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NATURAL HISTORY MUSEUM OF DENMARK

Assessment of the restoration of River Suså with special focus on the European bullhead (*Cottus gobio*) and European minnow (*Phoxinus phoxinus*)

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Submitted: November 1st 2021

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Abstract

Due to pollution in River Suså in the 1950s and 1960s the European bullhead (*Cottus gobio*) were exterminated and the population of European minnow (*Phoxinus phoxinus*) were severely reduced. In 2018 the first minnows and bullheads were released into the stream in an attempt to re-establish the populations and the following years even more fish were released. This paper examines whether or not the restoration project is a success through various field methods such as electrofishing, snorkelling and shrimp push net, and compares the morphology of preserved bullheads from the old Danish population with preserved specimens from Stockholm, Basel and Băile Herculane. The surveys showed that the bullheads in River Suså are breeding and have migrated both upstream and downstream from their release site. The minnows have also migrated but have not established as well as the bullheads and no juvenile minnows were found. Most of the minnows were found using electrofishing at a site modified by the local municipality to help the fish, suggesting that modifications could help restore the population of minnows. The surveys also found large amount of signal crayfish (*Pacifastacus leniusculus*) which could cause problems, especially with the bullheads due to competition for shelter. The morphological comparison showed no difference between the Danish specimens and the Swedish and a slight difference compared with the bullheads from Basel and Băile Herculane. Further work to restore River Suså could include removal of signal crayfish and establishment of shelters for the bullheads. Pools and slow moving backwaters could be created for the minnows since they lack resting sites.

Introduction

In the 1950's River Suså in Denmark was getting more and more polluted which lead to the disappearance of the European bullhead (*Cottus gobio*) and a severe reduction for the common minnow (*Phoxinus phoxinus*) (Carl & Møller, 2012). This in turn led to the decline of the thick-shelled river mussel (*Unio crassus*) which relies on the two fish for reproduction (Lopes-Lima et al., 2017). The mussel larvae spend 3-4 weeks attached to the gills of the fish while they grow after which they fall off the gills and settle on the bottom (Lundberg &

Österling, 2016). Without the fish the mussel will eventually disappear (Lopes-Lima et al., 2017). The mussel was thought extinct in Suså until a few living specimens were found along with empty shells. In 2017 and 2019 the remaining living mussels were tagged with PIT tags and some of them moved to a different location. No juvenile mussels or larvae were found during the examinations and the results suggest that there has been no recruitment for several years (Schneider & Zülsdorff, 2017, Schneider, 2019). Through funding from EU an attempt to recreate the original population has begun. This also includes restoring populations of the two host fish, European bullhead and common minnow, beginning with the release of 300 fish of each species in 2018. Since then both minnow and bullhead has been released in large numbers every year in the hopes that a large population of host fish can help restore the population of thick-shelled river mussel (see table 1 and 2). Furthermore an attempt at restoring the stream has been made to improve the conditions of the released fish. Large boulders have been placed in the river to create slow-moving backwaters and create some variation in the flow of the stream.

In this study the populations of European minnow and European bullhead will be examined in an attempt to determine if they have established reproductive populations and if so how far they have expanded from their original release site. Furthermore future improvements to the restoration project will be discussed. Lastly an attempt will be made to determine whether or not the newly released bullheads are of a similar population to the old one by using preserved specimens from the collection at the National History Museum of Denmark.

European bullhead (*Cottus gobio)*

The European bullhead (*Cottus gobio*) is a small bottom dwelling freshwater fish. It is found all over Europe and part of Siberia, from Spain in the south to Finland in the north (Riffel and Schreiber, 1998, Philaja et al., 1998). In Denmark it is only found in the river Suså. European bullhead typically lives in fast flowing streams and rivers buried beneath or lying on top of rocks. It spawns from February to June and up to four times in a season depending on the environmental condition with warmer temperature being more productive (Dorts et al., 2012). They spawn in small caves on the bottom where the female attaches the eggs to the cave and the male then protects and oxygenate them till they hatch (Kottelat & Freyhof, 2007). During spawning the males become very territorial and will attack other males in competition for the best spawning caves (Davey et al., 2005). A male will protect eggs from multiple females if possible and will take over eggs from another male if they get the chance to evict them from a good nesting site (Kottelat & Freyhof, 2007).

The life history and reproduction tactics of the European bullhead is strongly influenced by environmental conditions such as food availability and temperature (Abdoli et al., 2007, Dorts et al., 2012). The temperature affects the growth pattern of European bullhead. Higher temperature leads to increased growth in the young generations and faster maturation but it also results in slower growth in older generations and a shorter lifespan (Abdoli et al., 2007). Dorts et al. found that a 4°C increase in temperature lead to earlier spawning in females but negatively altered spermatogenesis in males. This could result in a reduced reproductive success.

