Evaluation of filter and extraction kits for eDNA-based quantification of Northern pike density

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Abstract

Multi-mesh gillnets are the most common gear in Swedish fish monitoring programs. The method works for many fish species but has limitations. The popular game fish Northern pike (*Esox lucius*) represents one of the species where the gillnet strategy has difficulties to reliably estimate density and to accurately provide data for reliable status assessments. Environmental DNA (eDNA) could be a useful complementary tool to standard surveys if it can provide reliable estimates of pike density.

The primary aim of this study was to investigate if eDNA could be used to quantify different densities of pike. This was tested in an experimental setup, using two outdoor tanks. One tank contained one pike and the second tank had eight pikes. Furthermore, another aim of the study was to investigate which filter combination and extraction kits were the best to use for the quantification of pike density. The underlying premise of quantitative eDNA is that there is a correlation between the amount of eDNA detected in the water and the actual biomass or abundance of the species.

The results revealed that it was possible to separate different pike densities, regardless of filter combination and extraction kits. The experiment also revealed that adding a GF/A filter to a CN filter did not yield more compare to what a single CN filter retrieved. This facilitates future studies due to faster filtration when using one filter and not two. Moreover, the experiment showed that Chelex as an extraction method yielded the most eDNA compared to the other two kits: Blood and Tissue and PowerWater. Nevertheless, these kits can be good choices for projects that do not have time for method development. PowerWater was the only extraction kit that did not need to be diluted and could perform qualitative amplifications in the qPCR, while Blood and Tissue was slightly faster and cheaper than PowerWater. Taken together, the benefits of Chelex makes it a strong candidate method for future monitoring of pike densities. Although there are several questions to address before eDNA can practically be used for monitoring pike.

Keywords: Blood and Tissue, Chelex 100, cellulose nitrate nembrane filter, COI, extraction kit, filter, glass microfiber filter, pike, PowerWater, quantitative eDNA

Popular science summary

Quantitative eDNA as a monitoring tool for Northern pike populations without the need to kill, harm or trap a single individual!

The use of DNA techniques have increased in the field of biology during the last decades and is now used for many different purposes. Not only in medical sciences but also for nature and resource management, which can now track species with the help of DNA. This method of sampling DNA in nature is called environmental DNA (eDNA). The new trend in aquatic research is to use the eDNA as a method to detect fish species from water samples, without seeing or catching a single individual. This is made possible by tracing the DNA that species release into the environment. This project tried to investigate the possibility to detect differences in Northern pike (*Esox lucius*) density, using eDNA as a quantitative method. The results revealed that it was possible to quantify differences in pike density in an experimental setup with two tanks containing different pike densities. These findings show that there is a potential for using eDNA for future monitoring of pike populations.

The method is straightforward. First, a certain amount of water was sampled, in this project, I filtrated 1 L of water per sample. The water was filtered through a filter that collected the eDNA particles. The next step was to extract the eDNA from the filter, which was done with equipment called commercial extraction kits. Next a machine, in this case qPCR, was used to amplify the extracted eDNA so that the amount of eDNA from the samples could be quantified. There are various types of filters and extraction kits. Therefore, the second aim was to investigate which extraction kit and filter combination yielded the highest eDNA concentration. The results revealed that using multiple filters did not collect more eDNA than a single filter. This is good news, future studies can save money and time, as it is faster to filtrate the water through only one filter. The results from the three extraction kits I tested revealed that the more advanced and expensive extraction kit performed the poorest. The one with the highest yield, which performed the best, was the least advanced and most inexpensive extraction kit.

In conclusion, quantitative eDNA can be used to detect differences in Northern pike density. Non-invasive monitoring methods have several benefits, and the results presented in this study highlight that eDNA methods have the potential to become a complementary fish monitoring tool. Especially for species where other monitoring methods are inadequate or lacking. This study has also highlighted the importance of using the right equipment to collect as much eDNA as possible for quantitative eDNA studies of fish.

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Abbreviations

BSA	Bovine Serum Albumin
BT	Blood and Tissue extraction kit
CH	Chelex 100 Resin extraction kit
CN	Cellulose nitrate membrane filter
COI	Cytochrome Oxidase 1
eDNA	Environment DNA
e.g.	For example
GF/A	Glass microfiber filter
i.e.	Meaning/that is
PW	PowerWater extraction kit
qPCR	Real-Time PCR system
SLU	Swedish University of Agricultural Sciences
SYBR Green	Fluorescent dye system
Tank A	Low density (1 pike)
Tank B	High density (8 pikes)
TaqMan	Probe based fluorescent dye system

1. Introduction

Management and conservation of wildlife are dependent on accurate monitoring of population statuses. For aquatic organisms, such as fish, there are extra dimensions that make monitoring a challenge. Many fish species are scattered over large areas in lakes and seas, both in horizontal- and vertical directions (Lacoursière-Roussel et al. 2016a; Cowart et al. 2018), which makes them harder to monitor. However, it is possible to follow the trends of fish stocks, such as for Atlantic cod (*Gadus morhua*), (Jonsson et al. 2016), brown trout (*Salmo trutta*), (Lobón-Cerviá 2009) and bluefin tuna (*Thunnus thynnus*), (Bravington et al. 2016). Although many methods are possible, a common approach is to use an index for describing population trends, measured as the catch per unit effort (CPUE) collected in monitoring. Another method is mark-recapture, which is based on probabilities of recapturing previously marked individuals to estimate total population sizes (Bravington et al. 2016). Importantly, tools used for monitoring should be standardized in order to be able to compare population development over time.

In Sweden, multi-mesh gillnets are the most common standardized fish monitoring gear in lakes and coastal waters (Ådjers et al. 2006; Olsson 2019). The method works for many fish species but, as all methods, it has limitations. Firstly, the fish need to actively swim into the nets. Secondly, gillnets are also size-selective, meaning not all age and size classes of the population are caught in the nets. Thirdly, the gillnets are also species selective (Ådjers et al. 2006). Thus, gillnets only catch fish in the area where it is set and active species of a specific size range, due to the set-up of mesh sizes of the gillnet (Teixeira-de Mello et al. 2014). Another factor that needs to be taken into consideration when monitoring with gillnets is that it is only performed in a limited number of places, meaning that only a few targeted fish stocks are monitored. This has resulted in some fish species being poorly represented in monitoring programs, which is a challenge for efficient management. In Sweden, there are about 150 fish species (Vanhoenacker 2015). Based on coastal monitoring using multi-mesh gillnets about 10 species, out of 38, have been caught in sufficient quantity to be able to perform reliable analysis (Ådjers et al. 2006). Examples of fish species where the gillnet strategy has difficulties to reliably estimate density and to accurately provide data for reliable status assessments are the Northern pike (*Esox Lucius*) and brown trout (Ådjers et al. 2006). Thus for pike

the traditional monitoring methods need to be complemented or replaced with new methods, not just because of the current lack of long-term monitoring data but also of declining trends in the Baltic Sea (Ådjers et al. 2006; Olsson 2019). The exact reasons for the decline are not completely known but have been coupled to large scale changes in the Baltic Sea and increased stickleback populations (Eriksson et al. 2009; Ljunggren et al. 2010; Eklöf et al. 2020)

1.1. The importance of the Northern pike

Pike lives in freshwater and brackish habitats across the temperate zone and is regarded as important, not only for ecosystem function but also for both recreational and commercial fisheries (Pierce 2012; HaV 2020). As a solitary and stationary keystone predator, the pike is a regulating factor in the food web (Eriksson et al. 2009; Crane et al. 2015; Larsson et al. 2015; HaV 2020). In Sweden, pike is one of the most popular fish species for recreational fishing (HaV 2019, 2020) and is especially targeted as a game fish due to, its potentially large size (Pierce 2012; Forsman et al. 2015; HaV 2020). Today coastal recreational fishing lands ten times as much pike per year as commercial fisheries, while landings are approximately similar in the five largest lakes in Sweden (HaV 2020). The majority of pike caught in recreational fisheries are however released back into the water. The national estimate is, that the release rate is greater than 80% (SCB 2019).

