UNIVERSITY OF GOTHENBURG

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THE USE OF REGURGITATED PELLETS FROM THE GREAT CORMORANT TO DETECT THE PRESENCE OF THE ROUND GOBY

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Photo of a great cormorant colony taken by: Karl Lundström (SLU)

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ABSTRACT

The round goby (*Neogobius melanostomus*) is an invasive alien species that poses one of the greatest threats to biodiversity and ecosystem services in Sweden today. The round goby originates from the Black Sea and Caspian Sea but has in recent decades rapidly spread with global shipping traffic to new areas in Europe and North America. To protect indigenous species and habitats there is a need for early detection methods of round goby. The great cormorant (*Phalacrocorax carbo*) is despised by the public because it competes with the fishing industry and its feces kills the vegetation at its breeding sites. However, the great cormorant can potentially provide valuable information regarding the spread of invasive fish species. The purpose of this pilot project is to investigate whether the regurgitated pellets of great cormorants can be used to detect the presence of the invasive alien species round goby. Regurgitated pellets were collected from Medholmarna and Norra Röden. The QIAGEN DNeasy Blood & Tissue Kit protocol was followed to lyse and extract DNA, while the Qubit fluorometer and digital PCR (dPCR) were used to detect and quantify the amount of round goby DNA. The DNA concentration in the pellets from Medholmarna ranged from 14.4 ng/µl to 194 ng/µl (mean: 68.395 ng/µl), compared to 14.4 ng/µl to 150 ng/µl (mean: 58.885 ng/µl) from Norra Röden. In the samples from Medholmarna, six out of ten pellets contained round goby DNA with an average concentration of 0.295 (± 0.305) DNA copies per µl. In the samples from Norra Röden, eight out of nine pellets contained round goby DNA with an average concentration of 32813 (± 83257) DNA copies per µl. The difference in round goby DNA concentration between the sites suggests that the round goby is more established in the area around Norra Röden than around Medholmarna. The results from this project indicate that the QIAGEN DNeasy Blood & Tissue Kit, Qubit fluorometer, and dPCR are three practical tools to analyze dietary samples and that great cormorant pellets can be used as an additional method to detect the presence of the round goby. Large-scale projects and analysis of great cormorant pellets can provide important information for future conservation efforts and the protection of marine ecosystems.

Keywords: Invasive species, Regurgitated pellets, Great cormorant, Round goby, dPCR

SAMMANFATTNING

Den svartmunnade smörbulten (*Neogobius melanostomus*) är en invasiv främmande art som utgör ett av de största hoten mot biologisk mångfald och ekosystemtjänster i Sverige idag. Den svartmunnade smörbulten härstammar från Svarta havet och Kaspiska havet men har de senaste decennierna spridit sig med hjälp av fartygstrafik till nya områden i Europa och Nordamerika. På grund av den svartmunnade smörbultens negativa effekt på inhemska marina ekosystem är behovet av metoder för tidig upptäckt av stort intresse. Storskarven (*Phalacrocorax carbo*) är en sjöfågel som är illa omtyckt av allmänheten eftersom dess avföring förstör vegetationen där den häckar och på grund av dess skador på fångst och fiskeredskap. Storskarven kan dock potentiellt tillhandahålla värdefull information kring spridningen av invasiva fiskarter. Syftet med detta pilotprojekt är att undersöka om storskarvens spybollar kan användas för att detektera förekomsten av den invasiva främmande fiskarten svartmunnad smörbult. Spybollar samlades in från Medholmarna och Norra Röden. QIAGEN DNeasy Blood & Tissue Kit protokoll följdes för att lysera och extrahera DNA. Qubit fluorometer och dPCR användes sedan för att identifiera och kvantifiera mängden svartmunnad smörbult DNA. Mängden DNA i spybollarna från Medholmarna varierade mellan 14.4 ng/µl och 194 ng/µl (medelvärde: 68.395 ng/µl) jämfört med 14.4 ng/µl och 150 ng/µl (medelvärde: 58.885 ng/µl) från Norra Röden. Vid Medholmarna innehöll sex av tio spybollar DNA från svartmunnad smörbult med en medelkoncentration på 0.295 (± 0.305) DNA kopior per µl. Av spybollarna från Norra Röden innehöll åtta av nio DNA från svartmunnad smörbult med en medelkoncentration på 32813 (± 83257) DNA kopior per µl. Skillnaden i DNA koncentration av svartmunnad smörbult i spybollarna mellan platserna tyder på att den svartmunnad smörbult är mer etablerad i området kring Norra Röden än kring Medholmarna. Resultaten från denna studie tyder på att QIAGEN DNeasy Blood & Tissue Kit, Qubit fluorometer och dPCR är tre praktiska analysverktyg för dietprover och att storskarvens spybollar kan användas som en ytterligare metod för att detektera förekomsten av den svartmunnade smörbulten. Storskaliga projekt och analys av storskarvens spybollar kan förse viktig information till framtida bevarande arbeten och skyddandet av marina ekosystem.

Nyckelord: Invasiva arter, Spybollar, Storskarv, Svartmunnad smörbult, dPCR

INTRODUCTION

ROUND GOBY

One of the major threats to biodiversity and ecosystem services today is the spread of invasive species. In Sweden, one species of concern is the round goby (*Neogobius melanostomus*). The round goby is a relatively small (10-25 cm) fish species with a robust body and a large head (Florin et al., 2021). Adult individuals have a murky brown color with small black spots along the sides and are characterized by the large black dot on the rear part of the front dorsal fin (Florin et al, 2021) (Figure 1). Similar to other gobies, their ventral fins are fused together into a suction disc and the round goby is therefore commonly mistaken for the black goby (*Gobius niger*) (Florin et al., 2021). However, the most noticeable difference between these two species is that the black goby does not have the black dot on the back edge of the front dorsal fin (Florin et al., 2021). In addition, the round goby also looks similar to the sand goby (*Pomatoschistus minutus*) (Florin et al., 2021). This species also has a black dot on the back edge of the front dorsal fin and is instead distinguished by its smaller size (max. 10 cm) and significantly shorter rear dorsal fin (Florin et al., 2021). Due to the morphological similarities between many species of gobies, the use of molecular methods and DNA analysis are often important to be able to distinguish the species from each other.

Figure 1. The round goby (*Neogobius melanostomus*). Photo: Anders Salesjö (SLU Artdatabanken, 2022)

SPREAD

The round goby originates from the Black Sea and Caspian Sea (Florin et al., 2021). From there the species has dispersed and invaded new locations and is now present in many river systems across Europe and North America (Figure 2) (Florin et al., 2021). The species was encountered in the Baltic Sea for the first time in 1990, in the Polish Bay of Gdansk (Florin et al., 2021). Since then, the species has continued to spread and is now well established along all coastlines within the Baltic Sea (Florin et al., 2021). However, the presence of the round goby in Swedish waters was not discovered until 2008 but is now commonly found in many harbors and archipelagos along both the Swedish east- and west coast (Florin et al., 2021).