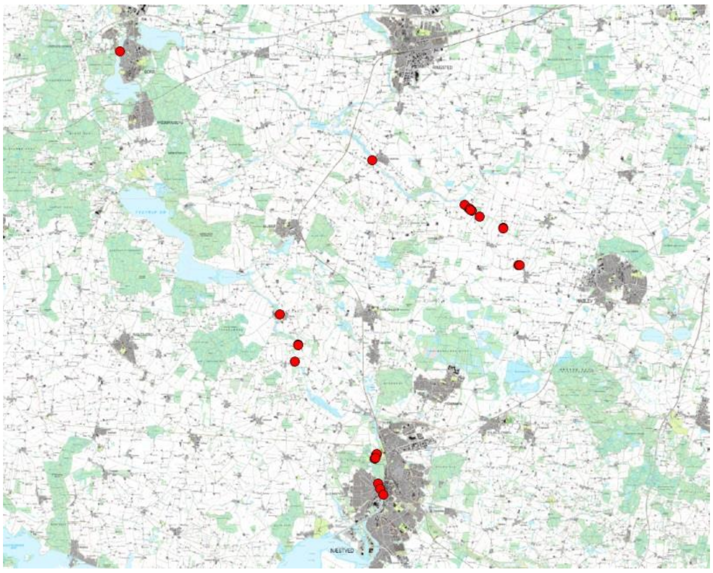

Figure 1. Map of every record of European bullhead by Natural History Museum of Denmark through time.

In Denmark the European bullhead is only known from River Suså. Old sources report it as a common fish in the lower part of the system around Holløse Mølle and Herlufsholm which is also where the preserved specimens in the collection at the Natural History Museum of Denmark are from (Carl & Mølller, 2012). The specimens from Holløse Mølle are from 1881 and 1896 and the specimens from Herlufsholm are from 1883. It went extinct from both sites in the 1950s or 1960s but two specimens were caught in the 1970s and 1970-80 far upstream at Nymølle Bro and at the inlet from Sorø Sø respectively. Surveys of the same sites in 2007 and 2008 were unable to find any bullheads (Carl & Møller, 2012).

European bullhead is a highly divers species which has been through several taxonomical revisions since the first description. Subspecies have been established only to be removed again later with the inclusion of genetic data (Sideleva et al., 2015). Variation within the species has resulted in morphological distinct populations, even within the same river system (Abdoli et al., 2007, Sideleva et al., 2015). Recently the European bullhead was revised into several new species (Freyhof et al., 2005). Some of the newly revised species are difficult to distinguish morphologically from each other. For species identification it is often the location that is used along with genetic identification (Kottelat & Freyhof, 2007). The biology of the species differs very little and often the biggest difference is adaptations to various temperatures. Some of the newly introduced species have not been studied thoroughly enough to describe them completely, but they are assumed to be highly similar to *C. gobio* (Kottelat & Freyhof, 2007).

European minnow (*Phoxinus phoxinus)*

The European minnow (*Phoxinus phoxinus*), also known as common minnow, is a small freshwater fish found in northern and central Europe along with northern Asia. It has a maximum length of 12-15 centimetres and feed on diatoms, filamentous algae, various plant matter and invertebrates. The feeding habits vary slightly depending on the size of the minnow with larger fish eating larger invertebrates. A slight variation in food preference is also found between minnows residing in lakes or streams and rivers, but this mostly comes down to variations in the species composition of the invertebrates in the two habitats (Frost, 1943). Depending on the habitat, the minnow has a seasonal migration pattern. In lakes the minnow are pelagic most of the summer and part of the spring and autumn but during winter they hide on the bottom beneath stones. During spawning in the spring the minnows migrate upstream into the inflowing streams. A similar pattern is found in minnows living in the streams and rivers but they prefer backwaters and slow moving water where they are often found in large schools. During warm summertime they tend to migrate towards cooler water if possible (Frost, 1943). In the Baltic Sea they are also found in brackish water. This population consist of three different migration patterns: permanent resident in brackish waters, short migration into streams during spawning in the spring or longer migrations into freshwater (Svigsden et al., 2018).

In Denmark the European minnow is quite common in Jylland although through the last 100 years the population have been reduced in many of the streams. At Fyn it is found in a few streams but pollution have reduced their population in some streams or exterminated the in others. At Sjælland it is quite rare and is only found in a few streams. Two streams had large populations, River Suså and Køge-Lellinge Å. The population in Køge-Lellinge Å was reduced around 1973-1976 but a few fish survived and a population still exists. The population in River Suså was severely reduced due to pollution and were only found in a small part of the stream however it did survive (Carl & Møller, 2012).

Figure 2. Map showing every recorded European minnow in the Suså river system by the Natural History Museum of Denmark through time.

For the restoration project in River Suså both European minnow and European bullhead have been released in large numbers. The bullheads have been released four times, three times at Nymølle Bro and once at Teestrup Bro.

Table 1. Dates, locations and quantity of European bullhead released.

Date	Location	Total number of fish released
21/11/2018	Nymølle Bro	300
10/10/2019	Nymølle Bro	600
09/10/2020	Nymølle Bro	400
04/10/2021	Teestrup Bro	230

The first three batches of bullhead were caught in three different streams in Sweden, Fyllå, Engbo and Nybro Ån, and the fish released in 2021 were produced by Fyns Laksefisk.