The important ecological role and large sport fishing interest emphasize the need for a more reliable monitoring of pike populations, both in coastal and freshwater habitats. As have been mentioned multi-mesh gillnets perform poorly for monitoring pike populations and according to yearly reports of Swedish fish monitoring in the large lakes, gillnets catch less than one pike in every 20th net (HaV 2020). The low catch rate from standard surveys is not sufficient to be able to follow the pike population structure to a satisfactory degree. New and less invasive techniques are currently being developed and one interesting tool is the use of DNA from the organism.

1.2. Environmental DNA as a monitoring tool

A new tool that might be able to monitor pike is environmental DNA (eDNA). EDNA is the DNA that organisms release into the environment (Klymus et al. 2015), for instance by secretion, feces, or shedding of body tissue such as skin or mucus (Takahara et al. 2012; Cowart et al. 2018; Tillotson et al. 2018). The DNA fragments that are released can be collected in water, sediment, or earth samples (Klymus et al. 2015), in both terrestrial and aquatic environments (Jelger et al.

2014; Klymus et al. 2015). The main uses have been the detection of species distribution and mapping of biodiversity and community composition (Cowart et al. 2018). The eDNA method performs well both for common and endangered species (Frøslev et al. 2019), as well as for micro- (Kaevska & Slana 2015; Abdelfattah et al. 2018) and macro organisms (Cowart et al. 2018; Majaneva et al. 2018a; Tillotson et al. 2018).

The eDNA sampling method has been proven to be more efficient than many visual detection monitoring strategies (Doi et al. 2017; Itakura et al. 2019). A study that compared fungi communities in soil showed that eDNA detected more species than traditional methods (Frøslev et al. 2019). Similar findings from aquatic communities, where eDNA methods also detect more species than other methods, such as detection of macro/meiofauna and invasive species in the West Antarctic Peninsula (Cowart et al. 2018). Another study by Itakura et al. (2019) compared electrofishing and eDNA to monitor the Japanese eel (*Anguillid japonica*), in Japanese rivers. The eDNA method detected the eels to a greater extent than the electrofishing in running water, where eDNA could collect samples in areas unavailable to electrofishing, for example in deeper water. The results also showed that the collection of eDNA was faster in comparison.

According to several aquatic studies, eDNA has benefits compared to traditional monitoring. Firstly, eDNA is more efficient to detect scarce species and species with large territory ranges (Cowart et al. 2018; Itakura et al. 2019). Secondly, there is no need to catch the fish which will save time and money for management (Lacoursière-Roussel et al. 2016a; Cowart et al. 2018; Itakura et al. 2019). Thirdly, from an animal welfare perspective, a lesser need to catch or kill individuals is in line with the three R's of animal research reduce, refine, and replace, (Curzer et al. 2016) which is especially relevant for endangered species or small populations (Tillotson et al. 2018; Itakura et al. 2019). In the United States, Pierce (2012) conducted a study on pike that highlighted the ethical dilemma with gillnets as a monitoring tool in lakes. The study showed that the frequent use of gillnets on small pike populations might lead to increased mortality of older pike compared to younger individuals, which in turn might affect the size structure of the population.

1.3. The new area of use, quantitative eDNA

The recent development of the eDNA technique is to try to follow fish stocks by measuring the eDNA concentration in water samples and compare it in relation to fish biomass/abundance, which is referred to as quantitative eDNA. Several international studies have tried to measure eDNA concentration in different aquatic environments to estimate the abundance and biomass of a broad range of species,

(Takahara et al. 2012; Thomsen et al. 2012; Lacoursière-Roussel et al. 2016b; a; Sassoubre et al. 2016; Doi et al. 2017; Itakura et al. 2019; Jo et al. 2019). The underlying premise is that there is a correlation between the amounts of eDNA detected in the water and the actual biomass or abundance of the species. (Thomsen et al. 2012; Lacoursière-Roussel et al. 2016b; a; Sassoubre et al. 2016; Doi et al. 2017). A clear example from northern Europe is a study that revealed a significant difference in the amount of eDNA of two amphibian species in the water. The reasons were size and activity, the toad larvae (*Pelobates fuscus*) were bigger and more active and therefore shedded more eDNA than the newt larvae (*Triturus cristatus*) (Thomsen et al. 2012). Another study showed a correlation between eDNA and fish abundance, by comparing eDNA levels in the water to the quantity of fish caught in multi-mesh gillnets (Lacoursière-Roussel et al. 2016a). In summary, several studies suggest that eDNA quantification can be related to biomass or abundance estimated with other monitoring methods.

1.4. Challenges with quantitative eDNA

Even though quantitative eDNA seems to have good potential to be used as a tool for estimating population abundance/biomass of pike, the method is under development and many factors need further research. Most of the current knowledge originates from eDNA studies on presence/absence of species, that have been modified to serve quantification purposes. Furthermore, there is an overlap between the different eDNA research groups where some studies combine biodiversity with quantification studies e.g. (Thomsen et al. 2012), while others focus only on biodiversity (Abdelfattah et al. 2018) or extraction methods (Majaneva et al. 2018b). Many studies use the same or similar equipment but study biodiversity (Abdelfattah et al. 2018) or species distribution (Cowart et al. 2018) which have different aims. However, the problems that exist within the 'eDNA community' are the same or similar and therefore other researchers' discoveries are of interest, to understand the complex interaction eDNA has to its environment (Jelger et al. 2014). Some examples that are not yet fully understood are how much DNA an organism releases to its environment, how fast it is released as well as the degradation rate (Lacoursière-Roussel et al. 2016a; Doi et al. 2017; Li et al. 2020). Moreover, the degradation rate may vary due to water temperature, (Lacoursière-Roussel et al. 2016b) and other abiotic factors (Li et al. 2020).

It is not yet possible to apply a standard method for quantitative eDNA for all aquatic environments (Deiner et al. 2015; Eichmiller et al. 2016; Piggott 2016). Except for the aforementioned reasons there are other examples, such as different aquatic organisms shed different amounts of eDNA (Thomsen et al. 2012), where fish has been observed to shed more than others, mainly in the form of mucus and

that fish have a relative big body size compared to many other organisms. This generally makes fish easier to detect through eDNA (Jelger et al. 2014). Klymus et al. (2015) conducted a tank experiment to gain a deeper understanding of the shedding of eDNA. They found different levels of eDNA in samples taken from the same individual fish. They hypothesized that the distribution of eDNA in the water from shedded materials such as feces or cells was not evenly spread. However, Thomsen et al. (2012) argued the contrary, that the eDNA was evenly spread in the pond they researched, based on the detection rate and biomass of fish and other aquatic- and semi-aquatic species from different taxa.

Other reasons that hinder the implementation of a standardized method for all aquatic organisms are that, depending on the aim of the project, researchers will face different trade-offs. Projects that want to gain high levels of eDNA may invest more money in premium equipment to obtain the most reliable results possible. Other projects might prefer the fastest or cheapest methods which might result in lower amounts of eDNA in the product but also cover larger study areas (Piggott 2016). Therefore, no consensus currently exists on which is the best way to collect eDNA for quantification purposes (Eichmiller et al. 2016). The amount of eDNA that is collected in a sample is referred to as yield, which has been defined as:

> "The total number of copies recovered in the DNA extract" (Eichmiller et al. 2016), p64)

At present, there is no single standardized method, and thus it is almost impossible to select the eDNA method for pike monitoring. Below I give some brief explanations and list some of the factors that affect the amount of eDNA yield, i.e. collection, extraction and amplification.

1.4.1. Collection

There is a trade-off at every step when it comes to eDNA projects (Eichmiller et al. 2016; Piggott 2016). For starters, the probability to detect the eDNA of the target species depends on the first decision, the collection of water samples, which is the amount of water volume that will be collected and the number of samples collected per site. For some projects, the best method is to collect a large amount of water per sample. For other projects, such as remote fieldwork or projects that want to cover larger areas, collecting a lower volume of water per sample, but more samples per site might be preferable (Piggott 2016). For quantitative eDNA, the aim is to collect as much eDNA as possible from the target species, which normally means that a larger volume of water needs to be collected from the study site, the bigger the volume of water that is filtered, the more eDNA is collected (Minamoto et al. 2016; Piggott 2016; Hunter et al. 2019; Li et al. 2020). Usually 1-2 L of water is filtered

per sample, where 1L has appeared to be a standard volume and used in several projects (Lacoursière-Roussel et al. 2016b; Piggott 2016; Majaneva et al. 2018a; Itakura et al. 2019; Capo et al. 2020b; a). Another factor to include in the sampling effort is, how many samples from each site that are needed. It depends on the project and the detectability of the species. In a pond experiment Piggott (2016) found that depending on the sampling method and the extraction kit that was used, the detectability of their target fish species differed between the methods, even with the same sampling effort.

