due to capture, storage and extraction all taking place within an enclosed capsule (Spens et al. 2017). Enclosed filtration has been shown to provide slightly higher detection rates of eDNA than open filtration (Spens et al. 2017, Li et al. 2018). If we were to use enclosed filtration in Gothenburg as well, this would probably make the relative signal strength between Norway and Sweden slightly more differentiated. This however only enhances the findings we have already found to a negligible degree according to literature (Spens et al. 2017, Li et al. 2018)

Another difference between filtrations in September and March is the time of the year. One concern for the project was that sampling in the end of March would show no results regardless of introduction, due to winter migration. Considering that studies from Denmark demonstrated however that some round gobies spend winters in estuaries and rivers (Christoffersen et al. 2019). Receiving eDNA signals in late March should therefore be expected. That being said, Christoffersen et al. (2019) suggests that a higher abundance and activity of round gobies (hence, more eDNA) is expected in rivers and estuaries during summer. Notably, round gobies are expected to spawn in the temperature range of 9-26°C (Charlebois et al. 1997). These two arguments should imply that our eDNA signals from the Oslo Fjord might be unrepresentatively weak compared to samples collected from Gothenburg in September.

4.2.2. Variations in Samples and Lab-triplicates

As illustrated in Figure 3.4, there were clear variations between water samples within stations. At the Gothenburg stations all water samples gave strong positive results. However, as Figure 3.4 illustrates the variation within samples from the same station were at times larger than the variation between stations. For instance, at station 1 all water samples (Slottsbrg. 1,2 and 3 in figure 3.4) were significantly different. Moreover, all the stations in Gothenburg have at least one sample that is significantly different from the other samples from the corresponding station. Which one of the three samples that are significantly different from the others seems to be random for each station. As sample no. 1 was always collected first and always filtrated through cup no.1 in the filtration set-up, this seems to be caused by something else than methodical errors. In a method that is performed similarly between all stations, a methodical error would likely create a pattern in the results.

The variation between lab-triplicates within the same sample is also illustrated in figure 3.4. This variation is interpreted from the box-lengths for each sample. While the variation in some lab-triplicates is negligible, there are extreme cases such as Saltholmens brygga 2 where copies/L spans from ca. 48 000 copies/L to 23 000 copies/L. As all samples should be homogeneous after filtration, this variation must have happened in the lab through either uneven mixing of template or the mixing of PCR mastermix prior to the droplet generation.

4.2.3. ddPCR and Data Management

ddPCR results ideally illustrate two clear clouds (one with negative droplets and one with positive droplets) with as few intermediate droplets between these clouds as possible (e.g., appendix A1). This makes it easy to differentiate positive and negative droplets when placing a threshold manually. This project struggled with intermediate droplets (commonly referred to as “rain”) arising from the negative clouds. Rain can be caused by a variety of factors, and can therefore be a challenging problem to tackle (Kokkoris et al. 2021). Examples of technical factors that can cause rain are variation in droplet size and fragmentation of DNA during extraction or storage (Meijerink et al. 2001, Dobnik et al. 2018). Rain can also occur when inhibitors, such as humic acids, delay or reduce amplification efficiency (Dingle et al. 2013, Kokkoris et al. 2021).

Although it was difficult to know which factors caused the rain specifically, several measures were considered to reduce the amount. According to Witte et al. (2016), increasing the number of PCR cycles, as well as annealing and elongation times, can reduce rain. As increasing annealing time and elongation time would be too time-consuming, increased cycles were the only measure applied to the assay. While this measure reduced the amount of rain, there are still samples where rain is pronounced (see appendix A). This made it difficult to read any apparent patterns between positive and negative clouds. With an apparent pattern, the threshold would normally be set just below the positive cloud, when it's not apparent, the threshold is set close to the negative control cloud to include all partially inhibited droplets (Dingle et al. 2013). In appendix A it is illustrated how all the thresholds were set according to Dingle et al. (2013).

4.2.4. Primer-specificity

As mentioned in section 2.5, the primers used in this experiment has been tested against several Ponto-Caspian gobies, other goby species, and Swiss native fish species (Adrian-Kalchhauser and Burkhardt-Holm 2016). In their paper, Adrian-Kalchhauser and Burkhardt-Holm (2016) notes that the specificity test is performed on concentrated and pure DNA, and that primers may show different specificity in environmental samples. It should also be noted that Adrian-Kalchhauser and Burkhardt-Holm (2016) did not test the primers against marine species and the primers are therefore not tested against e.g., black gobies. The black goby is a species native to Norway which is taxonomically closely related to the round goby (Thacker 2015). In 2018 the Norwegian Institute for Water Research (NIVA) performed an extensive study on primer-specificity for numerous non-indigenous species (Andersen et al. 2018). Their study tested two different sets of primers for round gobies: SL_eDNA_NM (used in our project) and Neo_Mel_COI (Andersen et al. 2018). While Andersen et al. asserts to have tested both assays, they only present the results from the Neo_Mel_COI primer-set and mentions that SL_eDNA_NM was not used in the final analyses of water samples. In other words, the primers used in this project has no published specificity test data against marine species in water samples. We did however run our primers in PrimerBLAST (See Appendix E for details) and found only two matching results *in silico*: *Neogobius melanostomus, Apollonia melanostoma* (previous scientific name for round goby and thus an older synonymous name for the same species). This strengthens the reliability of the primers we used but the results would benefit from a test with tissue-samples and in enviormental conditions.