The minnows have been released ten times at different sites. In 2019, 2020 and 2021 it was attempted to infect all the fish with larvae from the thick-shelled river mussel to hopefully have some of the larvae settle and help the population of mussel.

Table 2. Dates, locations and quantity of European minnows released.

Date	Location	Total number of fish released
23/05/2018	Tvede Bro	300
30/04/2019	Teestrup Bro	1000
30/04/2019	Lunden	1000
19/05/2020	Assendrup	1100
19/05/2020	Teestrup Bro	1000
19/05/2020	Lunden	1000
21/05/2021	Lunden	2500
21/05/2021	Assendrup	2500
21/05/2021	Teestrup Bro	2500
21/05/2021	Sørup	2500

The minnows released in 2018 were caught in Hågerup Å at Fyn while the rest were produced at Fyns Laksefisk. All data concerning the release of fish have been provided by Næstved municipality.

Environmental DNA (eDNA)

Environmental DNA (eDNA) is a method using the continued release of DNA from various lifeforms to the surrounding environment to detect the presence of the different species. The method can be useful for detection of rare species or species known to be difficult to observe trough other methods (Jane et al., 2015; Thomsen & Willerslev, 2015; Goldberg et al., 2016).

In an aquatic environment the DNA is dispersed with the current before it is degraded. How fast the DNA is degraded depends on various environmental factors but varies between a few days to a few weeks. Compared to a terrestrial environment where DNA can persist for decades, aquatic DNA is degraded fast (Thomsen & Willerslev, 2015). The fast degradation in the aquatic environment gives a more up to date result of species presence or absence compared to terrestrial tests. eDNA sampling in a pond can show 80-100% of the species present while sampling in running water is closer to 50% (Thomsen et al., 2012). In flowing water the detection depends on various factors such as flowrate of the stream and distribution and concentration of specimens. The flowrate affects how far DNA is transported from the animal releasing it but also the concentration of DNA. Jane et al. (2015) found that higher flowrate resulted in a lower concentration of DNA downstream of the source but DNA was detected further away compared to a low flowrate. The lower flowrate resulted in higher concentration close to the source compared to high flowrate but after 239 meters the DNA was not detectable. Jane et al. (2015) also found that increased concentration of biomass increased the amount of DNA in the water close to the source. Higher DNA concentration results in a higher detection rate.

In streams and rivers the distribution of DNA can vary widely in the same system depending on the movement of the water. To improve the chance of obtaining enough DNA for detection, the ecology of the target species should be considered (Goldberg et al., 2016). Various stretches of a stream or river could contain different concentration of biomass and therefore concentration of DNA. Just downstream of deeper pools with slow moving water could contain more DNA from species adapted to sheltering in the pools while the opposite might be the case for species adapted to faster flowing water.

Methods

Fieldwork

The fieldwork consists of several different methods to supplement each other; electrofishing, manual fishing with shrimp push net, snorkelling and night surveys done with powerful flash lights. Each method has advantages and disadvantages but by combining them a better result is achieved. Samples for eDNA detection was collected at the different sites in May and September, but were not included due to time restrictions. Eight different sites were sampled (see figure 3) but not every method was used at every site due to lack of accessibility. An initial test sampling was done with shrimp push net at Nymølle to examine the site and the method.

Figure 3. A map of the sampling sites. The sampling site at Nymølle was the release site of the bullheads. The European minnows were released at Assendrup, Teestrup Bro, Tvede Bro (not on the map), Sørup (close to Vandværket) and slightly downstream of Hjelmsølille.

The electrofishing was done over three periods, two days in May, one in June and one in September. In May, water levels and powerful currents made a thorough examination difficult. On two of the sites, Suså Landevej and Vandværket, the water level was too high to wade in and the fishing had to be done from shore. This meant that part of the stream was out of reach. Furthermore unclear water made it easy for stunned fish to be washed away before we were able to see them. At each site approximately 50 meters were fished with electrofishing, while the banks along the same stretch of stream were fished manually with a shrimp push net. The net fishing were done by scraping the net along the bottom towards the bank, by placing the net and kicking the bottom and plants towards the net or by scraping the net trough the plants along the bank.

Due to the warmer water in June snorkelling was easier and five different sites were searched (Vetterslev, Hjelmsølille, Nymølle, Tornelundsvej and Teestrup Bro). In June and September the stream was very shallow compared to May and it was easier to do electrofishing. In June only a small stretch of the site at Nymølle were fished in an attempt to find juvenile bullheads. In September three sites were fished for a stretch of 50 meters, Teestrup Bro, Tornelundvej and Hjelmsølille. Each stretch was fished twice and all fish collected and counted. The fish were released after the stretch had been fished twice. The sites fished were chosen based on accessibility, distance to the initial release and habitat conditions. The sites were supposed to be the most likely places for the bullheads and minnows to spread to from the release site.