The most used method to collect the eDNA from the water is, filtration through membrane filters (Kaevska & Slana 2015; Hinlo et al. 2017; Spens et al. 2017; Majaneva et al. 2018a; Capo et al. 2020b). There are different types of filters and the filters have various pore sizes. It can be a trade-off between the combinations of water volume, filter type and the pore size of the filter. For example, smaller pore size, as 0,2 µm, cannot filtrate large water volumes without risk of clogging (Minamoto et al. 2016; Hinlo et al. 2017; Hunter et al. 2019; Capo et al. 2020b). However, a smaller pore size seems to yield more eDNA (Minamoto et al. 2016). The pore size should be determined according to water quality. Filter with larger pore size is favourable for water samples with flowing water or when the water is turbid and contains lots of organic compounds (Turner et al. 2014; Kaevska & Slana 2015). Another method is to collect smaller volume of water, to minimize the risk of clogging if sampling with small pore size in those water conditions (Turner et al. 2014). An important factor is that larger pore sizes are capable to filtrate larger water volumes which increases the chance to collect the eDNA from the target species and lessens the risk of clogging. Nevertheless, smaller eDNA molecules, such as fragmented eDNA molecules will be lost, with the use of larger pore size (Deiner et al. 2015). The solution to this problem has been to find the optimal filter type with the right membrane that absorbs the most eDNA molecules. There are two types of filter categories that are used that seem to bind the most eDNA; the surface and depth filters. Surface filters are filters where particles only attach at the surface of the filter, for example, polyethersulfone- and polycarbonate track-etched filters. Depth filters have the same attribute but eDNA particles are also maintained within the filter membrane. Examples of such filters are cellulose nitrate-, glass fiber- and mixed cellulose ester filters. These filters collect more eDNA (Hinlo et al. 2017) and are commonly used (Eichmiller et al. 2016; Piggott 2016; Sepulveda et al. 2018; Capo et al. 2020b).

Studies indicate that various filter types differ in efficiency and that cellulose nitrate filters with a medium pore size yield more eDNA than other filters (Hinlo et al. 2017; Majaneva et al. 2018a). However, Capo et al. (2020b) argue that the Sterivex enclosed filter with medium pore size 0,45 µm outperformed Cellulose filters. The

difference in results is not necessarily only due to the efficiency of the filters but that the studies are different from each other. One experiment uses tank or aquarium water (Hinlo et al. 2017) while another uses field water samples, which leads to different results (Minamoto et al. 2016). There is an awareness among eDNA researchers that similar experiments might get contradictory results. It appears that eDNA has a complex relationship to the environment and that seemingly small differences in the sampling strategies that are used, such as choice of filter, pore size, water volume, or other choices are important factors (Deiner et al. 2015; Eichmiller et al. 2016; Hinlo et al. 2017; Majaneva et al. 2018a).

1.4.2. Extraction

After filtration of samples, the next step is the choice of strategy for extracting the eDNA. There are many different extraction methods and products available. Each choice affects the amount and quality of eDNA extracted from the samples. The most common extraction methods are commercial extraction kits, PCI and precipitation (Deiner et al. 2015; Piggott 2016; Sassoubre et al. 2016). The focus here will be on the extraction method using commercial extraction kits. When choosing extraction kit, it is important to know which kit will benefit the aim of the project. For example, one extraction kit that is cost- and time efficient might suit projects where fast result are desired. Another extraction kit might be more expensive and extracts more eDNA and will do better for projects that require high eDNA yield (Schiebelhut et al. 2017). Another favourable trait is that the extraction kit is robust against inhibition (see section 4.5. Inhibition), especially for samples collected from flowing- or turbid waters. One example can be found in Majaneva et al. (2018b), whom compared the performance of extraction kits in a metabarcoding project and concluded that the PowerPlant extraction kit was the best choice compared to the other two extraction kits tested, due to PowerPlant had higher resistance against inhibition. For quantification studies inhibition is a problem that must be addressed, otherwise, it may lead to misleading results and an underestimation of eDNA levels in the samples (Eichmiller et al. 2016).

1.4.3. Amplification

After extraction, the amount of eDNA in the sample will not change. However, how much of the eDNA that will amplify depends on various factors. A few will be mentioned and explained here briefly. Real-time/Quantitative polymerase chain reaction (qPCR) is a commonly used technique in eDNA studies (Thomsen et al. 2012; Klymus et al. 2015; Olsen et al. 2015; Eichmiller et al. 2016; Cowart et al. 2018). QPCR, as well as traditional PCR, rely on thermal cycling, where the targeted DNA sequence becomes copied in a series of cycles of heating and cooling. This enables, in real time, a detection and estimation of the number of copies of the

chosen eDNA sequence that are in the samples (Lederman 2009; Tajadini et al. 2014). The detection of eDNA copies is performed using dyes that contain a fluorescent, which contains dye or probes that bind to the sequence of the nucleic acids and immediately gives off light. This light gets detected by the qPCR for each PCR cycle (Tajadini et al. 2014).

It is important to choose the right gene that is easy to amplify but still species specific to avoid amplification from other species (Tekaya et al. 2017). It is also important to have the right primers for the chosen gene sequences, preferably short ones. There is a greater chance that fragmented eDNA contains the exact short sequence, which most likely will result in higher detection of the dyes and increase amplification levels (Thomsen et al. 2012).

The two most common dyes for qPCR are SYBR Green and TaqMan (Tajadini et al. 2014; Gomes et al. 2017). SYBR Green is less expensive and is a nonspecific DNA-binding dye that attaches to double-stranded DNA. TaqMan is a sequence-specific hydrolysis probe with a fluorophore that attaches to the specific single-stranded DNA (Tajadini et al. 2014). The choice of TaqMan, SYBR Green, or other fluorescents have an influence on how well the targeted eDNA will be amplified or be detected due to the different tolerance of inhibitors (Glover et al. 2016). Inhibitors interfere with the amplification process and can mask high copy numbers (Jane et al. 2015). For quantification projects, it will be impossible to follow population density/abundance if inhibition changes the amplification success from year to year or for each batch that is run by the qPCR. Therefore, it is important to choose the right equipment for the project and try to include all the complex interactions when dealing with eDNA. The recommendation is to do pilot studies before any real samples are collected (Jelger et al. 2014).

1.5. The aim of this study

This study was motivated by the need to develop efficient and non-invasive monitoring methods for pike and other ecologically important fish species that are hard to monitor with standard surveys. Recent developments in eDNA techniques suggest that quantitative estimates can be obtained (Thomsen et al. 2012; Lacoursière-Roussel et al. 2016b; a; Sassoubre et al. 2016; Doi et al. 2017), but if this is also true for Swedish pike remains unknown. Hence, the primary aim of this study was to investigate if it is possible to use quantitative eDNA as a method to measure different densities of pike. Due to the lack of a consensus on which equipment combinations are best to use, this study also tried to develop more robust protocols in terms of which filter combination and extraction kits are best to use for

the quantification of pike density. These questions were addressed by comparing eDNA levels from the high and low density of pike in two tanks.

The hypotheses were:

I. Quantitative eDNA can be used to measure differences in pike density.
II. A single filter will catch less DNA than a combination of different filter types.
III. The most advanced extraction kit should extract more eDNA than other less advanced extraction kits.

2. Materials and methods

2.1. Experiment setup

The project was conducted at the Institute of Freshwater Research of SLU, Stockholm, Sweden. The project consisted of two parts; first to prepare water with pike eDNA and to collect water samples; second to extract samples and amplify eDNA (Figure 1).