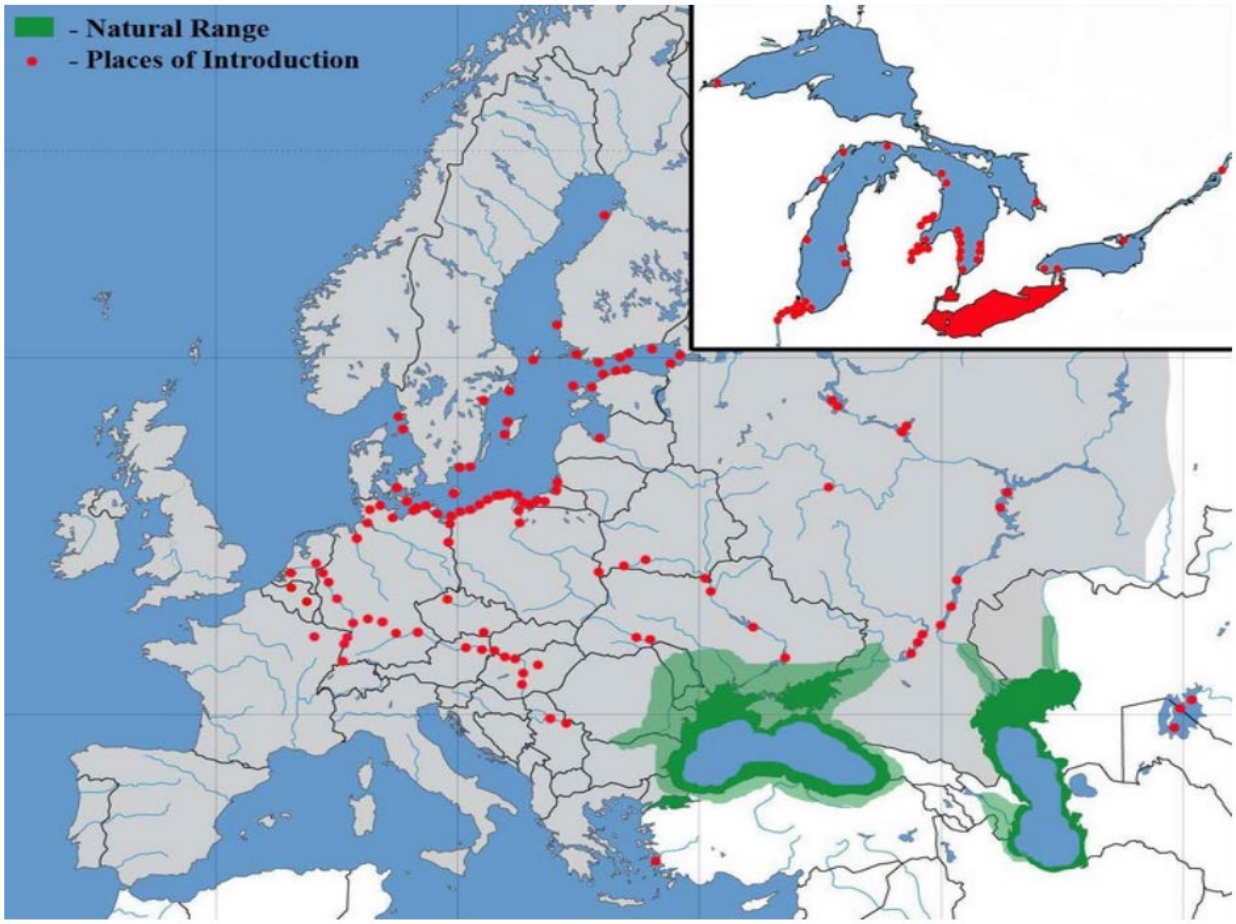

Figure 2. The distribution of the round goby in Europe, Asia and North America (large lakes in the top right corner). The round goby natural range is marked in green and places of introduction are marked in red. The map is not complete and all marks are not verified. (Source: Kwach 2014, retrieved from Florin et al., 2021).

The species becomes sexually mature at 2 years of age for females and 3 years of age for males (Kornis, 2012). Under favorable conditions, mating can take place several times during a season (Kornis, 2012). During the mating season, the males build nests where the females can later lay their eggs (Florin et al., 2021). After the eggs have been fertilized the males protect them until hatching, which leads to high egg survival rate and rapid population growth (Kornis, 2012).

The natural process of dispersal of the round goby is relatively slow. Adults remain stationary and usually do not travel long distances except for seasonal migration or when there is competition for food and shelter (Björklund & Almqvist, 2010). The spread of the round goby is therefore dependent on the transport of larvae by water currents in the connecting rivers systems (Florin et al., 2021).

However, the spread of the round goby in recent decades has been greatly accelerated by human activities in terms of global shipping (Kotta, 2016). When cargo ships fill their tanks with ballast water in one port, round goby larvae sometimes follow (Hensler & Jude, 2007). At the arrival at the new location the cargo ships empty their tanks, which leads to the transport of the round goby and other marine species worldwide (Hensler & Jude, 2007). The transportation of the species can also be facilitated by the attachment of roe to the hulls of ships (Kotta, 2016) and leisure boats (Florin et al., 2021). The success of establishment of the new species depends on the frequency of invasion and its physiological tolerances to the new habitat (Kotta, 2016). As a result of transoceanic shipping, coastal areas close to harbors and estuaries have become some of the most heavily invaded environments in the world (Kotta, 2016).

THREATS

The round goby has many characteristics of an invasive species. It can adapt to many different environmental conditions, reproduce rapidly, and has a dominant territorial behavior (Florin et al., 2021). As a result, the presence and establishment of the invasive round goby may have negative effects on the marine ecosystems and services.

The round goby is a benthic species that can live both in coastal brackish water environments but can also migrate up into freshwater rivers during the summer to reproduce (Florin et al., 2021). In the ocean, it prefers to live among vegetation in shallow, rocky protected areas (Kornis, 2012). However, the round goby can also be found around sand and clay bottoms (Kornis, 2012). The round goby is resistant to fluctuations in temperature (-1 °C to +30 °C) and can withstand high salinity levels (> 30 PSU) (Florin et al., 2021).

In addition, the round goby is an opportunistic feeder and adapts to the local and seasonal abundance of prey (Emmrich, 2011). In Sweden, the round goby poses a threat to other benthic fish species which occupy similar habitats such as the black goby (*Gobius niger*), the viviparous eelpout (*Zoarces viviparus*) and the European plaice (*Pleuronectes platessa*) (Florin et al., 2021). The round goby can also decrease the biomass of other fish species by preying upon their roe and fry (Chotkowski & Marsden, 1999). In addition, mussel banks are also decreasing in biomass due to the intense grazing pressure from the round goby (Florin et al., 2021). Since there will be fewer mussels to filter and purify the water, it may lead to an increased risk of eutrophication (Florin et al., 2021). The spread of the round goby with ballast water to new geographical areas may also lead to the spread of harmful diseases and parasites such as the eel parasite *Anguillicola crassus* (Kvach et al., 2019).

ACTIONS AND REGULATIONS

Despite the round goby's negative ecological effect both in Europe and North America, the round goby is not covered by the EU regulation of invasive alien species since it originates from areas around the Black Sea (Florin et al., 2021). Invasive species that are only of importance to specific member states are not regulated (Florin et al., 2021). To preserve indigenous species and habitats that are of importance to Sweden, guidelines and measures by national conventions need to be established (Florin et al., 2021).

The most economical approach to reduce the negative impacts of invasive species is to prevent their establishment (Florin et al., 2021). One of the most effective ways to do so is by purification of cargo ship's ballast water (Florin et al., 2021). However, this solution comes with a high cost so countries of interest might seek alternative methods. Maintaining strong predatory fish stocks and promoting selective commercial fishing are two cheaper alternatives (Florin et al., 2021). Although it may be too late to prevent the establishment of the round goby in Swedish waters, educating the public, reporting detection, and taking immediate action can help minimize further spread and negative consequences (Florin et al., 2021).

DETECTION OF INVASIVE SPECIES

Harbors, estuaries, and waterways are key areas of investigation for early detection of aquatic invasive alien species (Florin et al., 2021). However, traditional methods such as visual observation and direct sampling are often time consuming (Goldberg et al., 2016). Reliable and efficient monitoring surveys are therefore necessary to assess the health of ecosystems and the composition of species (Staehr et al., 2022). In recent years, the use of environmental DNA (eDNA) has gained attention as a promising tool for monitoring aquatic species and measuring the biodiversity (species presence, abundance, and community composition) in marine ecosystems (Dickie et al., 2018). Environmental DNA refers to genetic material that organisms release into the environment. It is a broad term and can include anything from feces, mucus, hair, or fish scales (Barnes & Turner, 2016). Since genetic material can be collected from bulk environmental samples such as soil and water, eDNA is a popular non-invasive method (Barnes & Turner, 2016). By avoiding traditional methods, the use of eDNA can greatly reduce the time and cost for monitoring projects and simultaneously aid ecosystem conservation and management through improved detection of alien or endangered species that otherwise are not easily detectable (Staehr et al., 2022). New methods to study eDNA can therefore become increasingly important in the future to be able to protect endangered species and/or prevent the spread of invasive species in time. One of these ways is the analysis of great cormorant's regurgitated pellets.

THE GREAT CORMORANT

One of the main predators of round goby is the great cormorant (*Phalacrocorax carbo)* (Rakauskas, 2013). There are two types of great cormorant species in Sweden: The Atlantic cormorant *Phalacrocorax carbo carbo* and the continental subspecies cormorant *Phalacrocorax carbo sinensis* (Strömberg, 2012). However, it is only *Phalacrocorax carbo sinensis* that breeds in Sweden, whereas the Atlantic cormorant instead spends the winter in ice-free areas along the coasts and around lakes (Strömberg, 2012). In this paper, both subspecies will be referred to as the great cormorant *Phalacrocorax carbo*.

At the end of the 19th century, there were only a few colonies of great cormorants in Sweden, and it would take until the 1940s before the population started to rapidly increase (Strömberg, 2012). Between 1970 and 2000, the number of breeding Ph. *carbo sinensis* increased from 300 to 52 000 individuals in Sweden (Steffens, 2010). Sweden is now the country with the highest number of breeding cormorants in Europe (Strömberg, 2012). The majority of great cormorant in Sweden breed in the Baltic Sea and around the large lakes of Vänern, Vättern, Hjälmaren and Mälaren (Florin et al., 2021). Only 5% of the Swedish cormorant population breed on the west coast (Florin et al., 2021).

In recent decades, there has been a conflict of interest between the great cormorants and humans due to the bird's competition for fish resources and damage to fishing gear (Ågren, 2014). The great cormorant is a generalist and feeds on whatever fish that is available to them (Strömberg, 2012). Its predation can affect the fish stocks and potentially alter the ecosystems in the ocean, lake, or watercourse (Strömberg, 2012). In addition, the great cormorant's feces is corrosive so the islands of which the great cormorant breeds on can often be recognized by its lack of ground vegetation and presence of dead trees (Ågren, 2014). Due to their lack of teeth the cormorant also leaves behind regurgitated pellets containing bones, scales and other hard parts from the prey that are difficult to digest (Lunneryd & Alexandersson, 2005).

AIM

Populations of the invasive round goby (*Neogobius melanostomus*) have expanded rapidly in Swedish waters in recent years since it was first discovered in the Baltic Sea in the 1990s. Due to the round goby's negative effect on native marine ecosystems, methods of early detection are of special interest. The great cormorant (*Phalacrocorax carbo*) is an unpopular bird of great concern because its feces kills the vegetation where it breeds and because of the bird's competition with fishing industries. However, the great cormorant can potentially be a vital source and provide valuable information about the spread of invasive fish species. This pilot project aims to investigate the feasibility of using DNA extracted from the great cormorant's regurgitated pellets to detect the presence of the round goby within an area.