An attempt was made to observe the bullhead by night using powerful flashlights. Since the bullhead forage by night they should come out from their shelter beneath rocks. Reports from local fishermen said that they had seen juvenile bullhead swim around during dark, both by Nymølle and by Teestrup Bro.

Table 3. Dates for the various methods used at each site.

	Electrofishing	Net fishing	Snorkelling	Night surveys
Vetterslev	19/5/21	19/5/21	23/6/21	27/9/21
Suså Landevej	19/5/21			
Vandværket	19/5/21			
Hjelmsølille	19/5/21		23/6/21	27/9/21
Nymølle	18/5/21 – 24/6/21	18/5/21	23/6/21 – 24/6/21	8/10/21
Tornelundsvej	18/5/21 – 23/9/21	18/5/21	24/6/21	28/9/21 - 8/10/21 - 9/10/21
Teestrup Bro	18/5/21 – 23/9/21	18/5/21	24/6/21	28/9/21 - 8/10/21 - 9/10/21
Assendrup	18/5/21 – 23/9/21	18/5/21		

Morphological comparison

The reintroduced European bullhead was collected from three different populations in southern Sweden and therefore might not be identical with the original stock in River Suså. To test the morphology, eight specimens from the original population were examined and compared with fish from other populations around Europe. The eight fish were caught in 1881-1900 and kept in alcohol in the collection at the Natural History Museum of Denmark.

All fish where measured, using various meristic and morphometric characteristics. The meristic measurements where done as Kontula & Väinölä (2004) while the morphometric measurements where done as Riffel & Schreiber (1998) (see figure 4). All morphometric measurements where calculated as % standard length.

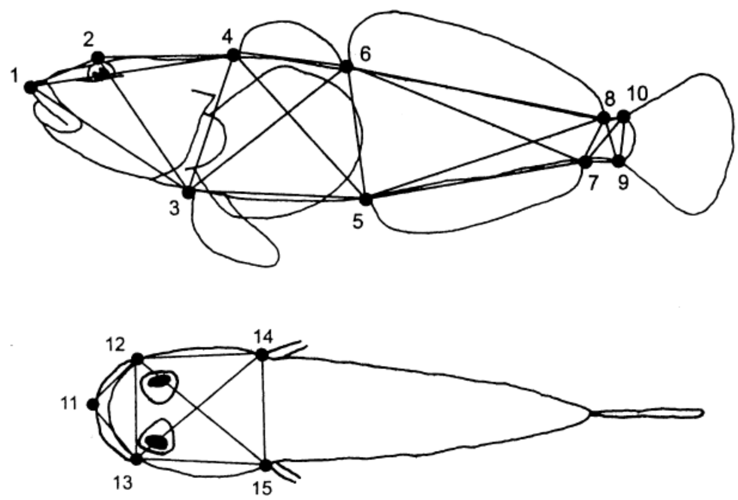

Figure 4. Morphometric measurements done on each of the bullheads. Source: Riffel & Schreiber (1998).

To compare the original Danish population with other populations, similar examinations were made on ten fish from around Europe. Of the ten fish, eight were from Băile Herculane from 1963. One fish was from Basel from 1882 and the last was from Stockholm from 2008. All the fish were from the collection at the National History Museum of Denmark. The original curators determined them to be *Cottus gobio* but phylogenetic studies on and a revision of the species has since changed that for the fish from Basel and Băile Herculane. They could potentially be *Cottus haemusi* or *Cottus rhenanus* based on a book by Kottelat and Freyhof (2007). Since the fish from Basel have been conserved since 1882 and the fish from Băile Herculane since 1963 some, characteristics are extremely difficult to distinguish. Due to the low amount of fish released in the River Suså for the project it was not possible to use them for the morphological studies. As many fish as possible had to be released to increase the chance of creating a stable population.

Results

Field observations

Between May 18th and September 23rd four days were spent electrofishing various stretches of the river searching for European bullhead and European minnow. The initial surveys in May only showed bullhead at the initial release site and no European minnows were caught. Ten other species of fish were caught with perch (*Perca fluviatilis*) and spined loach (*Cobitis taenia*) being the most common and found on almost all sites. None of the fish were caught at all the sites in May.

In June only the site Nymølle was fished in search of juvenile bullheads. The result was four bullheads of which one was a juvenile. This was the first time a juvenile bullhead was caught since the reintroduction. Some of the smaller bullheads caught during the surveys could potentially have spawned the previous year but since there is no record of the size of the released fish it is impossible to know.

In September only three sites were fished, two upstream of the release site for bullhead and one downstream. This time European minnow were found on two sites, one upstream and one downstream, with two and 19 fish caught respectively. One European bullhead was found at the downstream site approximately two kilometres from the release site. During fishing seven other species were caught with perch and spined loach being the most common. Perch was by far the dominant species with 535 fish caught compared with 92 spined loach. Almost all the perch had spawned the same year and were 4-6 centimetres long.

The complete list of fish caught using electrofishing can be seen in the tables below.