The experiment started on the 22nd of May 2020, with nine pikes of similar size and weight being collected from a local fisherman from Lake Mälaren. The pikes were separated in two densities in outdoor tanks, with one single pike (1,3 kg) in one tank (tank A) and eight pikes (mean 1.2±0.27 kg) in the other tank (tank B). The tanks were equipped with handmade artificial seaweed to reduce stress and function as sun cover. Each tank was covered with a net to prevent the pike from escaping. The tanks were filled with continuous flow through water from Lake Mälaren filtered through a sand filter. The water temperature was ≈ 9 °C. Each tank could accommodate 7000 L of water, only one third ≈ 2 300 L/tank were filled with water. Pikes were kept in the tanks for six days to acclimatize and were not fed, to avoid any differences in eDNA due to feces.

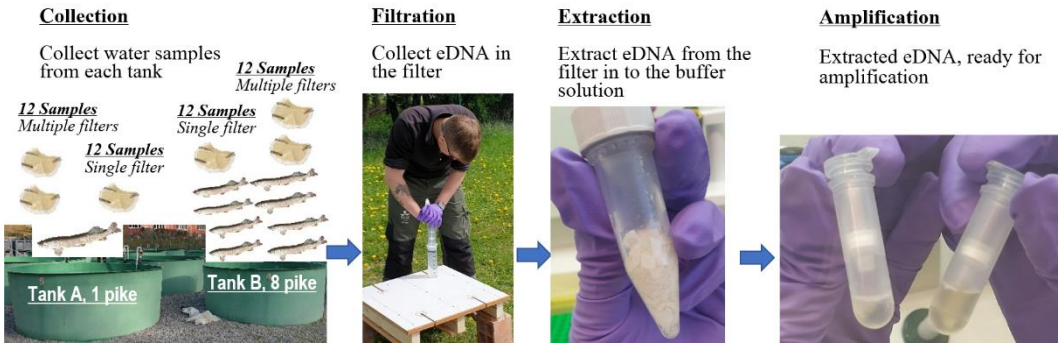

Figure 1. The main steps of the project, installing the pike in the tanks, collecting samples by filtration, extracting the samples with different extraction kits and amplification using qPCR.

2.2. Collection of water samples

Water samples were collected on the 28th of May. All samples were filtered in direct adjacency to the tanks. To avoid samples from tank A being contaminated by tank B, the samples from tank A were filtered first, which were hypothesized to have a lower concentration of eDNA than tank B. From each tank 30 L of water was collected. The assumption was that the eDNA in the tanks should be equally spread in the water because small eDNA particles should move around in the tank, due to the diffusion from the flow-through. Two filter types were used (see section, 2.3. Filter) and divided equally between the two tanks (Table 1). Twenty-four samples were collected from each tank. A total of forty-eight samples were collected. Each sample filtrated 1 L of water. The filtered samples were folded, individually placed in small zip-lock bags and directly placed in a portable freezer.

To avoid contamination gloves were changed between each sample. Other safety measures were to use new tweezers for each sample. Before use, filter holders and syringes were sterilized in a 10% chlorine bath. Moreover, there were a safety distance between the workplace for filtration, the storage for used equipment, the freezer and the tanks. After filtration, the samples were stored in a regular freezer -20 °C inside the laboratory.

2.3. Filter

Two filter types were used, cellulose nitrate membrane filter with 0.8 µm pore size (CN) as the single filter and glass microfiber filter (GF/A) together with CN as multiple filters (Table 1). These two filters have a good binding efficiency (Lacoursière-Roussel et al. 2016b; Hinlo et al. 2017), and therefore useful for quantification of eDNA. The filters were assembled on Swinnex filter holders. In turn, the filter holders were assembled on syringes that hold 300 mL of water.

2.4. Extraction

The samples were extracted by using three different extraction kits: Qiagen DNeasy® Blood & Tissue (BT), Qiagen DNeasy® PowerWater (PW) and Chelex® 100 resin (CH). Each extraction kit extracted sixteen samples each of a total of forty-eight samples. A crossed design was used to equally divide the combination of samples between the extraction kits. Moreover, each combination had four sample replicates, (Table 1).

Table 1. The number of samples used to investigate which of the different filter combinations and extraction kits were best suited to detect differences in pike density between the two tanks. Cellulose nitrate membrane filter (CN) and glass microfiber filter (GF/A)

Treatment, high/low density of pike	**Density** 1 pike	**Density** 1 pike	**Density** 8 pikes	**Density** 8 pikes
Filter combination	CN	CN + GF/A	CN	CN + GF/A
Extraction kit: Chelex	4	4	4	4
Extraction kit: Blood and Tissue	4	4	4	4
Extraction kit: PowerWater	4	4	4	4

Two of the three extraction kits followed a similar workflow but with individual differences in some steps and with added modifications for this project, which will be described later. The main steps were 1, lysis, 2, precipitation, 3, purification and 4, elution (Figure 2). The workflow was as follows; all samples were thawed at room temperature, cut to smaller pieces of ≈ 3 mm and stored in labeled tubes. The next step was lysis, where the samples were mixed with a buffer solution that dissolves the eDNA into the solution. The sample was centrifuged to separate solid particles and the filter from the solution. The solution was then transferred to a new tube and the solid material, called pellet, from the first tube was discarded along with the shredded filter. Next was precipitation, which collected DNA from the buffer. A liquid solution was added, the eDNA no longer became soluble and instead started to precipitate to the surface of the solution. The penultimate step was purification, which was to clean the eDNA from unwanted particles. Different solutions were filtered through a filter inside the spin column tube, the eDNA particles were absorbed by the filter membrane so that particles such as minerals or organic materials were removed. With the aim to make the samples contain as pure eDNA as possible. In the final step, elution, a buffer was added that released the eDNA from the filter membrane into the solution in the tube. Now, the samples were ready for amplification (Figure 2).

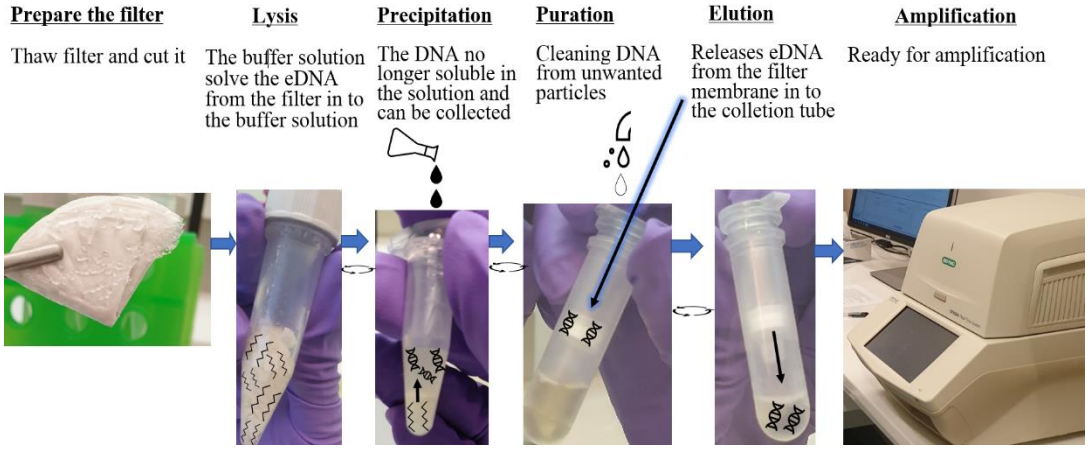

Figure 2. A basic flow chart of the main steps for the extraction. Between several steps were the samples centrifuged to separate solid particles which ended up on the bottom of the tube and the eDNA remained in the solution above the solid particles. Vortex was used to faster mix the different solutions with each other.

All samples for each extraction kit were extracted in two batches, half in the forenoon and the second half in the afternoon, due to limited workspace in the fume hood. Further, six negative controls were extracted, one for each batch. The extracted samples and negative controls were labelled and stored in the freezer at -20 °C.