4.3. Conclusion and Future Research

This project has provided indications that the round goby is present in the Oslo Fjord through our eDNA results. We found four stations around the Oslo Fjord with weak, but significant, positive eDNA signals. One of these is within, and one is near, earlier documented signal range found in established round goby populations. Even though we focused our last fishing campaign at and around stations with the strongest eDNA signal, we did not manage to catch the round goby. This indicates that the alleged introduction of round goby exists with low abundance and/or is patchy distributed.

This project has yet again proven eDNA-sampling as an asset for detecting the round goby, as we got strong signals in all our stations in Gothenburg. While many previous studies on the topic rightfully claim to find linear correlations between eDNA signal and round goby, this project has not the sufficient data to draw any conclusions about this.

How the introduction of round goby will affect Norwegian rivers, lakes and marine systems that are in both economical and conservational interest is hard to predict. Therefore, it should be an incentive for early detection to enable early mapping of impacts and planning of measures that limit the spread of round gobies. Future efforts to detect the round goby should consist of higher fishing efforts focused on stations with positive eDNA signals. More eDNA-sampling should also be executed to narrow down the search areas. To improve the chances of detecting eDNA, sampling should be performed between late spring and early autumn, as it is expected higher abundance of round gobies in the sea and a higher degree of released eDNA during this period. As mentioned in section 4.2.4., if the primers used in this project are to be applied in future research, primer-specificity should be tested against marine species like the black goby *in vitro* to ensure results of high quality. Alternatively, future research should consider the Neo_Mel_COI primers if the SL_eDNA_NM primers proves to be less reliable for marine environments.

During the project we experienced a lack of standardised protocols across eDNA research. To improve the quality of research, there should be agreed upon standardisations that would make studies more comparable. This is particularly important for neighbouring countries/countries with similar invasion risk, where comparable data can better help monitoring and detection attempts.