Table 4. The complete list of different species caught using electrofishing on 18/5/21.

18/5/21 Species	Nymølle	Tornelundsvej	Teestrup Bro	Assendrup
European bullhead	4			
Gudgeon	14	2		5
Spined loach	2			1
Ninespined stickleback	1	1		
Ide	2			
Perch		1	1	3
Roach			1	53
Common bream				11

Table 5. The complete list of different species caught using electrofishing on 19/5/21.

19/5/21 Species	Vetterslev	Suså Landevej	Vandværket	Hjelmsølille
Tench		2		
Ide				1
Perch	2	3	1	1
Pike	1			
Roach	1		1	1
Eel	1			

Table 6. The complete list of different species caught using electrofishing on 23/9/21.

23/9/21 Species	Tornelundsvej	Teestrup Bro	Hjelmsølille
European bullhead			1
Gudgeon	20	10	3
Spined loach	49	3	40
Ninespined stickleback	1	1	
European minnow	2		19
Perch	85	132	318
Roach			34
Pike	1	1	4
Tench		1	

Manual push net fishing turned out to be better than electrofishing for catching spined loach but the method were only used for the initial testing and in May. The initial testing caught one minnow, two bullhead and spined loach. Snorkelling were also effective for finding spined loach and bullhead. During the survey in June at the initial release site both electrofishing and snorkelling found four bullheads and snorkelling found eight spined loach compared to five with electrofishing.

Night surveys with a flashlight showed no bullhead or minnows at four different sites, two upstream and two downstream of release site. Bullheads were only found at the initial release site (see figure 5). However smaller pelagic fish such as minnow, common roach and common bream were difficult to distinguish from each other and could have resulted in assigning the wrong species to some of the fish. The observations were only recorded if it was possible to identify the

Figure 5. Bullhead lying in the open during night. The bullhead was approximately 4 centimeters long and is most likely spawned in the stream.

species with absolute certainty. If there were any doubt the fish wasn't noted.

Sport fishermen fishing in the stream report that they have seen small bullheads at Teestrup Bro during the night, before any fish having been released at the site. They also reported catching bullheads further downstream of the release site towards Vetterslev. No confirmation in the form of pictures or caught fish has been provided, however the same fishermen have caught and provided pictures of bullheads from Nymølle Bro and they should be able to identify the fish correctly. These reports show that the bullheads have moved approximately six kilometres upstream from the initial release site.

All the surveys also showed large amount of signal crayfish (*Pacifastacus leniusculus*) at all the sites. No quantitative surveys were done on the signal crayfish but sites with more mud and less gravel and rocks seemed to contain fewer crayfish than sites filled with gravel and smaller rocks. The size did not seem to differ between sites and varied between 2-14 centimetres body length.

In June both electrofishing and snorkelling were done at the initial release site and both methods showed juvenile bullhead, proving that the released bullheads are breeding. It is unknown whether or not this is the first time it has happened since there is no record of the size of the released bullhead. Small bullheads were caught but it is impossible to tell if they are part of the fish released in the end of 2020 or if they were spawned in the river.

Morphological comparison

In total eight fish from the original Danish population and ten fish from other populations were measured. The size of the fish varied between 40 mm standard length for the smallest and 88 mm standard length for the largest. The number of second dorsal fin rays varied slightly between the Danish and Swedish bullheads and the bullheads from Basel and Băile Herculane with the northern populations having an average of 17 fin rays and the southern populations having an average of 19 fin rays. Due to the low number of fish no actual statistics have been made on the data. The latteral line was slightly longer for the bullhead from Stockholm (71,43% of standard length), Basel and Băile Herculane (average 70,57% of standard length) compared to the Danish bullhead (65,09% of standard length) but the

Danish population varies between 56,82% to 72,60% of standard length. Therefor both the Swedish bullhead and the average from Basel and Băile Herculane are within the same range as the original Danish population.

The average of the morphometrics by Riffel & Schreiber (1998) have been collected into figure 6 below. The bullheads from Basel and Băile Herculane were calculated together and the bullhead from Stockholm was added by itself for comparison with the Danish bullheads.