To avoid contamination of the samples all work in the laboratory was performed under a fume hood. Before starting the extractions and between different batches the workspace was sterilized with bleach of 10% and RNase Away. Further, gloves were frequently replaced between batches of samples and if any solution was spilled. Non disposable equipment such as scissors and tweezers were instead sterilized with 95% ethanol and burner between each sample. Another safety measure was that work with extraction kits took place in one room and work with PCR and products with high DNA concentration were carried out in another room.

2.4.1. Modification of the PowerWater protocol

The DNeasy® PowerWater protocol was followed for PW samples with two modifications, to extract more eDNA from the samples. First, the vortex time of the bead tube was extended to 10 min instead of 5 min. The second change was in the last step, where the EB Solution was reduced to 70 µL per sample instead of 100 µL.

2.4.2. Modification of the Blood and Tissue protocol

For the extraction with BT the Qiagen DNeasy® Blood and Tissue protocol was used with some modifications. The filters did not fit into the original eppendorf

tubes and instead, 5mL eppendorf tubes were used. The ATL buffer was increased to 370 µL instead of 180 µL and for the proteinase K increased to 30 µL instead of 20 µL, to cover the whole samples. The lysis stage was performed overnight the night before extraction for all the samples.

2.4.3. Modification of the Chelex protocol

For the extraction with CH more deviations were made from the original protocol. The modified protocol of Chelex® 100 resin, see Appendix 1. The CH samples were prepared the day before extraction. The frozen samples were placed individually in sealed coin envelopes. The coin envelopes were placed in ziplock bags filled to one third with Silica Gel. The samples were labeled and stored at room temperature overnight to dry. This was done to standardize the sample volume as CH, unlike the other methods, did not include a step where DNA was concentrated with a specific µL amount of buffer at the end of the process. For extraction, each sample was placed in a 5ml eppendorf tube containing 1500 µl 5% Chelex® 100 resin solution.

2.5. Amplification

To estimate eDNA levels in the samples the mitochondrial gene Cytochrome Oxidase 1 (COI) was used targeting a 94 base pair long DNA sequence. The primers and TaqMan probe used were developed by Olsen et al. (2015, 2016) and have previously been used successfully in pike monitoring (Dunker et al. 2016). The primer and probe sequences were:
F-primer: 5'-CCTTCCCCCGCATAAATAATATAA-3',
R-primer: 5'- GTACCAGCACCAGCTTCAACAC-3', for the
probe 5'- FAM-CTTCTGACTTCTCCCC- BHQ1- 3' (Olsen et al. 2015, 2016; Dunker et al. 2016). The reaction buffer (assay) was used from Dunker et al. (2016) protocol with slight modifications. Each well was filled with; 4,4 µL of sterilized H_2O, 7,5 µL TaqMan Environmental Master Mix 2.0, 0,03 µL forward primer, 0,03µl reverse primer 0,03 µL Probe (200nM final concentration each), and 3 µL of DNA template for a total well volume of 15 µL. The assay was pipetted in a Hard-Shell® 384-Well PCR plate.

Furthermore, a standard curve was made from a piece of pike tissue extracted using the standard DNeasy® Blood & Tissue protocol. A serial dilution protocol in 1:10 was made in eight steps. Each sample was filled in four wells as technical replicates. The eight samples from the standard curve did as well have four technical replicates each. To measure the contamination four wells were filled with purified H2O and functioned as negative controls. The prepared plate was fitted in the thermal cycler ready for amplification. A Bio-Rad CFX384™ Real-Time PCR system (qPCR) was

used, the thermal cycling protocol was; activation one cycle 95°C for 10 min, quantification 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Temperature and time that was set according to primers and probe for amplification to occur.

Several protocols were made with different levels of the buffer, where some plates were run in the qPCR with Bio-Rads SsoAdvanced™ Universal SYBR® Green Supermix (SYBR Green). The SYBR Green assay could not produce amplification for most of the samples (data not shown), likely due to inhibition. Therefore, a TaqMan assay was used instead. The TaqMan assay performed better even though Chelex- and BT samples had to be diluted with a level 1:8 to further alleviate the problem of inhibition. The dilution gave three displacement steps of the cycle in the qPCR.

2.6. Technical equipment

The computer programs that were used; CFX Maestro™, visualized the amplification from the qPCR and converted the data to Excel files. R (The R Foundation) and Rstudio (RStudio Team) along with the car package (Fox & Weisberg 2019) were used to analyse the Excel data from CFX Maestro. For the full list of materials that were used see appendix 2.

2.7. Statistical analysis

The statistical analysis was done in two steps with two separate models due to a handling error in the laboratory during the extraction process. Although the samples were extracted, the identification of the samples was lost and it became unknown to which treatment they belonged. Therefore, these samples were excluded from the analyses (Table 2).

For both analyses, the technical replicates of each sample were first aggregated and the mean values were used. Second, to meet the assumption for the ANOVA test, log transformation was required (using the natural logarithm) to fit the standardized residuals. That was followed up with Shapiro-Wilk's test for normality and Levene's test for homogeneity of variance. For both analyses, there was no statistical significance for either of the groups, the assumption was that they were normally distributed and there was homogeneity of variance within different groups. Thirdly, the analyses started with a full model including all possible interactions, which was subsequently simplified by reducing non-significant interactions. The models used eDNA concentration as the response variable and three factors: pike density, filter and extraction kit as explanatory variables. For BT

and CH, a three-way ANOVA was used to evaluate if filter, extraction kit and pike density could explain the eDNA concentration levels (Table 3). While a two-way ANOVA was used for PW separately (Table 4).

Table 2. Realised number of samples used in the analyses, per extraction kit, filter and pike density. The two filter combinations were cellulose nitrate membrane filter (CN) and glass microfiber filter (GF/A)

Number of samples for each extraction kit			
Factor		**Pike density**	
Extraction Kit	*Filter combination*	*High, Tank B*	*Low, Tank A*
Blood and Tissue	CN	4	4
	CN+GF/A	4	4
Chelex	CN	4	4
	CN+GF/A	4	4
PowerWater	CN	3	2
	CN+GF/A	1	2

3. Results

After amplification, negative controls from the extraction and from the qPCR plate revealed that none of them contained any detectable amount of pike DNA. The results from the amplification have credibility and shows that the measures taken against contamination were sufficient.

3.1. ANOVA results for Chelex and Blood and Tissue

The three-way ANOVA was reduced to main effects and showed that the different types of extraction kits had an effect on eDNA concentration. Further, pike density also had an effect on eDNA concentration (Table 3). The samples from tank B contained higher levels of eDNA for both extraction kits than the samples from tank A, which had lower levels of eDNA concentration (Figure 3).

*Table 3. The three-way ANOVA table on main effects shows statistical significance for pike density (high and low) and the same for extraction kit (Blood and Tissue and Chelex). There was no difference in eDNA concentration regardless filter type (*cellulose nitrate membrane- *and* glass microfiber filter*).*

	Sum Sq	***Df***	***F***	***P***
Intercept	87.65	1	368.87	<0.001
Extraction kit	4.00	1	16.84	<0.001
Pike Density	165.85	1	697.99	<0.001
Filter	0.075	1	0.317	0.578
Residuals	6.653	28		

3.2. ANOVA results for PowerWater

The two-way ANOVA for PW showed an effect of pike density on eDNA concentration. Filter did not affect the eDNA concentration (Table 4). The samples from tank B contained higher levels of eDNA than the samples from tank A, which had lower levels of eDNA concentration.