METHODS AND MATERIALS

SAMPLING

The first batch of the great cormorant's regurgitated mucus coated pellets were collected on the 28th of March 2023 from Medholmarna (Coordinates: 57° 58' 0" N, 11° 43' 59" E, Sweden) in Hakefjorden outside Kungälv. Medholmarna was specifically targeted for two reasons; it houses a large colony of breeding great cormorants and the presence of the round goby in the area is unknown. The second batch of regurgitated pellets were collected from Norra Röden (Coordinates: 57° 45' 29" N, 11° 44' 30" E, Sweden) in Nordre Älvs Fjord outside Torslanda, on the 20th of April 2023. Similar to Medholmarna, this island was selected because it holds a large colony of breeding great cormorants. However, the presence of the round goby is known around Norra Röden. Norra Röden could therefore be used as the reference group and Medholmarna as the experimental group.

A boat was rented from Stenungsund Marina to be able to reach Medhomarna, whereas a boat was rented from Björkö Marina to be able to reach Norra Röden. Once on the islands, the regurgitated pellets were found within one meter of the nests. To ensure accurate molecular analysis of dietary samples, proper handling was crucial since all subsequent molecular work relies on the quality of DNA extracted from the collected samples (Thalinger et al., 2022). Plastic disposable gloves were used to collect the regurgitated pellets and changed in between the collection of each one to prevent cross-contamination. Each pellet was placed in a 50 ml falcon tube filled with ⅔ of 96% ethanol. In total, 13 regurgitated pellets were collected at Medholmarna in comparison to 38 regurgitated pellets collected from Norra Röden. The samples were then stored in a freezer at -21°C until being transferred to the Zoologen building at Gothenburg University one week later for further analysis. Freezing DNA below -20°C slows down the rate of enzymatic activity and prevents degradation, which is important to preserve DNA samples for future analysis (Bahl et al., 2012). After collection, the regurgitated pellets were exposed to cell lysis, DNA extraction and digital PCR amplification to be able to quantify the concentration of round goby DNA. Out of the 38 collected regurgitated pellets from Norra Röden, only 21 were lysed and 9 were analyzed in dPCR due to time constraints and financial limitations.

CELL LYSIS

There are various protocols and commercial kits available for DNA extraction. Regarding dietary samples from the great cormorant, DNeasy Blood and Tissue Kit from QIAGEN has shown to be highly effective (Thalinger et al., 2022). DNeasy Blood & Tissue Kits are designed for fast and reliable purification of DNA (QIAGEN, 2020). Samples are lysed with proteinase K which enables the breakdown of nucleases and isolation of intact DNA and RNA molecules (QIAGEN, 2020). Buffers are then used to wash the mixture and to create optimal DNA-binding conditions (QIAGEN, 2020). During centrifugation, the buffers ensure specific DNA binds to the silica-based membrane while contaminants and enzyme inhibitors pass through the DNeasy Mini spin column (QIAGEN, 2020). Afterwards, the DNA is eluted and ready to be analyzed (QIAGEN, 2020).

All test tubes from Medholmarna and Norra Röden were marked (both the lid & the container) with numbers and location of collection to prevent a mix-up. To avoid individual bias, test tubes were randomly selected for the analysis and marked while the tubes still remained in the transportation box. The workspace was sterilized using chlorine and aluminum foil, scalpel, and tweezers (both straight- and curved pointed) were gathered. The aluminum foil was used as a tray where the regurgitated mucus coated pellets could be dissected using the scalpel and the tweezers (Figure 3). Small pieces of the regurgitated pellets (1 ml in total) were cut out and placed in a 5 ml microcentrifuge tube. The pieces were cut out from different parts of the regurgitated pellets to make sure no DNA was left out. After the regurgitated pellets had been dissected, the remaining parts were returned to its original tube with ethanol and stored in the freezer at -21°C. In between each regurgitated pellet, the aluminum foil, scalpel, and tweezers were disinfected using chlorine. In addition, the scalpel and tweezers were also held over a Bunsen burner to sterilize the tools and remove any potentially remaining organic matter. The used plastic gloves were also changed prior to each regurgitated pellet to prevent contamination.

Figure 3. An overview of the workspace for the dissection of the regurgitated pellets. The marked sample tubes are shown in the top of the picture and at the center of the picture is the aluminium tray with the scalpel and tweezers. Photo: Tim Dorup

Once all the regurgitated pellets had been cut out and placed in a microcentrifuge tube the process to dissolve the pieces began in accordance with procedures from a similar study (Thalinger et al., 2016). To begin with, 3 ml of TES Buffer (0.1 M TRIS, 10mM EDTA, 2% Sodium dodecyl sulphate; pH 8) and 15 µl proteinase K was added to each microcentrifuge tube. A 0.5-5 ml finnpipette was used to transfer the TES buffer and a 10-100 µl finnpipette was used to transfer proteinase K to the microcentrifuge tube. A new pipette tip was used for each solution and sample to prevent contamination. A blank control was also included, where 3 ml of TES buffer and 15 µl proteinase K was mixed but without any regurgitated pellet. Afterwards, all samples were vortexed and incubated on a rocking platform overnight at 56 °C to be able to dissolve the regurgitated pellets.

DNA EXTRACTION

After the pellets had been dissolved, the procedures from the blood protocol from QIAGEN DNeasy & Blood & Tissue Kit were followed to extract the DNA (QIAGEN, 2020). First of all, 440 µl of sample concentration was transferred from the 5 ml microcentrifuge tube to a new 2 ml microcentrifuge tube. Afterwards, 400 µl lysis buffer AL was added to each sample using a 100-1000 µl finnpipette. To prevent contamination, a new pipette tip was used for each tube and the old one was discarded. Once the buffer had been added, the samples were thoroughly mixed by vortex. Once again, a blank extraction control was made to be able to test for cross-contamination later. The extraction blank control followed the same procedures as for the pellet samples, however, 440 µl of the blank control from the lysis step was used instead of DNA concentrations from dissolved pellets.

In the next step, 400 µl of 99% ethanol was added to the samples and mixed by vortex. The addition of ethanol allowed DNA to precipitate. Each solution (620 µl) was transferred into a 2 ml collection tube with a DNeasy Mini spin column in it. The cap of the DNeasy Mini spin column was marked with number and location of collection to prevent a mix-up. The DNeasy mini spin columns with the collection tubes were then centrifuged at ≥6000 x g (8000 rpm) for 1 minute. This step helps to catch the DNA in the filter of the spin column (QIAGEN, 2020). Afterwards, the flow-through, which had passed through the filter, was discarded in a chemical waste bottle. The DNeasy Mini spin column was then placed back in the same collection tube and the remaining DNA concentration solution (620 µl) of each sample was transferred using a 100-1000 µl finnpipette. The samples were then once again centrifuged at ≥6000 x g (8000 rpm) for 1 minute. The flow-through was discarded in a chemical waste bottle and the collection tube was discarded in a plastic bin. The spin column was then inserted into a new 2 ml collection tube.

For the next step, 500 µl buffer AW1 was added to each spin column to wash the DNA. Once again, the samples were centrifuged for 1 minute at ≥6000 x g (8000 rpm). Samples with high DNA concentration tended to clog the filter in the DNeasy Mini spin column. To solve this, a pipette tip was gently scraped on the filter and the samples were centrifuged once more at 10 000 rpm. The DNeasy Mini spin column was then placed in a new 2 ml collection tube and the old collection tube was discarded in a plastic bin and the flow-through was discarded in a chemical waste bottle. Before the third centrifugation step, 500 µl buffer AW2 was added to the spin column to wash the DNA and remove other biomolecular debris. This time the centrifuge was set on a three minute timer at 20,000 x g (14,000 rpm). After the centrifugation, the spin column was once again placed in a new 2 ml collection tube. The old collection tube was discarded in a plastic bin and the flow-through was discarded in a chemical waste bottle.

In the final step, 200 µl buffer AE (10 mM Tris-Cl 0.5 mM EDTA; pH 9.0) was added to the center of the spin column to be able to elute the DNA. The buffer AE helps to rinse the DNA through the spin column filter and into the collection tube. The samples were then set to rest in room temperature for 1 minute before being placed in the centrifuge for 1 minute at ≥6000 x g (8000 rpm). The DNeasy Mini spin column was later discarded and the liquid in the collection tube, which had passed through the filter, was stored.

MEASUREMENT OF DNA CONCENTRATION

A Qubit fluorometer is a valuable tool for quantifying DNA, RNA, and protein (Invitrogen, 2017), which is crucial for checking DNA quality before various applications such as next-generation sequencing (NGS) and polymerase chain reaction (PCR) (Nakayama et al., 2016). Fluorescent dyes are used to selectively stain double stranded DNA, RNA, and proteins (Invitrogen, 2017). The machine can then measure the DNA concentration based on the intensity of the fluorescent dye that is bound to the target molecule (Nakayama et al., 2016), which minimizes the effect of the possible presence of small amounts of contaminants (Invitrogen, 2017).