5. Literature

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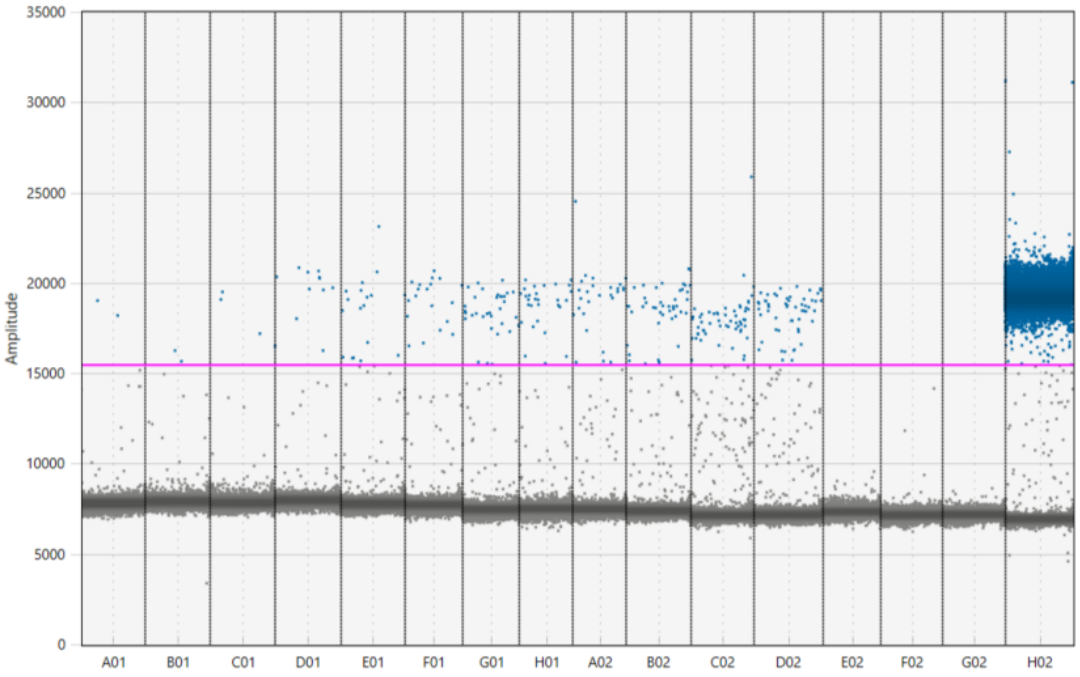
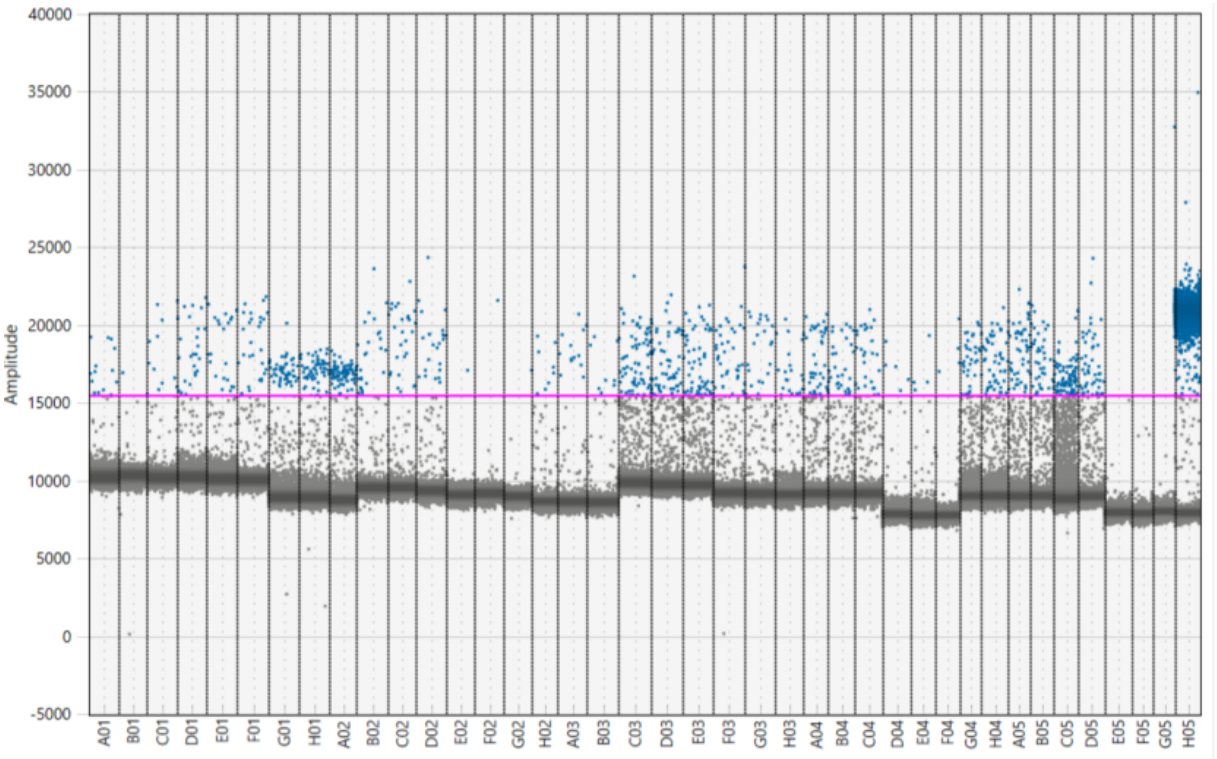
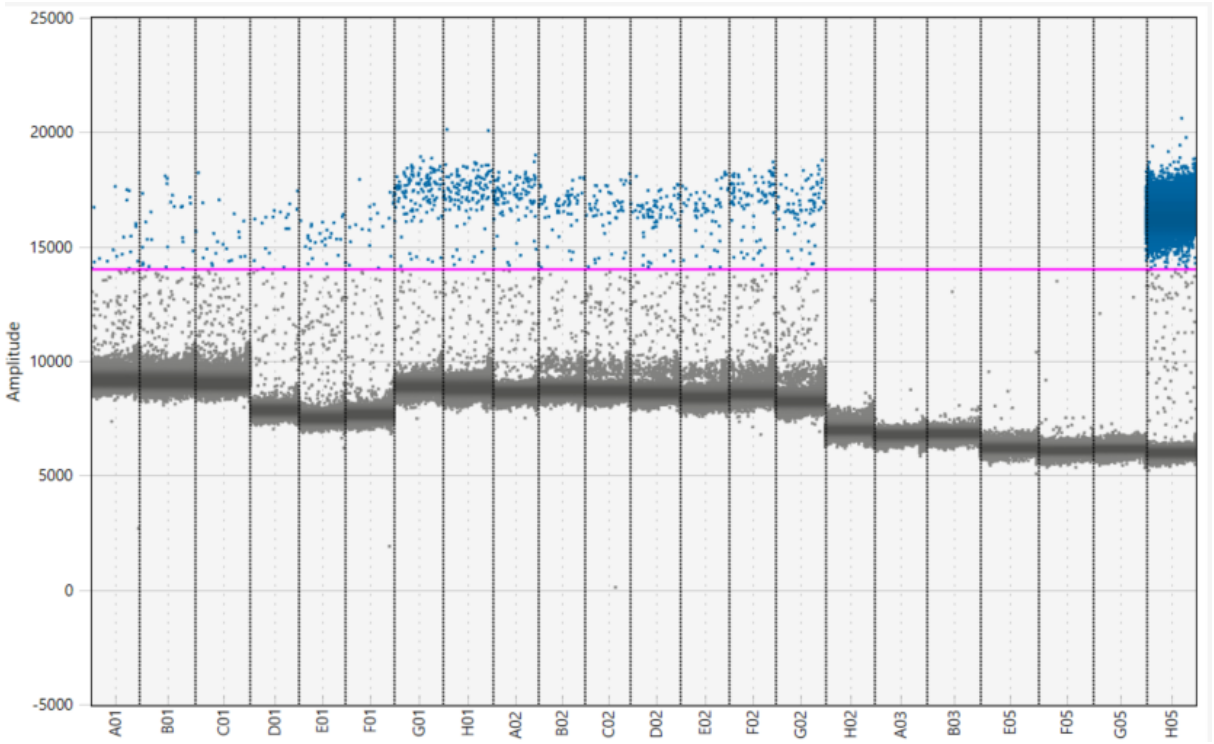
Appendix A – All ddPCR Outputs With Thresholds