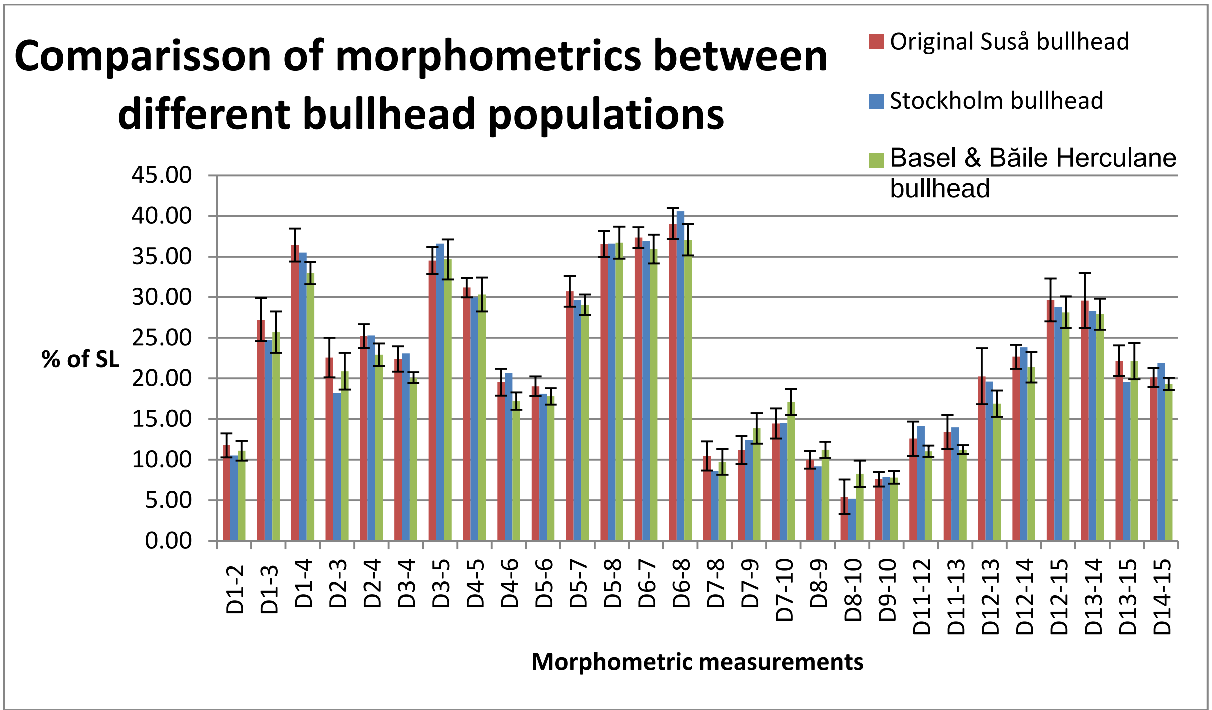

Figure 6 shows the average of the morphometrics by Riffel and Schreiber (1998) seperated into the Danish bullheads, the bullhead from Stockholm and the bullheads from Basel and Băile Herculane. The standard deviation is shown for the Danish bullheads and the bullheads from Basel and Băile Herculane. All data are shown as percentage of standard length.

Figure 6 shows the highly similar morphometrics of the different populations. Most of the measurements are the same between populations but for measurement D7-9, D7-10, D8-9 and D8-10 Basel and Băile Herculane are slightly higher. All four measurements concern the length from the second dorsal fin or the anal fin to the tail fin. The bullheads from Basel and Băile Herculane seem to have a slightly longer area between the fins and the tail although the standard deviation for the two populations overlaps slightly.

All the morphometric variables can also be combined in an ordination plot (Figure7). The plot below shows an ordination plot with all the morphometric variables included. The variables consist of the measurements done as shown in figure 6 above and the length of the lateral line. All data are calculated as a % of standard length. The plot is made using the package "Vegan" in R version 1.3.1073.

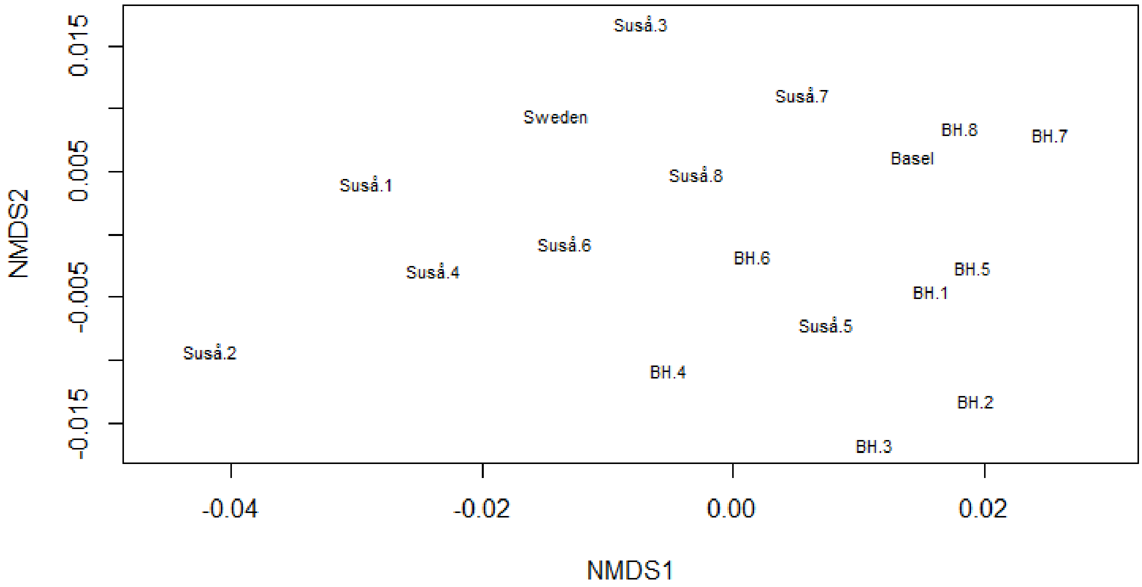

Figure 7 shows an ordination plot of the different preserved European bullheads. The plot is based on the data from 29 different morphometric variables.