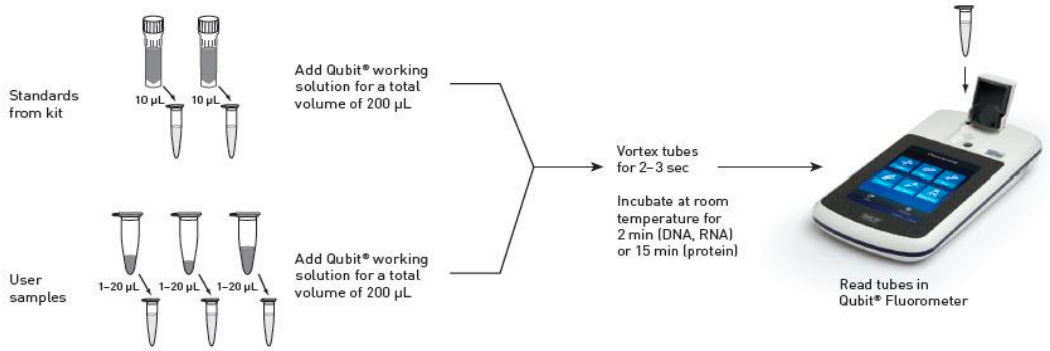

Figure 4. The workflow of Qubit dsDNA BR Assay Kit and Qubit Fluorometer analysis (Thermo Fisher, 2015).

Once the DNA had been extracted from all samples, the DNA concentration of each was measured using the Qubit 3.0 Fluorometer in accordance with the manufacturer's protocol (Invitrogen, 2017). To be able to perform this, two standards had to be prepared to set the upper and lower limit (Figure 4). The two standards calibrate the fluorometer, which enables it to generate concentration data (Invitrogen, 2017). The two standards were mixed in accordance with the manufacturer's instructions from the Qubit dsDNA BR Assay Kit (Invitrogen, 2022). In brief, a master mix was prepared by mixing 1 µl Qubit reagent with 199 µl Qubit buffer. One 500 µl Qubit assay tube was then filled with 190 µl of the master mix and 10 µl of standard 1 and another 500 µl Qubit assay tube was filled with 190 µl of the master mix and 10 µl of standard 2. To be able to measure the DNA concentration of each sample, 195 µl of master mix were mixed with 5 µl DNA solution in a 500 µl Qubit assay tube (Figure 4). A blank control was also prepared for the Qubit test by mixing 195 µl of master mix with 5 µl of extraction blank. A new pipette tip was used for each solution to prevent cross contamination. Once complete, the total volume in each Qubit assay tube should be 200 µl. Each Qubit tube was then vortexed for 5 seconds and set aside to rest for 2 minutes.

Afterwards, the samples, blank control and standards were measured one by one in the Qubit 3.0 Fluorometer (Figure 4). The settings were set on dsDNA, broad range and 5 µl of DNA sample size. The two standards were first measured, followed by the blank. The DNA concentration of the samples were then measured in numerical order.

DIGITAL PCR AMPLIFICATIONS

Digital PCR (dPCR) is an advanced nucleic acid detection and quantification method (Behind The Bench Staff, 2015). Digital PCR involves dividing DNA, complementary DNA (cDNA), or RNA samples into microreactions, which are then analyzed through PCR amplification (Behind The Bench Staff, 2015). A microfluidic plate can be used to facilitate the analysis of thousands of microreactions at the same time (Behind The Bench Staff, 2015). Each well of the plate is loaded with a mixture of sample, master mix, and reagents, which are distributed into microreactions (Behind The Bench Staff, 2015) (Figure 5). After the dPCR, each microreaction is individually counted and scored as either positive or negative based on the presence of the targeted nucleic acid (Pecoraro et al., 2019) (Figure 5). The fraction of negative microreactions is then used to determine the absolute quantity of target molecules present in the sample (Behind The Bench Staff, 2015). Consequently, dPCR is particularly beneficial for detecting small amounts of target nucleic acid among samples.

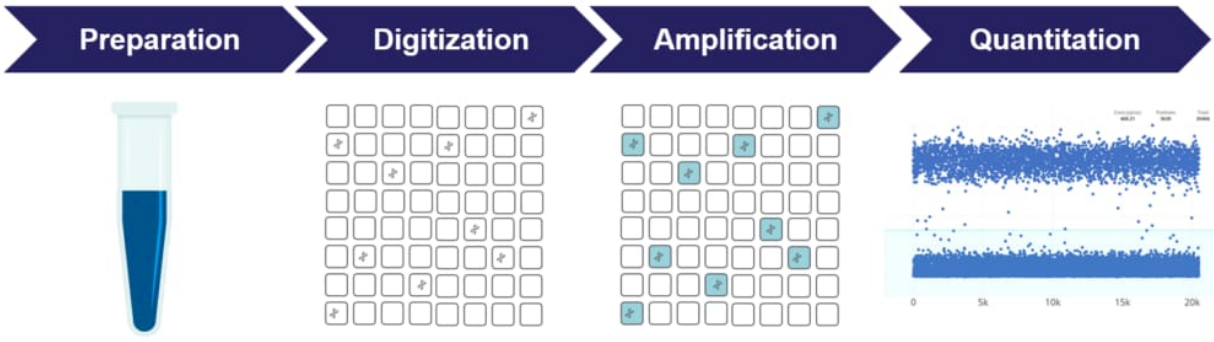

Figure 5. The workflow of digital PCR analysis (Behind The Bench Staff, 2015).

To begin with, a master mix was prepared for each nanoplate. The master mix consisted of QIAGEN probe mix and round goby dPCR assay in a 10:2 ratio. The probe mix contains DNA polymerase, buffer and dye for the reference channel in TE buffer with low EDTA (0.1 mM), whereas the round goby dPCR assay contains target specific forward primer, reverse primer, and a fluorescent-labeled probe. Primers amplify the gene fragment, the probe anneals to it and releases the FAM dye that is registered by the dPCR machine. For each nanoplate, 260 µl of QIAGEN probe mix and 52 µl of round goby dPCR assay, which targets a fragment of the 12S mitochondrial gene, was transferred to a 1.5 mL centrifuge tube and mixed by vortex (Table 1). A 100-1000 µl finnpipette was used to transfer the probe mix and 10-100 µl finnpipette for the assay. A new pipette tip was used for each centrifuge tube to prevent contamination.

The solution was then distributed into twelve 1.5 ml centrifuge tubes with 24 µl in each one (Table 1). Nine of them were assigned to regurgitated pellet DNA samples and the other three were divided into one DNA extraction blank, one no template control (NTC) and one positive PCR control (PPC). The no template control contains water instead of template DNA and is used to monitor contamination and false positive results (Wallinger et al., 2017). In contrast, the positive PCR control contains targeted DNA extracted from round goby tissue and is used to test the amplification success of the dPCR (Wallinger et al., 2017). The samples were then diluted with water. For the regurgitated pellet DNA samples and extraction blank, 36 µl of water was added, whereas for the positive PCR control and no template control 46 µl and 56 µl of water was added respectively (Table 1). At last, 20 µl of DNA regurgitated pellet mix samples were added to its respective centrifuge tube (Table 1). No DNA was added to the no template control and 10 µl of diluted round goby DNA was added to the positive PCR mix (Table 1). For the blank centrifuge tube, 20 µl of the blank extraction solution was added. For transfers of each solution, a 10-100 µl finnpipette was used whereas a new finnpipette was used for each sample and solution to prevent cross contamination. In the end, each microcentrifuge tube should have a total volume of 80 µl.

Table 1. The total amount (µl) of QIAGEN probe mix, round goby assay, DNA and water for the DNA sample mix, non-template control mix and positive PCR control mix.

DIGITAL PCR MIX SAMPLES			
	DNA SAMPLE MIX (µl)	NON TEMPLATE CONTROL MIX (µl)	POSITIVE PCR CONTROL MIX (µl)
QIAGEN PROBE MIX	20	20	20
ROUND GOBY ASSAY	4	4	4
DNA	20	0	10
H2O	36	56	46
TOTAL VOLUME	80	80	80

Once the solutions for the dPCR had been mixed, the process to fill the dPCR plate began. For this experiment a 26k nanoplate 24-well was used which allowed 12 samples to be tested for each plate (two wells for each regurgitated pellet DNA sample, blank extraction sample, no template control and positive PCR control). In a 26k nanoplate, each PCR reaction is divided into 26000 independent partitions. Number of valid partitions for each well in the plate is monitored using the reference dye in the dPCR mix. The PCR reaction mixture was pipetted into the wells one by one. To prevent cross-contamination, a new pipette tip was used for each sample. Once all the wells were filled, a rubber seal was applied to the top of the plate. A roller was then used to make sure the rubber seal stuck to the nanoplate. In total, three dPCR plates were analyzed: one for the regurgitated pellets from Medholmarna and two for the regurgitated pellets from Norra Röden. After the first dPCR test on the regurgitated pellets from Norra Röden, some of the samples were saturated and contained too much DNA of round goby. The saturated samples therefore had to be diluted (1:10) and run again in another dPCR test in order to be able to accurately measure the DNA concentration.

Before the nanoplate was inserted into the machine, the settings of the dPCR were adjusted on the computer. The priming profile was set on QIAGEN Standard Priming Profile, which is the standard priming for all sample types. Afterwards, the cycling profile was divided into two phases. First, the initial denaturation at 95 °C for 2 minutes followed by 40 cycles of denaturation at 95 °C for 30 seconds and annealing plus extension at 64 °C for one minute . For the imaging profile the exposure duration was set to 500 ms and 300 ms, gain 6 and the channel was set to use only color green (FAM dye). Each well was then labeled to give the machine an idea of the plate layout. For each well, the reaction mix, target DNA, type (positive control, sample or no template control) and sample name ("POS" for the positive PCR control, "NEG" for the no template control, "M1-M10/B" for Medholmarna and "NR1-NR9/B" for Norra Röden) was set. Once all of the settings were adjusted, the nanoplate was inserted into the machine and the dPCR process began. The process of digital PCR took in total 2.5 hours before the results could be analyzed.