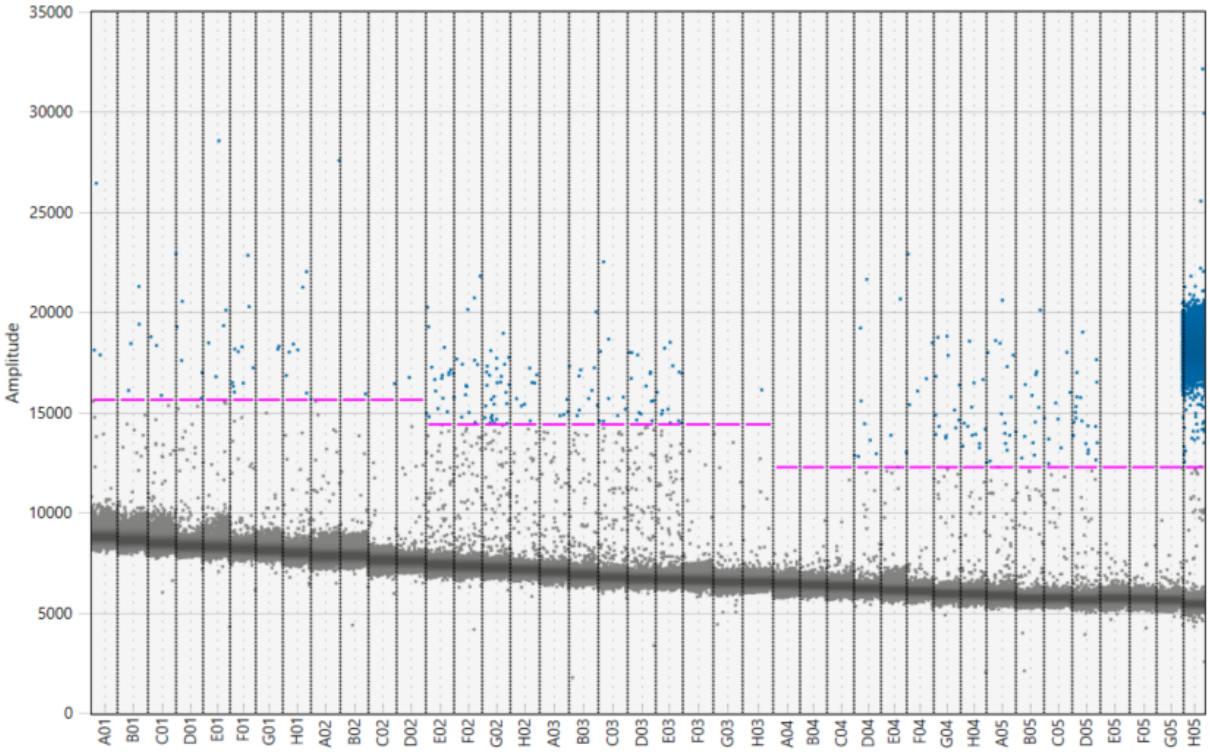
Appendix A1: Lab triplicates from PCR sample 1-4 (A01-D02) with negative PCR control triplicate (E02-G02) and Positive PCR control (H02).



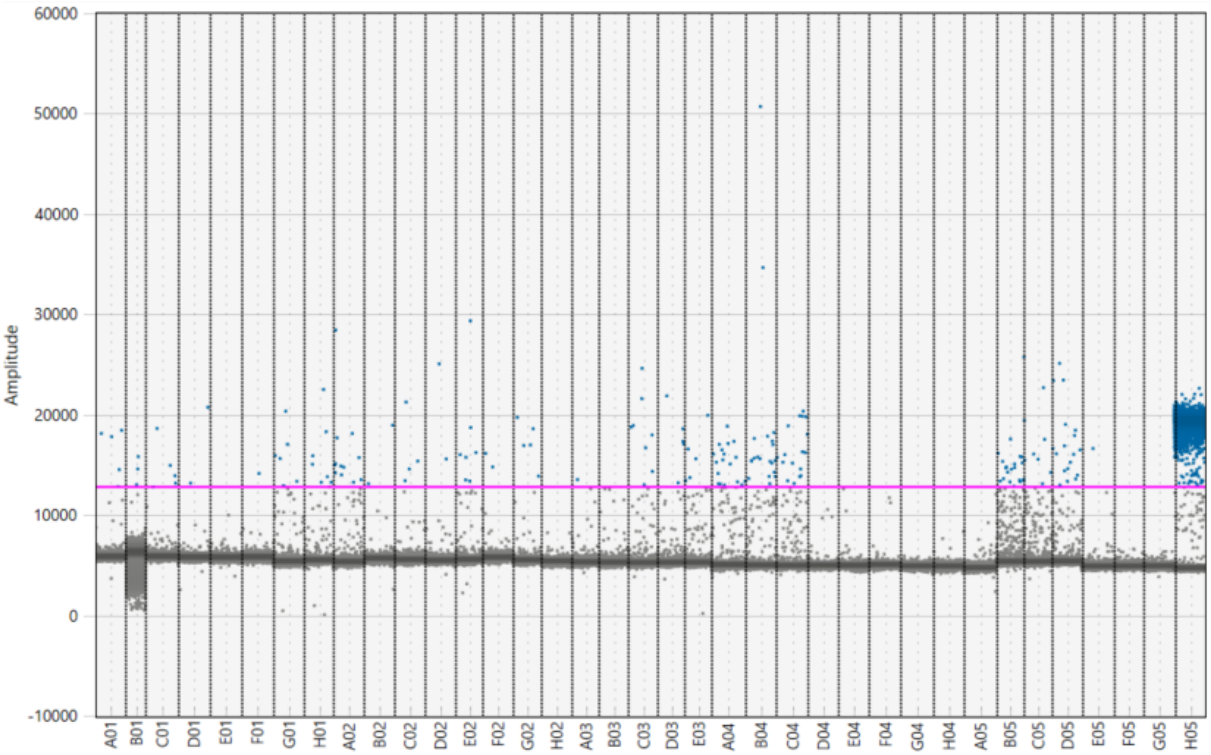
Appendix A2: Lab triplicates from PCR sample 5-16 (A01-D05) with negative PCR control triplicate (E05-G05) and Positive PCR control (H05). Note: E02-G02 is extraction controls and were labelled “sample 9”