The plot shows a mostly clear difference between the Danish and Swedish fish on the top left and the fish from Basel and Băile Herculane on the bottom right. However one fish from the River Suså is located in the bottom right.

Discussion

Evaluating the results

Electrofishing showed that both European bullhead and European minnow had moved from their initial release site. The bullhead had moved approximately two km and the minnow had moved slightly upstream or far downstream depending on which site they came from. Both species were found at the site Hjelmsølille where the local municipality had modified the stream in an attempt to restore it. Large boulders had been placed in the stream to provide shelter for the bullhead beneath and for the minnows behind the boulder. These improvement are planned several other places along the stream and could help both the bullhead and minnow establish a self-sustaining population.

The result suggests that some of the bullheads have moved approximately two km downstream. This could be due to lack of shelter at the release site, due to the fish being washed downstream during times with large amount of rain and a powerful current or simply migrating downstream. However Knaepkens et al. (2004 and 2005) showed that European bullhead in Belgium did not move more than 270m from their original capture site over the cause of half a year. Both studies by Knaepkens et al. showed that some bullheads move around while others stay within 10 metres of their capture site and some fish switched between being mobile and being stationary. Knaepkens et al. (2004) found that the fish moved around more during spawning season while Knaepkens et al. (2005) did not find any seasonal difference. Since the bullhead in River Suså had moved approximately two kilometres it might be more likely that it was washed downstream by an increased current than that it swam that far downstream. If this is the case, the boulders dropped in the stream could help prevent the bullheads from washing away. However since bullhead require some kind of shelter it could be that it had moved because all shelters at the release site were full. Either by other bullheads or by signal crayfish. If the latter is the case then it could cause a faster expansion of the population to try accommodating the lack of shelter. A way to help the bullheads would be to increase the amount of available shelters. This could be done by dumping large rocks into the stream as they are doing now but a more specific method could be to place custom made shelters for the fish. For instance ceramic roof tiles placed on the bottom could provide potential shelters for the bullheads. However an increase in shelters could also help the signal crayfish since their population density are at times limited by the amount of available shelters (Bubb et al. 2009).

The minnows were found at three different sites: Tornelundsvej, Nymølle and Hjelmsølille. At Nymølle one was found in February, at Tornelundsvej two were found in September and at Hjelmsølille 19 were found in September. The one at Nymølle were found using manual push net fishing and the minnows caught at the two other sites were caught using electrofishing. Since the minnows have been released at several different sites both upstream and downstream of the three sites, it is impossible to know how far they have moved. Most likely the fish at Tornelundsvej and Nymølle had been released further upstream and either they were washed downstream or intentionally searched downstream. Minnows are able to swim at relatively high speeds but they need slow moving backwaters or pools to rest (Holthe et al., 2009). River Suså runs through mostly farmlands and many alterations have been made to reduce the chance of flooding of the farmlands. At the site Assendrup where the minnows were released in 2020 the stream have been dug down and channelized. This provide little to no shelter for the minnows in the fast flowing water. Assendrup is also one of the places where the thick-shelled river mussels are. Releasing the minnows close to the mussels might be a good idea when trying to get them infected by the larvae of the mussel but without any cover they will quickly wash downstream till they find shelter. They could potentially also have been washed downstream till they reach the first lake approximately 16 kilometres further downstream from the nearest release site. Here they might be able to establish a population and stabilize over the next few years however currently no minnows are known to live in lakes in Denmark (Carl & Møller, 2012). If during that time the river is restored to a point where the minnows will be able to live here permanently, they might be able to colonize the river from the population in the lake.

Over the last four years more than 15000 minnows have been released in the river over a stretch of approximately 15 kilometres but only 22 have been caught again by electrofishing. Sports fishermen have seen and caught them in the area and other parts of the stream. However the lack of fish caught using electrofishing clearly shows a problem with the release method and it is most likely the lack of resting sites. At Hjelmsølille where most of the minnows were caught, rocks had been placed to provide some shelters behind them at it seemed to be working. The municipality planned to create shelters using large rocks anywhere they were allowed to. Even though it could help stabilising the population of minnows it might not be enough to help the mussels if the minnows are unable to spread to the entire river system. The minnows released in 2019, 2020 and 2021 from Fyns Laksefisk were all supposed to be infected with the mussel larvae in an attempt to spread the mussel to new parts of the river and introduce new genes and individuals. However parts of the river are subject to weed cutting every year which could damage new mussels. Furthermore the density of host fish is for most stretches of the river far below recommended. The recommended density of host fish for thick-shelled river mussel is 40 fish per $100m^2$ (Stoeckl et al., 2020) and considering only 22 minnows were caught during the surveys it is unlikely that any stretch of the river contains that density. Of course European bullheads are also a viable host fish but considering the amount of minnows released compared with the bullheads the intention is for the minnows to be the primary host fish. To improve the conditions for the minnow many more resting sites have to be created either through dumping of large rocks or through creation of pools or indentations in the edge of the river. This however is not necessary an easy solution considering it would require the cooperation of the farmers and landowners along the river. Creating backwaters would potentially encroach on the farmland and the owner would have to give up part of their land for the project. Large rocks could potentially create a blockade in the river along with weeds and branches if not maintained which could lead to local flooding of the fields along the river. All these factors have to be taken into account when trying to re-establish the minnow population.