CALCULATIONS

To accurately determine the absolute quantity of target DNA in digital PCR, a correction factor is necessary to account for microreactions that may have received more than one molecule of the target DNA due to random assortment (Behind The Bench Staff, 2015). The Poisson model is utilized to apply this correction factor, which takes into account the probability of multiple molecules in a single microreaction. For this to work, at least one negative reaction must be present to determine the probability (Behind The Bench Staff, 2015). The ratio of negative and positive reactions therefore plays a critical role in establishing the absolute quantity of target DNA present in each original sample. The following equations were used to calculate the target copies per partition as well as total DNA concentration of round goby per sample:

Equation 1

$$Target\ Copies\ Per\ Partition\ (\lambda) = -\ln\frac{Number\ of\ Valid\ Partitions - Number\ of\ Positive\ Partitions}{Number\ of\ Valid\ Partitions} \times 1000$$

Equation 2

$$Concentration\ of\ Target\ DNA\ (Copies/\mu l) = \frac{Target\ Copies\ Per\ Partition\ (\lambda)}{Total\ Sample\ Volume\ of\ Each\ Well\ (\mu l)}$$

RESULTS

TOTAL DNA CONCENTRATION IN THE SAMPLES

The twenty-one analyzed great cormorant regurgitated mucus coated pellets collected from Norra Röden all contained high amounts of DNA. However, the calculation of the DNA concentration by the fluorometer is not limited to the round goby as it measures DNA from all possible species. The total DNA concentration in the sample tubes from Norra Röden ranged between 14.4 ng/µl and 197 ng/µl (Mean: 68.395 ng/µl, Median: 50.90 ng/µl) (Table 2). However, in the nine regurgitated pellets analyzed in dPCR, the range of the DNA concentration was more narrow. The DNA concentration of the nine regurgitated pellets ranged between 21.5 ng/µl and 114 ng/µl (Mean: 56.12 ng/µl, Median: 49.60 ng/µl) (Table 2). The regurgitated mucus coated pellets from Medholmarna also contained high amounts of DNA. The results from the thirteen analyzed pellets showed a range between 14.4 ng/µl and 150 ng/µl (Mean: 58.885 ng/µl, Median: 30.80 ng/µl) of total DNA concentration (Table 3). Out of the ten pellets that were analyzed in dPCR, the highest DNA concentration was 150 ng/µl and the lowest was 14.8 ng/µl (54.05 ng/µl, Median: 27.85 ng/µl) (Table 3).

Table 2. The total DNA concentration of all regurgitated pellets collected from Norra Röden, including the blank sample (NRB). DNA concentrations below 0.2 ng/µl were referred to as out of range by the Qubit Fluorometer.

LOCATION	SAMPLE	TOTAL DNA CONCENTRATION (ng/µL)
Norra Röden	NR1	45.2
Norra Röden	NR2	114
Norra Röden	NR3	50.8
Norra Röden	NR4	88.4
Norra Röden	NR5	21.5
Norra Röden	NR6	49.6
Norra Röden	NR7	37.6
Norra Röden	NR8	54.0
Norra Röden	NR9	44.0
Norra Röden	NR10	14.8
Norra Röden	NR11	74.4
Norra Röden	NR12	36.3
Norra Röden	NR13	31.1
Norra Röden	NR14	197
Norra Röden	NR15	179
Norra Röden	NR16	100
Norra Röden	NR17	35.8
Norra Röden	NR18	70.0
Norra Röden	NR19	14.4
Norra Röden	NR20	58.4
Norra Röden	NR21	120
Norra Röden	NRB	< 0.2

Table 3. The total DNA concentration of all regurgitated pellets collected from Medholmarna, including the blank sample (MB). DNA concentrations below 0.2 ng/µl were referred to as out of range by the Qubit Fluorometer.

LOCATION	SAMPLE	TOTAL DNA CONCENTRATION (ng/µL)
Medholmarna	M1	14.8
Medholmarna	M2	24.9
Medholmarna	M3	55.6
Medholmarna	M4	18.0
Medholmarna	M5	103
Medholmarna	M6	150
Medholmarna	M7	17.4
Medholmarna	M8	30.8
Medholmarna	M9	108
Medholmarna	M10	18
Medholmarna	M11	125
Medholmarna	M12	85.6
Medholmarna	M13	14.4
Medholmarna	MB	< 0.2

ROUND GOBY DNA CONCENTRATION

For each well in the nanoplate, the fluorescence intensity of each partition is measured and plotted in a graph with the analyzed wells on the x-axis (Bio-Rad, n.d). Partitions that have the target DNA will generate a specific fluorescence signal (green in this case). The threshold is set to divide the positive partitions depicted by the blue dots versus negative partitions depicted by the grey dots. The threshold can be set manually or automatically by the machine itself. The actual amplitude of each partition is not important (Bio-Rad, n.d). Anything that is reasonably higher than the negative group and above the threshold is assigned as positive (Bio-Rad, n.d). The number of positive and negative partitions is then used to calculate the initial concentration of the target sequence in the sample (Bio-Rad, n.d).

The dPCR results from the regurgitated pellets collected from Medholmarna showed that six out of ten pellets were positive (contained DNA from the round goby) (Figure 6). Since the no template control (NTC) contained 1 positive partition in each well, all samples with ≤ 1 were regarded as negative (Figure 7b). Therefore, samples 5 (M5) and sample 6 (M6) were regarded as negative (Figure 7a & Figure 7b). Sample 9, however, was regarded as positive since one of the wells contained 4 positive partitions (Figure 7b). After adjustments for Poisson distribution, the average concentration of round goby per sample ranged between 0 copies/µl to 0.933 copies/µl (Table 4), which gave a total average concentration of 0.295 (± 0.305) copies/µl for all samples from Medholmarna (Table 5).

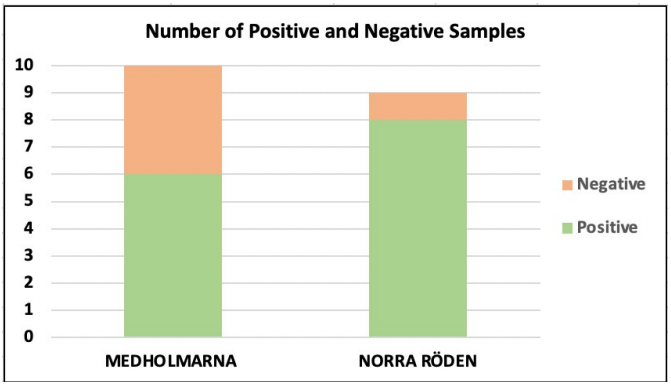

Figure 6. The number of positive and negative samples for Medholmarna and Norra Röden. The positive samples are colored in green, and the negative samples are colored in orange.

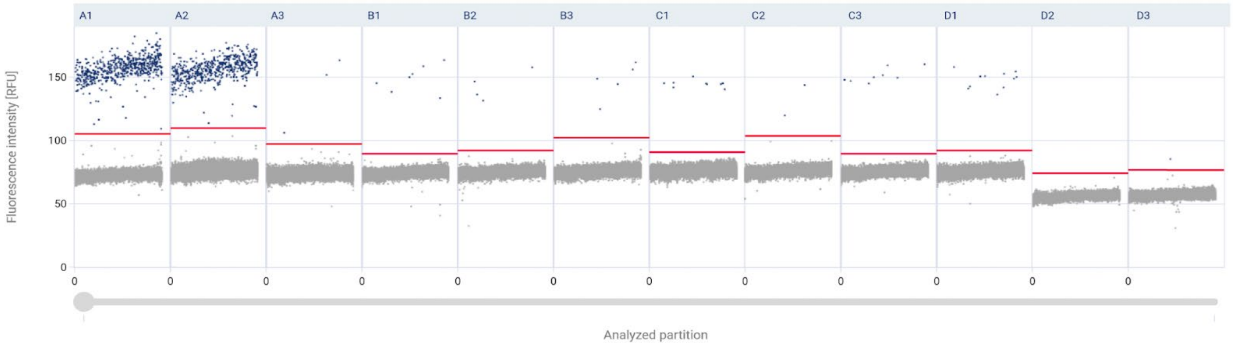

Figure 7a. The dPCR results from Medholmarna. On the y-axis is the fluorescence intensity and on the x-axis are the analyzed wells. The red line is the threshold which separates the negative partitions (depicted in grey) from the positive partitions (depicted in blue). A1-A2 is the positive control (PPC), A3-B1 is sample 1, B2-B3 is sample 2, C1-C2 is sample 3, C3-D1 is sample 4 and D2-D3 is sample 5.