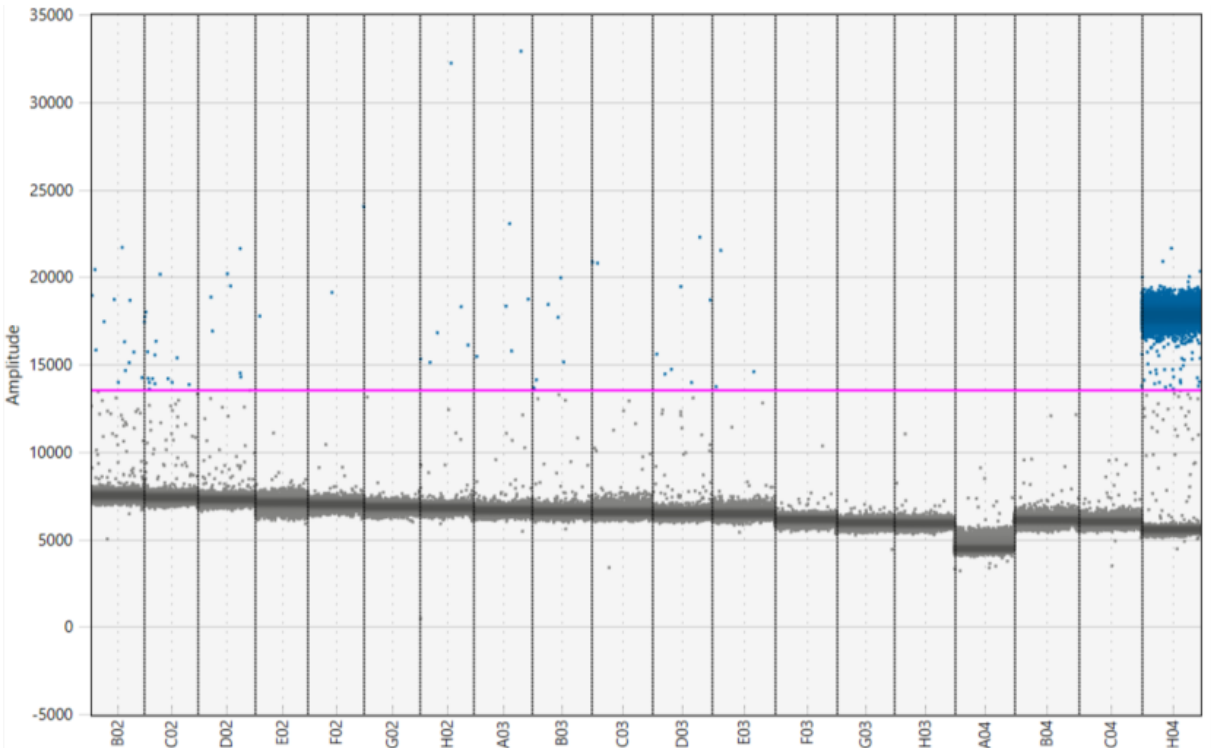
Appendix A3: Lab triplicates from PCR sample 17-22 (A01-B03) with negative PCR control triplicate (E05-G05) and Positive PCR control (H05). Note: H02-B02 is extraction controls and were labelled "sample 22"



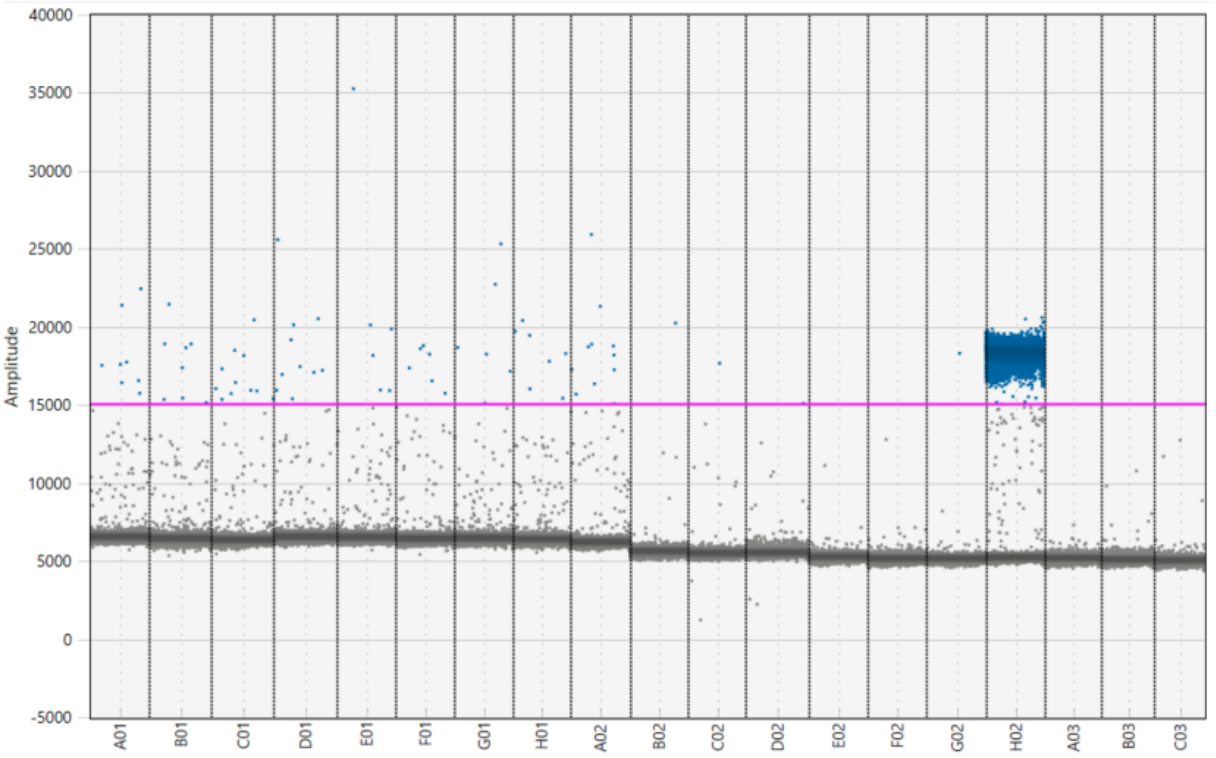
Appendix A3: Lab triplicates from PCR sample 23-34 (A01-D05) with negative PCR control triplicate (E05-G05) and Positive PCR control (H05). Note: A04-C04 is extraction controls and were labelled "sample 31". The threshold here was adjusted after the clear decline in amplitude from A01-H05. The highest threshold is set at Halden – Field control. The second threshold is set at Høysand – field control. The third is set at an extraction control.