*Table 4. The Two-way ANOVA table shows statistical significance for pike density (high and low) for PowerWater. There was no difference in eDNA concentration regardless filter type (*cellulose nitrate membrane- *and* glass microfiber filter*).*

	Sum Sq	***Df***	***F***	***P***
Intercept	160.62	1	29.60	<0.01
Pike Density	71.97	1	13.26	0.015
Filter	12.06	1	2.22	0.196
Residuals	27.13	5		

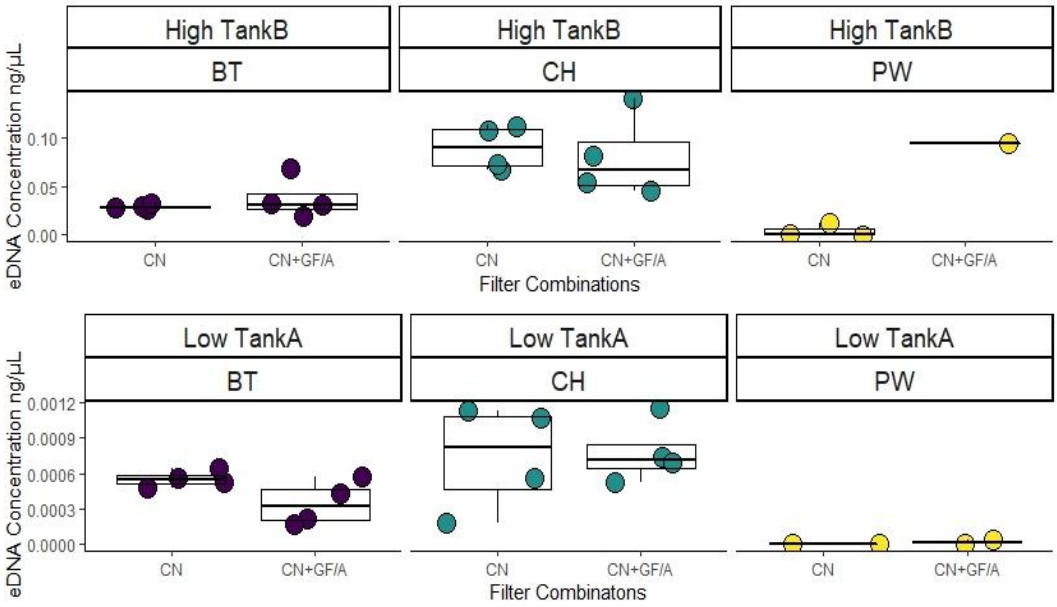

Figure 3. The boxplot shows how much eDNA each combination yielded in eDNA concentration (ng/µL), for the extraction kits: Chelex (CH), Blood and Tissue (BT) and PowerWater (PW) as well the efficiency of the two filter combinations for the two filter types: cellulose nitrate membrane filter (CN) and glass microfiber filter (GF/A). The upper row show samples collected from tank B with high density of pike and the lower row samples collected from tank A with low density of pike. The line in the boxes represents the median, the lower and upper ends of the boxes show quartiles (25th and 75th). The vertical line whiskers represent the lowest and highest observation points of the data set. The dots are the sample replicates (mean of four technical replicates).

4. Discussion

4.1. Quantifying eDNA concentration

The main aim of this project was to find out if quantitative eDNA could be used as a method to measure different densities of pike. Further, other aims were to investigate which extraction kit and filter combination yielded the highest eDNA concentration. The results revealed that the samples from tank B, with more pike, also contained higher levels of eDNA, which all three extraction kits and the two filter combinations did detect (Figure 3). This means that the density of pike can be separated by quantitative eDNA. This finding is in line with other similar eDNA studies of fish (Klymus et al. 2015; Lacoursière-Roussel et al. 2016b; Capo et al. 2020b; Di Muri et al. 2020). Even though it was not possible to analyse the three different extraction kits in one ANOVA model, the results showed that the quantification of pike density could be applied regardless of extraction kits. This finding is also consistent with other studies of quantification of fish abundance and biomass in controlled environment (Klymus et al. 2015; Lacoursière-Roussel et al. 2016b; Sassoubre et al. 2016). Furthermore, the amount of yielded eDNA for the three extraction kits differed from each other, which are in line with other studies (Ip et al. 2015; Djurhuus et al. 2017; Hermans et al. 2018; Brannelly et al. 2020).

4.2. Evaluation of filter combinations

The hypothesis that the GF/A filter together with the CN filter would collect more eDNA than a single CN filter, was rejected. Filter did not have an influence on the eDNA concentration for any of the extraction kits. Despite that the two filter types are among the highest-ranked when it comes to eDNA sample collection (Lacoursière-Roussel et al. 2016b; Minamoto et al. 2016; Majaneva et al. 2018a; Shu et al. 2020), combined did they not yield substantially higher eDNA concentration than a single CN filter. This result facilitates future studies. The filtration step will go faster when only one filter is used as it took longer to filtrate

water through two filters than one filter. Further, the use of only one filter also reduces the costs.
Overall, the sample replicates varied and were not as grouped as expected, especially for CH. For example, the samples from tank B with CN+GF/A filters had seven units' differences between the highest and lowest sample replicates (Figure 3). A similar pattern was found for the sample replicates of tank A. Due to its simplicity, within the eDNA community lies the assumption that samples extracted with CH will be less reliable (Lienhard & Schäffer 2019). Samples extracted with CH, from both tanks, did not have any outliers though, which are illustrated in the boxplots (Figure 3). However, compared to BT, CH appears to have more variation among the sample replicates. If it is only due to CH as an extraction kit or if it also is due to heavy inhibition of the samples needs to be investigated further. Samples extracted by BT could give a hint of a potential pattern to be seen, where sample replicates from CN+GF/A, from both tanks, had more variation than the CN sample replicates (Figure 3). This pattern was not seen using CH. Further studies are needed to pin down the reasons for this; if it was just a coincidence, handling error in the lab or whether the two filters block each other's efficiency when they were combined.

For PW, however, there was an indication of difference between filters from the samples taken from tank B, with a high density of pike (Figure 3). Regrettably, only one sample replicate with CN+GF/A filter could be used for PW and therefore interactions could not be analysed for PW nor could any conclusion be made.

Currently, few studies have tested if multiple filters yield more eDNA than a single filter. Therefore, these results cannot be put into the context of other similar studies. To my knowledge, there is only one study Hunter et al. (2019), that tested a multi-filter isolation technique, showing a higher eDNA yield using four combined filters compared to a single filter. According to their findings, the four filters yielded 10% more eDNA than a single filter. Their study was not similar to my study, so parallels are hard to draw. However, that four filters only yielded 10% more eDNA than a single filter, indicates that it is sufficient to only use one filter. Thus, my results contribute to more knowledge in this area.

4.3. Clogging

Another reason to use multiple filters was to avoid clogging the filters due to particle rich and turbid water. Only one sample with a CN filter clogged, filtrating around 800 mL of water before clogging. The sample was included in the analysis (tank B, CN for BT) and performed similarly to the others, (Figure 3). This illustrate that to detect differences in eDNA concentration in a sample 200mL water is not

enough. It was unexpected that not more filters clogged due to the high levels of particles that were on the filter surfaces after filtration. One reason for the low clogging rate could be that CN filters are capable of filtering larger water volumes and clog less compared to other filter types (Sepulveda et al. 2018). It could be due to its properties as depth filter (Hinlo et al. 2017). Lastly, the pore size of these filters was in the upper range of medium pore size, 0,8 µm, compared to the most used size in eDNA studies 0.45 µm (Shu et al. 2020). As has been mentioned, filters with larger pore sizes can filter more water with less risk of clogging (Minamoto et al. 2016; Capo et al. 2020b; a). My experiment highlighted that CN filter resist clogging well and there is no need to use multiple filters, not even during spring with extra turbid water.

4.4. Evaluation of the extraction kits

4.4.1. PowerWater

My hypothesis that PW, as the more advanced and expensive extraction kit, should perform best of the three was rejected. However, the interpretation of the result was difficult since PW deviated from the other two extraction kits in two aspects. First, as mentioned, only half of the samples of PW could be used in the analyses. Second, the samples from PW were not diluted. Nevertheless, the results suggest that PW was not the most efficient extraction kit out of the three used in my study (Figure 3).

An experiment by Hinlo et al. (2017), comparing the performance between BT and PW, showed that BT yielded more eDNA than PW. One explanation was that the methodology of BT favoured the collection of eDNA because the filters lie in a buffer for a long time, up to several hours. The buffer is able to penetrate the filters and to solve eDNA into the buffer. Compared to PW, which uses a mechanical step, crushing the filter for 5-15 min, which gives the buffer less time to solve the eDNA within the filter. Another explanation why PW was inferior to BT was that PW protocol involved more steps where the sample solutions were transferred between different tubes, which can lead to eDNA loss and the tube might also absorb some eDNA in the inner walls (Gaillard & Strauss 1998).