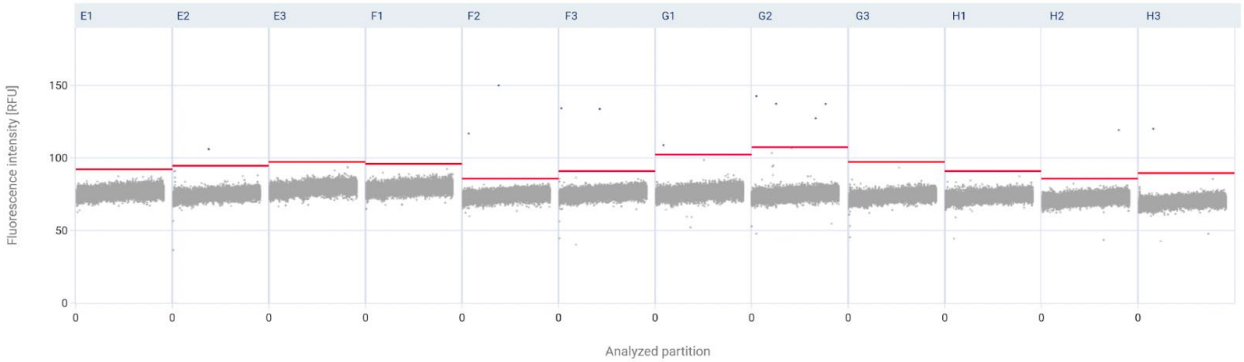

Figure 7b. The dPCR results test from Medholmarna. On the y-axis is the fluorescence intensity and on the x-axis are the analyzed wells. The red line is the threshold which separates the negative partitions (depicted in grey) from the positive partitions (depicted in blue). E1-E2 is sample 6, E3-F1 is sample 7, F2-F3 is sample 8, G1-G2 is sample 9, G3-H1 is sample 10 and H2-H3 is the no template control (NTC).

Table 4. The average target copies per partition and average DNA concentration of round goby per sample, including the blank (MB) of the regurgitated pellets collected from Medholmarna.

LOCATION	SAMPLE NAME	TARGET	AVERAGE TARGET COPIES PER PARTITION	AVERAGE DNA CONCENTRATION OF ROUND GOBY (COPIES/µL)
Medholmarna	POSITIVE (PPC)	NM12S	0.02184	54.605
Medholmarna	M1	NM12S	0.00020	0.492
Medholmarna	M2	NM12S	0.00018	0.442
Medholmarna	M3	NM12S	0.00022	0.540
Medholmarna	M4	NM12S	0.00037	0.933
Medholmarna	M5	NM12S	0.00002	0.049
Medholmarna	M6	NM12S	0.00002	0.049
Medholmarna	M7	NM12S	0.00000	0.000
Medholmarna	M8	NM12S	0.00008	0.196
Medholmarna	M9	NM12S	0.00010	0.246
Medholmarna	M10	NM12S	0.00000	0.000
Medholmarna	MB	NM12S	0.00000	0.000
Medholmarna	NEGATIVE (NTC)	NM12S	0.00004	0.098

Table 5. The total average DNA concentration of round goby in all regurgitated pellets collected from Medholmarna and Norra Röden, including the standard deviation and standard error.

LOCATION	TOTAL AVERAGE ROUND GOBY DNA CONCENTRATION (COPIES/µL)	STANDARD DEVIATION	STANDARD ERROR
Medholmarna	0.295	0.305	0.096
Norra Röden	32813	83247	27749

In contrast, eight out of the nine analyzed regurgitated pellets from Norra Röden were positive (contained DNA from the round goby) (Figure 6). However, the blank sample (NRB) contained between 18 and 22 positive partitions in both dPCR trials made on Norra Röden (Figure 8b & Figure 10b). Samples with ≤ 22 positive partitions were therefore regarded as negative. All positive samples had more than 22 positive partitions, so none had to be neglected due to risk for contamination. The only pellet without any positive partitions was pellet number 8 (NR8) (Figure 8b). After Poisson calculations, the total round goby DNA concentration per sample ranged between 0 copies/µl and 253380 copies/µl (Table 6). The results from the first dPCR plate from Norra Röden showed that sample 1 (NR1), sample 6 (NR6) and sample 9 (NR9) were oversaturated (Figure 8a & Figure 8b), i.e., contained few negative partitions. In the second dPCR test on the regurgitated pellets from Norra Röden, sample 1 (NR1), sample 6 (NR6) and sample 9 (NR9) were diluted (1:10). Despite this, only sample 6 (NR6) and sample 9 (NR9) were altered on the graph whereas sample 1 (NR1) remained oversaturated (Figure 10a). After dilution, the total average DNA concentration of round goby for all analyzed samples from Norra Röden was 32813 (± 83247) copies/µl (Table 5).

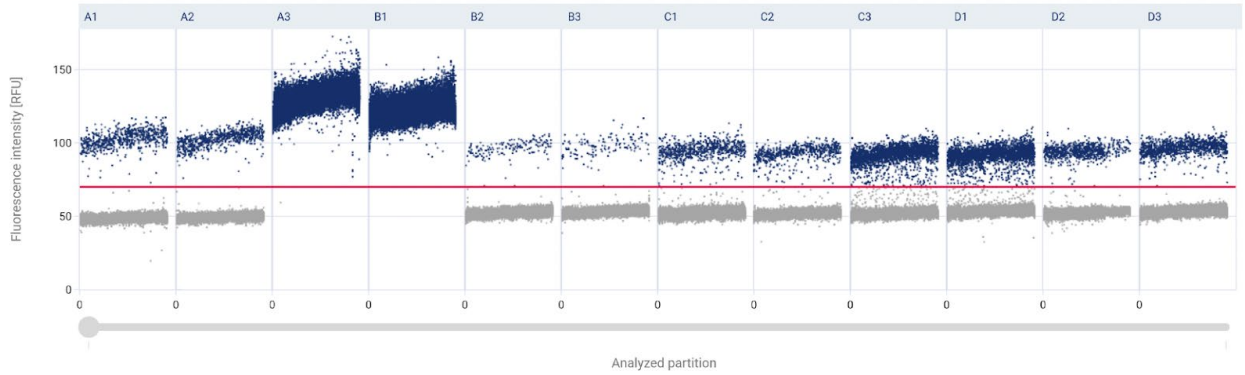

Figure 8a. The results from the first dPCR test from Norra Röden. On the y-axis is the fluorescence intensity and on the x-axis are the analyzed wells. The red line is the threshold which separates the negative partitions (depicted in grey) from the positive partitions (depicted in blue). A1-A2 is the positive control (PPC), A3-B1 is sample 1, B2-B3 is sample 2, C1-C2 is sample 3, C3-D1 is sample 4 and D2-D3 is sample 5.

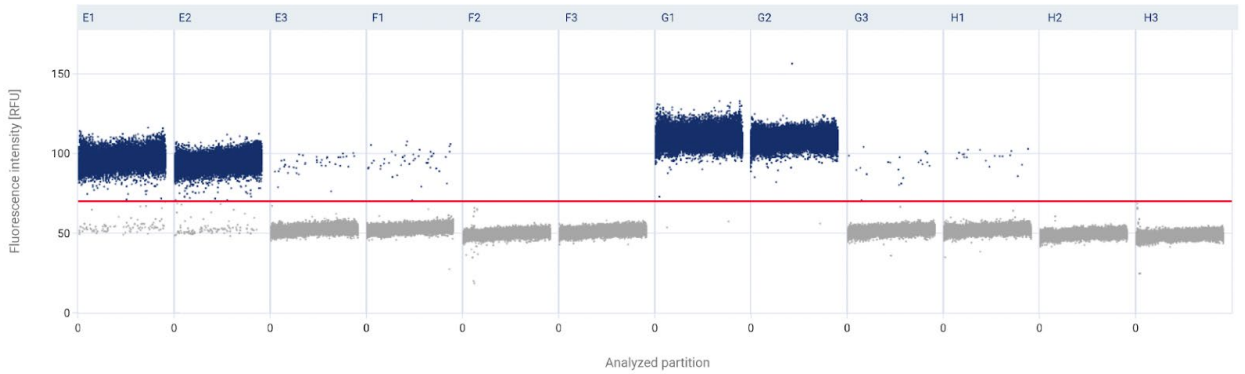

Figure 8b. The results from the first dPCR test from Norra Röden. On the y-axis is the fluorescence intensity and on the x-axis are the analyzed wells. The red line is the threshold which separates the negative partitions (depicted in grey) from the positive partitions (depicted in blue). E1-E2 is sample 6, E3-F1 is sample 7, F2-F3 is sample 8, G1-G2 is sample 9, G3-H1 is the blank sample and H2-H3 is the no template control (NTC).

Table 6. The average target copies per partition and average DNA concentration of round goby per sample (including the blank NRB) of the regurgitated pellets collected from Norra Röden.

LOCATION	SAMPLE NAME	TARGET	AVERAGE TARGET COPIES PER PARTITION	AVERAGE CONCENTRATION OF ROUND GOBY (COPIES/µL)
Norra Röden	POSITIVE (PPC)	NM12S	0.02729	68.217
Norra Röden	NR1	NM12S	101.35194	253379.858
Norra Röden	NR2	NM12S	0.00538	13.442
Norra Röden	NR3	NM12S	0.02559	63.974
Norra Röden	NR4	NM12S	0.10728	268.206
Norra Röden	NR5	NM12S	0.03837	95.928
Norra Röden	NR6	NM12S	5.96395	14909.871
Norra Röden	NR7	NM12S	0.00153	3.830
Norra Röden	NR8	NM12S	0.00000	0.000
Norra Röden	NR9	NM12S	10.63163	26579.082
Norra Röden	NRB	NM12S	0.00081	2.014
Norra Röden	NEGATIVE (NTC)	NM12S	0.00000	0.000

DISCUSSION

DNA EXTRACTION

All pellets collected from Medholmarna and Norra Röden had high amounts of DNA concentration which indicates that the QIAGEN DNeasy & Blood & Tissue Kit is an efficient and reliable method to extract DNA from dietary samples. However, step 1 in the protocol (QIAGEN, 2020) was not to recommend for the lysis of regurgitated pellets from the great cormorant. Buffer ATL in combination with proteinase K was not enough to break down the pellets and instead created a gel-like substance. As a result, it was difficult to pipette any liquid into a separate centrifuge tube. Step 1 in the protocol therefore had to be changed and buffer ATL was replaced by TES buffer in accordance with procedures from a similar experiment (Thalinger et al., 2016). With TES buffer the regurgitated pellets were better dissolved, and the solution could more easily be transferred into a new centrifuge tube. Despite this, some parts of the regurgitated pellet still remained in the bottom of the centrifuge tube. A 3:1 ratio of TES buffer and regurgitated pellet might not be enough for complete breakdown. For future projects, it would instead be recommended to use 5 ml of TES buffer, which is equivalent to the volume needed for medium sized regurgitated pellets (Thalinger et al., 2016).