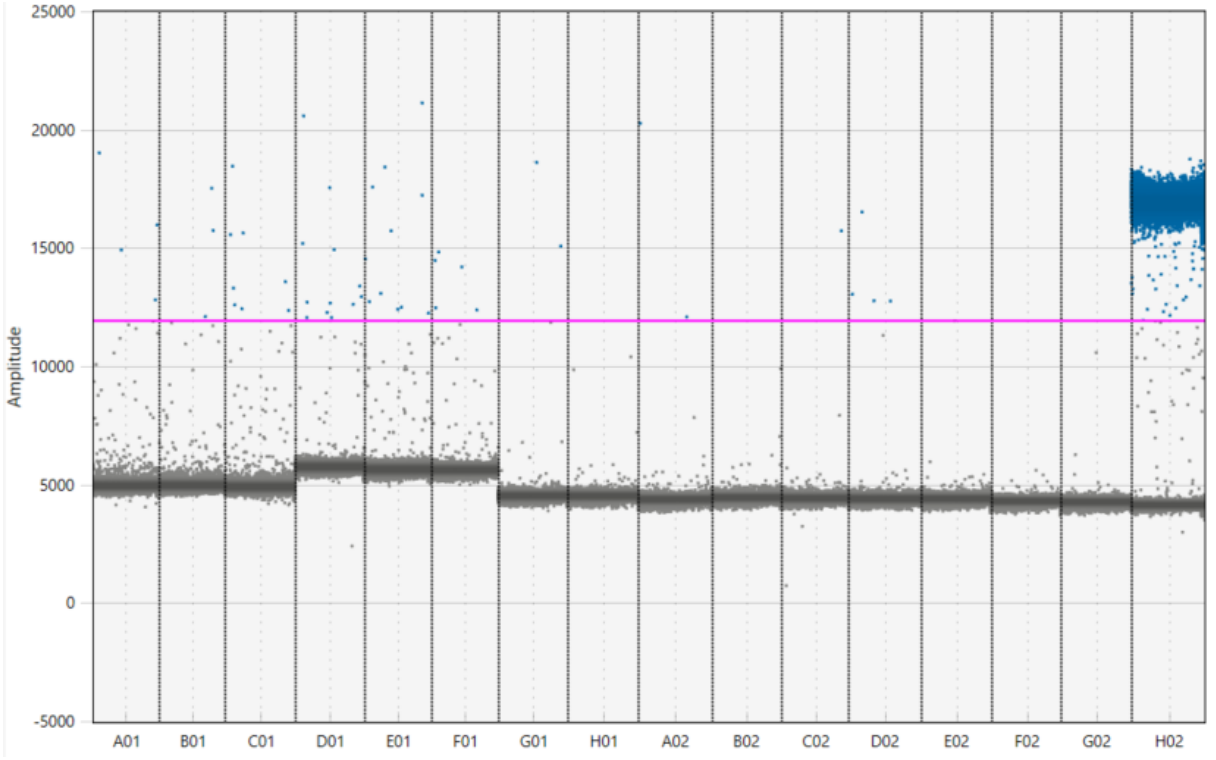
Appendix A4: Lab triplicates from PCR sample 35-46 (A01-D05) (except samp. 42 due to no call) with negative PCR control triplicate (E05-G05) and Positive PCR control (H05). Note: D01-F01 + G04-A05 is extraction controls and were labelled "sample 36" and "sample 45" respectively.



Appendix A5: Lab triplicates from PCR sample 50-54 (B02-H03) with negative PCR control triplicate (A04-C04) and Positive PCR control (H04). Note: F03-H03 is extraction controls and were labelled "sample 54".



Appendix A6: Lab triplicates from PCR sample 55-59 (A01-G02) with negative PCR control triplicate (A03-C03) and Positive PCR control (H02). Note: E02-G02 is extraction controls and were labelled “sample 59”.