Moreover, it seemed that PW also suffered from inhibition or that eDNA levels were reduced during the extra cleaning steps PW has, to reduce inhibition. However, one important thing that cannot be overlooked is that PW was the only extraction kit that could amplify in the qPCR without being diluted, both with TaqMan and with SYBR Green, during the first try-out (data not shown). This gives a signal that PW has another dimension. Despite the poor performance in this

experiment, it is the second most used extraction kit in eDNA projects (Shu et al. 2020). The study of Hermans et al. (2018) pointed out that PW produced more quality DNA than three other extraction kits out of six that were tested and the targeted organisms were from fish, mammals, plants, fungi and bacteria. Additionally, PW is recommended to be used for water samples containing inhibitors such as sediment, humic substance, or algae due to its inhibitor removal step (Shu et al. 2020).

4.4.2. Blood and Tissue

The result revealed that BT, the second most advanced extraction kit, had the second highest yield. The samples of BT were the most stable and produced equivalent values regardless of the combination compared to the other two extraction kits in this experiment (Figure 3).

Nonetheless, the samples of BT had to be diluted for successful amplification, even though the extraction process had several cleaning steps, similar to the cleaning steps of PW. In theory, BT should have resist inhibition at least better than CH, which was not the case. According to Eichmiller et al. (2016), BT lacks an inhibitor removal cleaning step. That could have been the reason why BT needed to be diluted. Other experiments that compared different extraction kits had to dilute BT samples as well due to inhibition (Eichmiller et al. 2016; Majaneva et al. 2018b). However, BT has been used successfully in many field projects of aquatic organisms where dilution was not needed (Thomsen et al. 2012; Cowart et al. 2018; Sepulveda et al. 2018; Capo et al. 2020b). BT is one of the most used extraction kits, optimal for eDNA extractions due to its non-toxic solutions, it is cheaper than many other advanced extraction kits such as PW. Another advantage for BT is that it is simple to use (Shu et al. 2020).

4.4.3. Chelex

The least advanced extraction method yielded the most eDNA. CH outperformed both the established extraction kits PW and BT although beforehand it was considered the least advanced extraction kit. One explanation could be that CH lacks cleaning steps in the extraction process. The trade-off between reducing inhibition and high eDNA yield has shown to affect the recovery of eDNA in samples. Extraction kits with extra inhibition cleaning steps have a lower yield compared to extraction kits that lack inhibition cleaning steps (Eichmiller et al. 2016). The benefit CH has in relation to PW and BT is its simplicity and that in this study CH also resulted in the highest yield, which is a coveted trait for quantification eDNA studies.

Nevertheless, more time for method development is needed for CH to investigate if the sample replicates can be as precise as the one for PW or BT, for future studies of eDNA quantification of pike, with samples containing high inhibition. CH is not commonly used in quantification studies. In more than 140 fish studies of eDNA extraction methods that have been published, only one article exists about CH (Shu et al. 2020). One reason could be that CH was not made for eDNA extraction. CH is a styrene divinylbenzene copolymer, which means its properties bind copper, iron, and other heavy polyvalent metal ions (Ip et al. 2015). It has for example been used for the removal of copper and (Perez et al. 2016) phosphate from water (Wu et al. 2007). Other research areas have been in criminology to reduce humic acids from bones (Sutlovi & Gojanovi 2007), in microorganism studies and it also successfully extracted DNA from tissue samples (Casquet et al. 2012; Lienhard & Schäffer 2019).

The reason why CH is interesting to use as an extraction tool for quantification is its simplicity. Only water needs to be added and be mixed with the resin. The next step is to add the CH solution to the samples and then separate the filter with the solution and the extraction part is done. The sample is ready for amplification, no cleaning steps require. According to Lienhard & Schäffer (2019), there are assumptions such as, extractions from CH are not suited for long storage and that the extracted long storage samples would not be stable for amplification. Other objections were that CH has issues with inhibitions due to the lack of cleaning steps (Ip et al. 2015). Recent studies have proved that CH is stable after a year of storage (Casquet et al. 2012) as well after twenty freeze-thaw-cycles (Simon et al. 2020). Similar findings were found during my experiment where CH was stored for several months in the freezer and been thawed and frozen a couple of times between extraction and amplification (data not shown). CH yielded the highest eDNA levels compared to the more established extraction kits BT and PW and did so in this experiment as well.

Nevertheless, inhibition is a factor that was a problem in this project. There are different solutions to reduce inhibition in CH samples. One way is to use a DNA Fast Flow centrifugal filter (Microcon) that reduces inhibition (Ip et al. 2015). Another way is to use the 1.5X bovine serum albumin (BSA). Which is a protein that isolates substances blocking the polymerase chain reactions. BSA was used for a mosquito project and it even increased the eDNA yield for CH (Musapa et al. 2013), no extra step was needed there either. There are several different approaches to deal with inhibitors for CH and several of them do not need to increase the steps of the protocol (Musapa et al. 2013; Pîrlea et al. 2017). This means that the advantage CH has, as a potential cheaper and faster extraction kit (Ardura et al.

2010; Musapa et al. 2013; Ip et al. 2015; Pîrlea et al. 2017; Lienhard & Schäffer 2019), will remain.

4.5. Inhibition

As have been mentioned, all extraction kits did suffer from inhibition with the try-out with SYBR Green. Undiluted CH and BT suffered greatly from inhibition even with TaqMan. The samples were collected during spring, which is known to contain more organic materials and other inhibitory substances, than other seasons (Jane et al. 2015). The main reasons are high compound levels from plants and soil that can colour the sample solution darker to more of a tea colour (Jane et al. 2015; Hunter et al. 2019). Some of the samples of CH and BT had darker colour (Figure 1) which suggests that the water contained high levels of plant substances such as tannins and phenolics (Hunter et al. 2019). A project of Jane et al. (2015) discovered that inhibition masked around 2000 eDNA copies for samples with high eDNA concentrations. In this study, the samples were highly diluted and the possibility that samples that yielded high levels of eDNA could have been masked.

An alternative to avoid samples with high inhibition could be to avoid sampling in spring. For pike monitoring however, spring is the most interesting time to monitor pike stocks, as they are not scattered or stationed in their territories. The spring migration gathers all sexually mature individuals in a population as well the elusive older pike when they migrate to their spawning areas (Pierce 2012; HaV 2020). The best solution for the future monitoring of pike stocks with eDNA must be to solve the inhibition problem rather than switch to another sampling season.

There are several ways to solve or reduce inhibition. Firstly, one way to avoid the problem is to use more inhibition tolerance assays, which elevates the detection of eDNA copies during amplification (Jane et al. 2015), such as TaqMan Environmental Master Mix 2.0, which is more expensive (Tajadini et al. 2014). Secondly, to use PCR facilitators (Jane et al. 2015) such as BSA (Musapa et al. 2013). Thirdly, use the clean-up column. The trade off with this method is that to include extra cleaning steps, less inhibition compounds will be in the sample but at the cost of there also being less eDNA in the sample (Eichmiller et al. 2016). Fourthly, diluting the samples. The level of inhibition will be reduced for diluted samples, but generally it also leads to eDNA loss in terms of that the sensitivity of the assay to detect the lower eDNA levels will be reduced (Sepulveda et al. 2018; Sidstedt et al. 2020). The last, and final solution is to use ddPCR. It has been shown that qPCR suffered more from inhibition than ddPCR (Sidstedt et al. 2020). Despite the advantage, eDNA rich and dirty samples can suffer from inhibition even when using ddPCR (Hinlo et al. 2017). However, ddPCR assay costs more than qPCR

assay (Shu et al. 2020). As has been illustrated it is not easy to reduce inhibition for a more successful amplification without any loss of eDNA from the samples.

In this project, several actions were taken in order to reduce inhibition. First, a clean-up column was tested to reduce inhibitors, but this did not work. Second, switching to an inhibition tolerant assay, TaqMan Environmental master mix 2.0. It resulted in preferable amplifications but could not overcome the high levels of inhibitors, for CH and BT. Last, to dilute the samples which resulted in successful amplification, but still contained some inhibition though in lower levels. For future projects, PCR facilitators may be an option to reduce the inhibition without diluting the samples, which might lead to higher yield.