DIGITAL PCR

As subspecies of round gobies are often difficult to distinguish morphologically from each other, molecular methods are to be preferred. In previous studies, molecular approaches have shown to outperform morphological identification of hard parts (otoliths, pharyngeal bones, and jaw structure) regarding the detection of prey (Oehm, 2017). In this study, the use of digital PCR was shown to be a successful method to detect the presence of round goby DNA in regurgitated pellets of great cormorants. Round goby DNA was found in six (60%) of the analyzed pellets from Medholmarna and in eight (88.89%) of the analyzed pellets from Norra Röden (Figure 6). The dPCR worked best on samples from Medholmarna with low levels of target DNA. With lower concentration the distribution of molecules in the partitions were better. As the concentration increased the probability that two or more molecules of interest ended up in the same partition increased, which could be seen in sample 1, sample 6 and sample 9 from Norra Röden (Figure 8a & Figure 8b). Samples that are saturated therefore need to be diluted otherwise the dPCR is unable to differentiate the positive and negative partitions and accurately calculate the DNA concentration. In this experiment, saturated samples were diluted 1:10. A dilution of 1:10 was enough for sample 6 and sample 9, however, sample 1 remained saturated (Figure 10a & 10b). Saturated samples should be diluted until there is enough negative partitions for Poisson calculations to be made.

Classification of partitions as positive or negative is based on the threshold, which should be set just above the cluster of negative partitions. In some dPCR reactions, straightforward classification of partitions as either positive or negative can sometimes be difficult. These partitions are referred to as nonspecific rain (Figure 8a) (Pecoraro et al., 2019). Rain represents partitions with intermediate fluorescence levels which can be caused by inhibitors (Pecoraro et al., 2019). Rain does not seem to have a pronounced distribution, but it is often equally spread between positives and negatives (Pecoraro et al., 2019), which can be seen in sample 4 from Norra Röden (Figure 8a). The use of a positive PCR control (PPC) and a negative no template control (NTC) is therefore vital for dPCR analysis. The positive PCR control contains DNA from the target species and is used to prevent false negative results and to test whether the amplification process worked as expected (Pecoraro et al., 2019). In all three dPCR tests performed in this study, the positive control did not show any defects on fluorescence amplification (Figure 7a, Figure 8a & Figure 10a). In contrast, the no template control samples, with only negative partitions, were used to check for contamination and help set the threshold (Pecoraro et al., 2019). Neither no template control samples for the dPCR tests on regurgitated pellets from Norra Röden showed any signs of

contamination so the threshold could be set manually. However, the no template control sample for Medholmarna had one positive partition in each well which indicates that contamination had occurred in that sample. Because of the high amplitude of the positive partition in the no template control (Figure 7b), the threshold for the dPCR test on Medholmarna could not be adjusted manually since it would risk misclassification partitions in other samples. The automatic threshold generated by the dPCR machine was therefore not altered.

In addition, a blank DNA control should always be used in a dPCR experiment to test for cross-contamination and false positives results (Pecoraro et al., 2019). The blank control (NRB) for Norra Röden contained between 18 and 22 positive partitions in the dPCR results which indicates that cross-contamination has occurred somewhere during procedures (Figure 8b & Figure 10b). Although the fluorometer assigned the DNA concentration to the blank control as out of range, it cannot be excluded that contamination has occurred during cell lysis and DNA extraction since the fluorometer cannot detect DNA concentrations below 0.2 ng/µl. However, all pellets with positive partitions from Norra Röden had ≥ 22 positive partitions which means that none of them had to be regarded as negative. The importance of a blank control can be exemplified with the dPCR tests on the regurgitated pellets from Medholmarna. The blank control for Medholmarna had no positive partitions which indicates that no cross-contamination has occurred (Figure 10b). However, if the blank at Medholmarna would have been the same as for Norra Röden, all the pellets from Medholmarna would have been regarded as negative. The blank control therefore does not only check for cross contamination but also ultimately the credibility of the results.

SAMPLING

Sampling of the great cormorant's regurgitated mucus coated pellets is a time-efficient method. Once a colony of great cormorants has been localized, the pellets can be easily collected with minimal disturbance to the population (Barrett, 2007). Regurgitated pellets are found close to the nests and are easily distinguishable from feces. Once a human goes ashore the island, the great cormorant colony leaves their nests and spectates from a distance, which allows for multiple pellets to be collected at each visit. However, the collection of regurgitated pellets is weather dependent. Stormy days with a lot of rain and strong winds makes the islands, such as Norra Röden and Medholmarna, difficult to dock. Bad weather can also cause degradation of the regurgitated pellets and/or make them less visible. The weather was one of the main reasons why the sampling of regurgitated pellets occurred four weeks apart between Medholmarna and Norra Röden. Medholmarna had to be visited before the end of March since access to the island was prohibited between April 1st to 31 of July for bird protection (Länsstyrelsen, 2023).

Another reason to wait to visit Norra Röden was because only a few regurgitated pellets were found at Medholmarna and the ones which were collected did not contain much bone remains from fish (Table 7). The lack of regurgitated pellets was assumed to be related to the timing of arrival of great cormorant to the breeding site. According to Bregnballe et al (2006), the date of arrival to a breeding site can vary up to 2.5 months. On average, 20% of the colony arrives between January and February, 60% in March and 20% during the first two weeks of April (Bregnballe et al., 2006). The timing of arrival is mainly based on the distance to the colony, but weather conditions and food availability were also two important factors (Bregnballe et al., 2006). Great cormorants that spend their winters far away tend to rely on endogenous factors and change of daylength to know when to migrate (Bregnballe et al., 2006). In contrast, locally wintering birds can instead react and adjust more to the variable and unpredictable weather (Bregnballe et al., 2006). If weather conditions start to deteriorate, cormorants sometimes leave the colony and do not return until weeks later when the conditions are more favorable for breeding (Bregnballe et al., 2006). This migration behavior of great cormorants could potentially explain the difference of regurgitated pellets found at Medholmarna in late March in comparison to Norra Röden in late April.

FORAGING AREA

Although the regurgitated pellets were collected from Medholmarna and Norra Röden, the exact location of which the round gobies had been caught by the great cormorants is unknown. Based on previous studies, the great cormorant has a mean maximum of 25 ± 10 km foraging area (Thaxter et al., 2012). However, during the breeding season the foraging area tends to be smaller with a mean maximum range of 12.4 ± 4.0 km (Fijn, 2022). Based on these assumptions, the area of which the round gobies have been caught can be estimated. Figure 9 shows the estimated area of where the round gobies might have been caught from Medholmarna. The map of Medholmarna shows that the round goby can have been caught anywhere from Orust to Björkö. If that is the case, the great cormorants could in theory have caught the round gobies around Norra Röden. However, that is highly unlikely since the great cormorant is an opportunistic predator and often feeds on what is of high abundance close to them (Grémillet, 1997). Although round goby DNA was found in regurgitated pellets from both Medhomarna and Norra Röden, the higher DNA concentrations in regurgitated pellets from Norra Röden in comparison to Medholmarna suggests that there is a difference in round goby population between the two locations (Table 5).

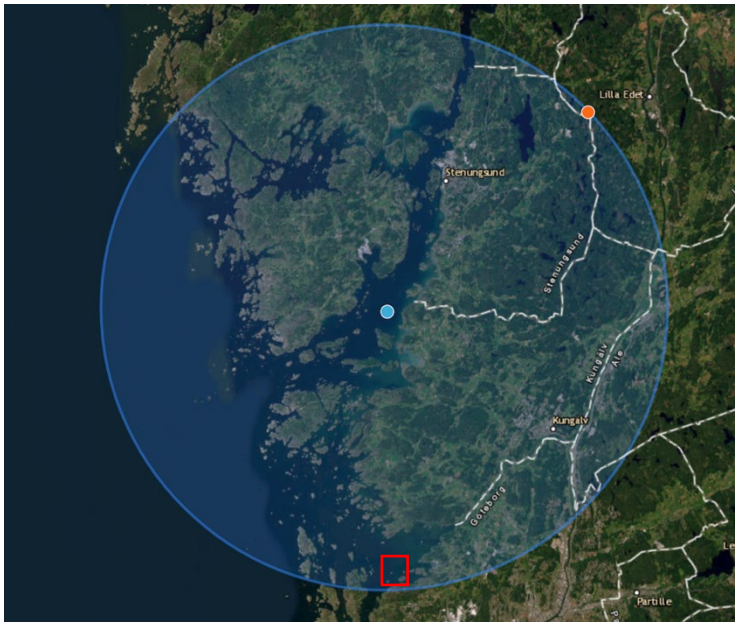

Figure 9. A satellite view of Medholmarna. Depicted in blue is the radius of 25 km which represents the foraging area of the great cormorant. The red square is the location for Norra Röden.