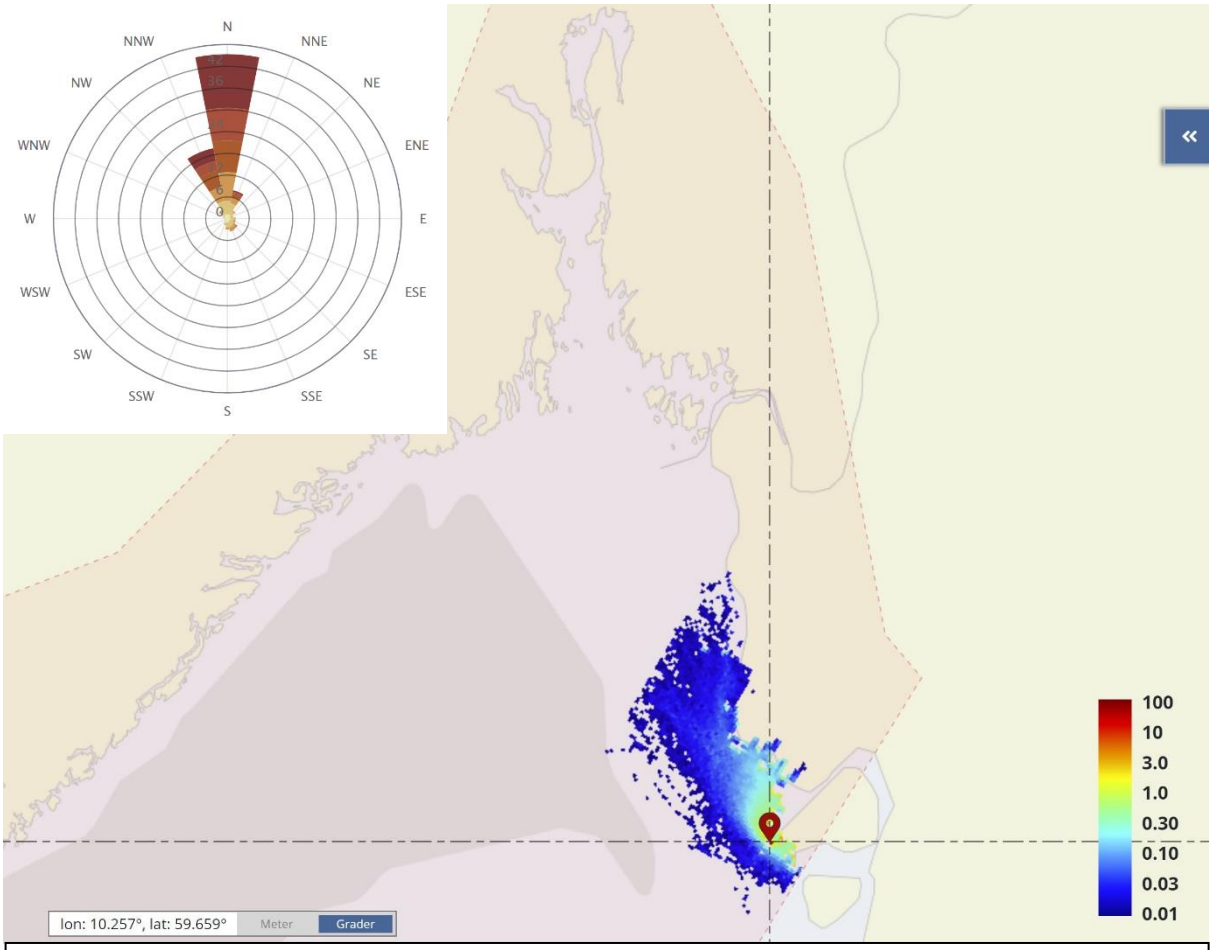
Appendix A7: Lab triplicates from PCR sample 42 (A01-C01) and 47-49 (D01-D02) with negative PCR control triplicate (E02-G02) and Positive PCR control (H02).

Appendix B – P-values Between All Stations (P<0.05 in bold)

Contrast	P-value
Fredrikstad Norway - Halden Norway	1.0000
Fredrikstad Norway - Hoysand Norway	1.0000
Fredrikstad Norway - Larvik Norway	1.0000
Fredrikstad Norway - Raade Norway	1.0000
Fredrikstad Norway - Sande Norway	1.0000
Fredrikstad Norway - Sandvika Norway	1.0000
Fredrikstad Norway - Tonsberg Norway	1.0000
Fredrikstad Norway - Laangedrag Sweden	0.2818
Fredrikstad Norway - Maasholmen Sweden	**0.0001**
Fredrikstad Norway - Nya Varvet Sweden	0.8302
Fredrikstad Norway - Saltholmens brygga Sweden	**0.0315**
Fredrikstad Norway - Slottsberget Sweden	**0.0135**
Halden Norway - Hoysand Norway	1.0000
Halden Norway - Larvik Norway	1.0000
Halden Norway - Raade Norway	1.0000
Halden Norway - Sande Norway	1.0000
Halden Norway - Sandvika Norway	0.9999
Halden Norway - Tonsberg Norway	1.0000
Halden Norway - Laangedrag Sweden	0.1401
Halden Norway - Maasholmen Sweden	**<.0001**
Halden Norway - Nya Varvet Sweden	0.6038
Halden Norway - Saltholmens brygga Sweden	**0.0127**
Halden Norway - Slottsberget Sweden	**0.0053**
Hoysand Norway – Larvik Norway	1.0000
Hoysand Norway - Raade Norway	1.0000
Hoysand Norway - Sande Norway	1.0000
Hoysand Norway - Sandvika Norway	1.0000
Hoysand Norway - Tonsberg Norway	1.0000
Hoysand Norway - Laangedrag Sweden	0.1965
Hoysand Norway - Maasholmen Sweden	**<.0001**
Hoysand Norway - Nya Varvet Sweden	0.7159
Hoysand Norway - Saltholmens brygga Sweden	**0.0194**
Hoysand Norway - Slottsberget Sweden	**0.0082**
Larvik Norway - Raade Norway	1.0000
Larvik Norway - Sande Norway	1.0000
Larvik Norway - Sandvika Norway	1.0000
Larvik Norway - Tonsberg Norway	1.0000
Larvik Norway - Laangedrag Sweden	0.1636