4.6. Future mesocosm research

Whether or not the inhibition levels will be reduced by laboratory equipment or by the natural annual variations of organic compounds, there remains several steps before one has a reliable field sampling protocol. Moreover, lower eDNA levels can be expected in natural environments than in this tank experiment. Nevertheless, during spawning, levels might be higher (Thalinger et al. 2019) when pike of different age- and size classes gather together in shallow areas (Pierce 2012).

Whether or not this protocol can be applied directly to the natural environment as a monitoring tool for pike, needs to be investigated further. However, there is evidence from Alaska that it can detect pike in the wild (Dunker et al. 2016; Sepulveda et al. 2018). Pike is regarded as an invasive species in Alaska and needs to be monitored for that reason. The study of Sepulveda et al. (2018) used eDNA to detect the distribution and density of pike between different sites. They used BT and GF filter with satisfying results. This means that at least one of the combinations used in this project should be able to detect differences in pike density in the natural environment.

Before similar monitoring can be done as the recently mentioned study of pike in Alaska, some concrete steps need to be investigated further such as, to get a better understanding of the normal eDNA concentration of pike in Swedish waters. Other areas are how fast is the degradation rate of the pike eDNA? A mesocosm tank experiment of Sassoubre et al. (2016) observed that the shed eDNA from different fish species behaved differently such as the mucus from mackerel floated at the water surface whereas the shed scale from sardines and anchovies sunk to the bottom. To know how the shed eDNA of pike behave could be useful for future water sampling to know if one collects more pike eDNA from surface samples than maybe samples collected further down the water column. Moreover, other questions

are: which time of the year and time during the day is the best sampling time? How low is the detection limit for the chosen equipment to detect pike eDNA? How many samples are needed for an area to detect differences between different sites and to be able to detect differences over time? Most answers to these questions can be collected through different experimental models such as mesocosms which seemed to work out well (Takahara et al. 2012; Thomsen et al. 2012; Skinner et al. 2020). Such as the study from Takahara et al. (2012), that did an abundance/biomass study on the common carp, (*Cyprinus carpio L.*). They could apply the theoretical hypothesis to the fish's natural environment. The first step was similar to my study, a tank experiment to get the basic information of eDNA from the fish. The second step advanced to take samples in regulated ponds to understand the eDNA biomass relation with bigger water volume. The third step was to sample in their natural environment, a lagoon in Japan, which resulted in a description of the relationship between biomass/abundance and eDNA concentration. This way many of the unsolved questions can be solved through controlled conditions that are determined by the aim of the project. Most likely, a similar three-step approach are needed for the study of pike to be able to estimate and establish a method that may work for different Swedish aquatic habitats.

4.7. Reflections

The knowledge of how to apply the eDNA approach for monitoring of pike has just begun and a lot of studies and work remain before reliable monitoring results can be accomplished. This includes conducting more mesocosm research, in tanks or controlled ponds, to be able to understand the eDNA-pike biomass relationship and investigating this in natural environments, e.g., by comparing with other sampling methodologies. However, this study has focused on detecting differences in two levels of pike concentrations and it was a small experiment with a small data set. Together with other eDNA experiments, it can contribute with a little piece to the complex puzzle of eDNA.

Nonetheless, in retrospect the results in my project give a base for some advice for future studies. First, take back-up samples that can be used if handling error happens in the laboratory. Backup samples could also have been useful to have during optimization of the protocol and maybe earlier had detect the inhibition problem. Second, more testing to see if the suggested inhibition reduction kits, such as the BSA, could have resulted in less variation between sample replicates and for successful amplification for undiluted samples of CH. Third, investigate how much effect inhibition had on the result of these samples. Finally, future projects could include more time for laboratory work especially for the creation of new protocols.

5. Conclusions

This experiment seemed to be one of the few that have tested the effect of multiple filters. This was a contribution to the complex puzzle to find the optimal method for eDNA monitoring of pike. This project revealed that it is possible to detect differences in pike density regardless of filter combinations or extraction kits. Another finding was that multiple filters did not perform better than single filters, even though the two highest ranked filters were combined. The reason why the filter combination performed equally is unknown and open for future research.

PW was the only extraction kit that did not need to be diluted and could still perform qualitative amplifications in the qPCR. Moreover, CH was the extraction kit that yielded the most eDNA. The benefit of CH is that it is a fast extraction kit that lacks cleaning steps, which reduces the costs and time invested in the laboratory. Another benefit is that there is no handling of chemicals using CH. The other two established extraction kits PW and BT are good choices for projects that do not have time for method development. The slightly faster and cheaper method, BT is recommended as a better choice. However, if the water is very turbid or the samples contain high levels of inhibition then PW is the best choice, with its extra inhibitor removal cleaning step.

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Appendix 1

Chelex 100 resin extraction protocol

Preparation of Chelex Mixture:

1. With Bleach, sterilize a dry reagent spatula, and a small magnetic stir bar.
2. Prepare a 5-10% by weight slurry of Chelex 100 Resin (Biorad part 143-3832,100-200 mesh Chelex, sodium form) and UV sterilized HPLC water. The most effective way to do this is to take a 50 ml sterile falcon tube, place it on a scale inside a small beaker and zero the scale. Then add 5 grams of Chelex and fill to 50ml mark with water. Precision is not critical. Sterile technique is.

3. Place sterile stirbar in tube and place on magnetic stirrer. Chelex settles quickly so if the slurry is not well mixed, your concentrations and results will be variable. Keeping the slurry well mixed, aliquot 300-500micro liters into 0.6 or 1.6ml eppendorf tubes (again, sterile) and cap immediately.

The extraction

Pre-heat a water bath to 100°C
Cut samples into 3x3 mm pieces and insert them in a 5ml Eppendorf tube with screw cap.
Put the Chelex solution on a magnetic stirrer and pipette X µL (1500) µL of solution into each tube.
Vortex the samples briefly and centrifuge at 12000 g for 1 min.
Vortex briefly and incubate the tubes at 100°C for 10 min.
Vortex briefly again and incubate the tubes at 100°C for another 10 min.
Vortex the samples briefly and centrifuge at 12000 g for 1,5 min.
Transfer supernatant to a new tube 1,5 mL Eppendorf tube. Repeat step 7-8 until all liquid has been transferred.
Centrifuge the 1,5 mL tubes at 12000 g for 1.5 min.
Pipette the supernatant to a new 1,5 mL tube. Avoiding pipetting any pellet of Chelex.
Repeat step 9-10 until there is no pellet of Chelex left in the samples.
Samples is now ready for analysis.

Appendix 2

Materials used for the project

Tank experiment

The equipment that was used for the collection of eDNA water samples were, fiberglass tank, brick, tarpaulin, steel mesh, 300 mL syringe, Swinnex filter holder, 30 L bucket, portable freezer, tweezers, Cellulose nitrate membrane filters 0,8µm, Glass microfiber filters and chlorine.

Laboratory materials

The equipment that was used for the laboratory part were, Vortex genie 2, Mini Centrifuge 6 Tube Adapters, ThermoMixer, Eppendorf Centrifuge 5417c/5430, Incubator, Thermoelectric Cooler and Warmer Mini Fridge, Bio-Rad CFX384™ Real-Time PCR system, DNeasy® Blood & Tissue kit, DNeasy® Power water kit, Chelex® 100 resin 50g, Probe, Primer-F, Primer-R, TaqMan Environmental Master Mix 2.0, Finntip™ filter 1000 µL, Finntip filter™ 200 µL, Finntip™ filter 10µL, Adjustable Volume Pipettes Single-channel pipettes, electronic pipette, Eppendorf Tubes® 5.0 mL with screw cap, Eppendorf Tubes® 1,5 mL, Magnetic stirrer, tweezers, Hard-Shell 384-well PCR plate, Silica gel of 2-5mm size, zip lock bags, syringes, filter holders, Purple Nitrile Powder-Free Disposable Gloves and coin envelope.