FUTURE RESEARCH

The results from this study indicate that the round goby is moving further north on the Swedish west coast. Future studies should therefore focus on regions north of Medholmarna to be able to detect the presence of round goby at an early stage and to prevent establishment in new areas. Great cormorant colonies also exist around the Koster archipelago which could be a key area for future research. Dietary sample analysis could be coupled with eDNA analysis on water samples in harbors and estuaries to provide a more detailed view of the presence of round goby. This can be especially important in regions where only a few round gobies exist which might not be enough to be detected in the regurgitated pellets. How abundant the round goby must be in an area for it to be detected in regurgitated pellet analysis remains uncertain so the sensitivity of this method could be an important area to investigate for future studies. GPS tracking of great cormorants in combination with analysis of regurgitated pellets could also provide information of where the round goby has been caught, which can facilitate future monitoring projects and management. Financial constraints limited this project to only 19 regurgitated pellets to be analyzed. Future large-scale projects should not only have a larger sample size but also sample pellets during different seasons for consecutive years to see if there is a trend in development of round goby populations. However, the use of regurgitated pellets of great cormorant does not have to be limited to round goby, the method could potentially also be used to detect the presence of other invasive fish species such as the black bullhead (*Ameiurus melas*) or prussian carp (*Carassius gibelio*).

CONCLUSION

The aim of this study was to investigate the usefulness of regurgitated pellets from great cormorants as a possible supplement to traditional monitoring techniques in detecting the presence of the invasive alien species round goby. As the result shows, lysis of regurgitated pellets and amplification of round goby DNA in dPCR was successful. High DNA concentrations were extracted from the pellets from both Medholmarna and Norra Röden. The majority of the samples from each location were positive and contained round goby DNA. However, the DNA concentration of round goby was significantly higher at Norra Röden in comparison to Medholmarna. This was as expected because the presence of round goby around Norra Röden was known since before but there were some question marks about its presence around Medholmarna. Although the exact location where the round goby was caught by the great cormorants cannot be determined, the concentration of round goby DNA in the pellets at Medholmarna still provides valuable insight into the presence of the species. Large-scale projects on great cormorants' regurgitated pellets can be an important addition for future conservation efforts and protection of our marine ecosystems.

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APPENDIX

Table 7. Estimated size and made observations from the dissected regurgitated pellets from Medholmarna and Norra Röden.

OBSERVATIONS		
LOCATION	**SIZE (ø cm)**	**DESCRIPTION**
Medholmarna	≤ 1	Small green/white pellet
Medholmarna	≤ 1	Small black/white pellet with a little bit of fish bones
Medholmarna	1 < X ≤ 2	Green/white regular size pellet
Medholmarna	≤ 1	Small white pellet with a little bit of fish bones
Medholmarna	1 < X ≤ 2	Green/white regular size pellet
Medholmarna	1 < X ≤ 2	Green regular size pellet with red content in the middle of the pellet
Medholmarna	1 < X ≤ 2	White/green regular size pellet
Medholmarna	≤ 1	Small green pellet
Medholmarna	> 2	Large pellet with a lot of fish remains and bones
Medholmarna	> 2	Large black pellet with black materia within with small amount of bones
Medholmarna	> 2	Large black/greyish pellet with a lot of fish bones
Medholmarna	≤ 1	Small green pellet
Medholmarna	≤ 1	Small green/white pellet
Norra Röden	> 2	Large grey pellet with a lot of fish bones and otoliths
Norra Röden	1 < X ≤ 2	Brown/red regular size pellet with a lot of bones and jaws.
Norra Röden	1 < X ≤ 2	Black regular size pellet with 2 stones and wood-like materia within
Norra Röden	1 < X ≤ 2	Black regular size pellet with stones and wood-like materia within
Norra Röden	1 < X ≤ 2	Black regular size pellet with a tiny bit of fish bones
Norra Röden	1 < X ≤ 2	Grey/white regular size pellet with a lot of fish bones
Norra Röden	> 2	Large grey/white pellet with a lot of fish bones
Norra Röden	1 < X ≤ 2	Brown regular size
Norra Röden	≤ 1	Grey/white small size pellet with a lot of fish bones
Norra Röden	1 < X ≤ 2	White/transparent regular size pellet with a lot of fish bones
Norra Röden	≤ 1	Small white pellet with a lot of fish bones
Norra Röden	1 < X ≤ 2	White regular size pellet with stones, red materia and a lot of fish bones within
Norra Röden	≤ 1	Small white pellet with a lot of fish bones and otoliths
Norra Röden	1 < X ≤ 2	White regular size pellet with a lot of fish bones within
Norra Röden	1 < X ≤ 2	Red/white/grey regular size pellet with some fish bones within
Norra Röden	> 2	Large red/brown/grey pellet with stones and a lot of fish bones within
Norra Röden	> 2	Large red/brown/grey pellet with lot of fish bones
Norra Röden	≥ 4	Really large red/grey pellet with worms and a few fish bones within
Norra Röden	≤ 1	Small red/green pellet
Norra Röden	≤ 1	Small brown/white pellet with stones within

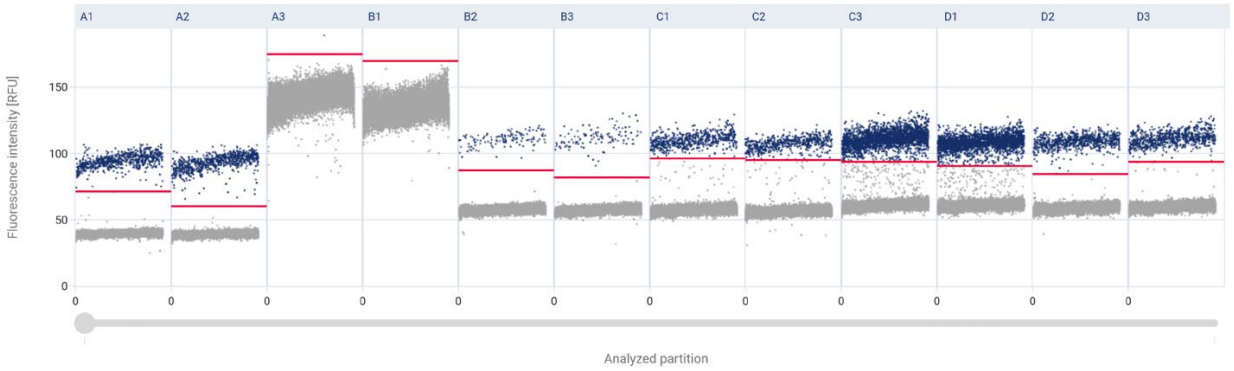

Figure 10a. The results from the second dPCR test from Norra Röden. On the y-axis is the fluorescence intensity and on the x-axis are the analyzed wells. The red line is the threshold which separates the negative partitions (depicted in grey) from the positive partitions (depicted in blue). A1-A2 is the positive control (PPC), A3-B1 is the diluted (1:10) sample 1, B2-B3 is sample 2, C1-C2 is sample 3, C3-D1 is sample 4 and D2-D3 is sample 5.

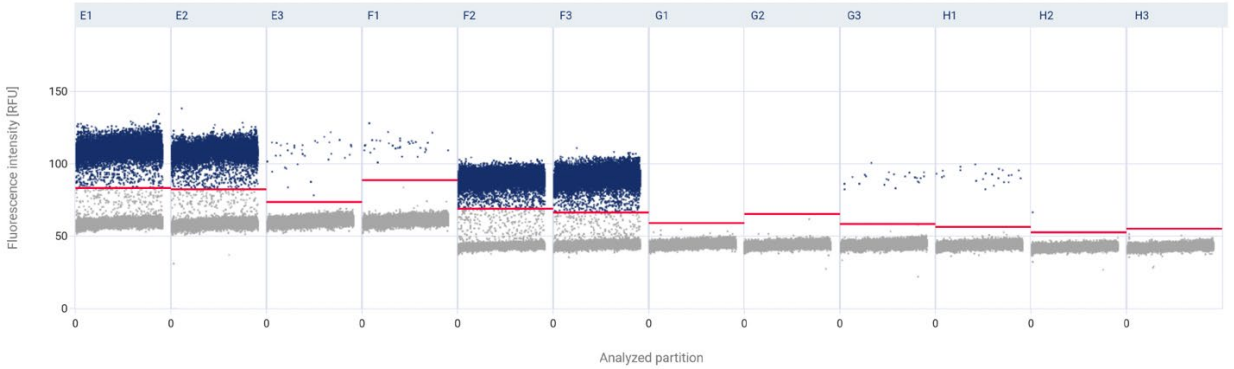

Figure 10b. The results from the second dPCR test from Norra Röden. On the y-axis is the fluorescence intensity and on the x-axis are the analyzed wells. The red line is the threshold which separates the negative partitions (depicted in grey) from the positive partitions (depicted in blue). E1-E2 is the diluted (1:10) sample 6, E3-F1 is sample 7, F2-F3 is the diluted (1:10) sample 9, G1-G2 is the blank sample from Medholmarna, G3-H1 is the blank sample from Norra Röden and H2-H3 is the no template control (NTC).