Larvik Norway - Maasholmen Sweden	**<.0001**
Larvik Norway - Nya Varvet Sweden	0.6550
Larvik Norway - Saltholmens brygga Sweden	**0.0153**
Larvik Norway - Slottsberget Sweden	**0.0064**
Raade Norway - Sande Norway	1.0000
Raade Norway - Sandvika Norway	1.0000
Raade Norway - Tonsberg Norway	1.0000
Raade Norway - Laangedrag Sweden	0.1736
Raade Norway - Maasholmen Sweden	**<.0001**
Raade Norway - Nya Varvet Sweden	0.6748
Raade Norway - Saltholmens brygga Sweden	**0.0165**
Raade Norway - Slottsberget Sweden	**0.0069**
Sande Norway - Sandvika Norway	1.0000
Sande Norway - Tonsberg Norway	1.0000
Sande Norway - Laangedrag Sweden	0.1558
Sande Norway - Maasholmen Sweden	**<.0001**
Sande Norway - Nya Varvet Sweden	0.6387
Sande Norway - Saltholmens brygga Sweden	**0.0144**
Sande Norway - Slottsberget Sweden	**0.0060**
Sandvika Norway - Tonsberg Norway	1.0000
Sandvika Norway - Laangedrag Sweden	0.4708
Sandvika Norway - Maasholmen Sweden	**0.0001**
Sandvika Norway - Nya Varvet Sweden	0.9533
Sandvika Norway - Saltholmens brygga Sweden	**0.0681**
Sandvika Norway - Slottsberget Sweden	**0.0306**
Tonsberg Norway - Laangedrag Sweden	0.2192
Tonsberg Norway - Maasholmen Sweden	**<.0001**
Tonsberg Norway - Nya Varvet Sweden	0.7518
Tonsberg Norway - Saltholmens brygga Sweden	**0.0224**
Tonsberg Norway - Slottsberget Sweden	**0.0095**
Laangedrag Sweden - Maasholmen Sweden	0.0512
Laangedrag Sweden - Nya Varvet Sweden	0.9987
Laangedrag Sweden - Saltholmens brygga Sweden	0.9953
Laangedrag Sweden - Slottsberget Sweden	0.9555
Maasholmen Sweden - Nya Varvet Sweden	**0.0055**
Maasholmen Sweden - Saltholmens brygga Sweden	0.3936
Maasholmen Sweden - Slottsberget Sweden	0.6151
Nya Varvet Sweden - Saltholmens brygga Sweden	0.7033
Nya Varvet Sweden - Slottsberget Sweden	0.4770
Saltholmens brygga Sweden -Slottsberget Sweden	1.0000

Appendix C – Model of How a Passive Particle Would Disperse Over 48 Hours from the Nearest Documented Source of Round Goby eDNA.



Appendix C1: Figure made by Vivian Husa, data retrieved from the Norwegian Current Information System (IMR 2020).

A: Rose diagram showing strong currents Northwards and Nort westwards outside Orust

B: Dispersal map illustrating how a passive particle would disperse over 48 hours under conditions described in the rose diagram.

Appendix D – Overview for Qubit-values

Lo = values too low to detect,

FiC = Field control

ExC = Extraction control

P-1, -2 and, -3 = Tissue samples

Sample	Value	Sample (cont.)	Value (cont.)
1 (FiC)	0,378	32	0,448
2	6,05	33	0,688
3	7,46	34	0,988
4	9,92	35 (FiC)	0,0524
5 (FiC)	0,516	36 (ExC)	Lo
6	6,25	37	0,816
7	4,8	38	0,0716
8	5,93	39	0,66
9 (ExC)	Lo	40 (FiC)	0,0332
10 (FiC)	0,607	41	0,54
11	21,7	42	1,01
12	11,8	43	1,3
13	15,4	44 (FiC)	0,0532
14 (FiC)	0,42	45 (ExC)	Lo
15	19,1	46	3,58
16	18,4	47	4,28
17	16,7	48	0,336
18 (FiC)	1,6	49 (FiC)	0,42
19	5,93	50	2,14
20	21,4	51	0,284
21	16,1	52	0,536
22 (ExC)	Lo	53 (FiC)	0,792
23	0,282	54 (ExC)	Lo
24	1,34	55	13,6
25	1,09	56	11,6
26 (FiC)	0,0376	57	13,6
27	2,6	58 (FiC)	0,584
28	1,2	59 (ExC)	Lo
29	3,06	P-1	7,65
30 (FiC)	0,0576	P-2	12
31 (ExC)	Lo	P-3	17,8

Appendix E – BLAST Search parameters and other details

Search parameter name	**Search parameter value**
Number of Blast hits analysed	3090
Entrez query	
Min total mismatches	2
Min 3' end mismatches	2
Defined 3' end region length	5
Mismatch threshold to ignore targets	6
Max target size	4000
Max number of Blast target sequences	50000
Blast E value	30000
Blast word size	7
Max candidate primer pairs	500
Min PCR product size	58
Max PCR product size	1000
Min Primer size	15
Opt Primer size	20
Max Primer size	25
Min Tm	57
Opt Tm	60
Max Tm	63
Max Tm difference	3
Repeat filter	AUTO
Low complexity filter	Yes