However with the bullheads spreading upstream towards the mussels and the newly released batch at Teestrup Bro given time they might be able to establish themselves in the same area as the mussels. Here they could act as host for the larvae and improve the chances of a successful reproduction of the mussels. Of course that would require that the mussels reproduced. Surveys of the mussels in 2017 and 2019 found a few gravid mussels but only one showed signs of fertilization (Schneider & Zülsdorff, 2017; Schneider, 2019). The low number of mussels could result in too little sperm being released into the water and a low chance of fertilization. If this is the case then the mussel population has to increase before they can become a sustainable reproducing population. This can be done either through the release of infected host fish as done already or through introduction of new mussels bred in controlled environment. This would also be a way to increase the genetic diversity in the population. A reduction in the population such as this will create a genetic bottleneck and reduce the diversity drastically resulting in a less resistant population.

Crayfish causing problems

During the surveys large amount of signal crayfish were found at the same sites. Signal crayfish is an aggressive and invasive species that often outcompetes the natural crayfish and can have a negative impact on the rest of the local fauna. Especially smaller benthic fauna are affected by the signal crayfish (Galib et al., 2021).

Bubb et al. (2009) examined, ex situ, the interaction between European bullhead and two English species of crayfish (the invasive signal crayfish and the native white-clawed crayfish) in relation to shelter use. The study showed that both species of crayfish was dominant over the bullhead. If a crayfish entered a shelter occupied by a bullhead, the bullhead would leave almost every time. This reaction was slightly higher for the invasive signal crayfish compared to the native white-clawed crayfish. On rare occasions the bullhead and crayfish would share the shelter and mostly during the daytime. During the daytime shelter sharing happened significantly more with the white-clawed crayfish than with the signal crayfish. During the experiment two bullheads died, one attacked by a signal crayfish and the other from unknown causes. Crayfish predation on bullhead has been observed but consistent predation has not been examined thoroughly in natural conditions. During spawning the male bullhead protects the eggs in a shelter. If the male is not present the eggs will be eaten. If a large crayfish enters the shelter during this time the bullhead could be forced to leave the eggs to avoid getting attacked by the crayfish. While Bubb et al. (2009) only observed one bullhead killed by a crayfish, four other experiments showed minor damage to the caudal fin apparently caused by the crayfish.

Galib et al. (2021) examined the effect of the invasion by signal crayfish on the brown trout, chabot bullhead (formerly part of European bullhead (Freyhof et al., 2005)), stone loach and macroinvertebrates in 18 streams in north-eastern England over the curse of seven years (2011-2018). The survey examined seven uninvaded streams, eight pre-invaded streams and three streams that were invaded during the seven years. They found that the population of benthic fish, bullhead and stone loach, declined significantly in pre-invaded streams from 2011 to 2018. In pre-invaded streams the population of benthic fish average declined in abundance was 83.2%. Especially the bullhead declined and in two streams they completely disappeared during the survey period. The study suggested that the large effect on bullhead could be associated with egg mortality and increased predation due to lack of shelter. In the pre-invaded streams the age distribution shifted from a large percentage of smaller juveniles to almost no juveniles. Consistent recruitment failure will lead to local extinction for the bullhead. While the chabot bullhead is not the exact same species as the European bullhead they share many attributes and aspects of their life cycle including egg protection. It is therefore reasonable to assume that the signal crayfish can have the same effect on the European bullhead as it has on the chabot bullhead.

In the context of re-establishing the population of European bullhead in River Suså, the signal crayfish can cause difficulty. Especially considering that the survey in England was on fully established populations of bullhead with invading crayfish while in River Suså it is the bullhead that has to establish a population. The signal crayfish can potentially make it difficult for the bullhead to expand the population further up- and down-stream from the initial release site. To help the bullhead establish a population in the stream, consistent removal of signal crayfish could be implemented to reduce their numbers and average size. An attempt at this has begun by encouraging the locals in Næstved municipality to begin collection of signal crayfish. Even though this initiative will never remove the signal crayfish completely, it could reduce the average size of the crayfish due to targeted collection of the biggest specimen (Iversen et al., 2011). However removal of the big crayfish could result in a higher growth rate for the remaining crayfish (Moorhouse & Macdonald, 2011). A smaller average size of the crayfish could help predators of the crayfish. Iversen et al. (2011) found that both perch and trout ate the signal crayfish in Alling Å but only the small ones. Ex situ they showed that large perch would eat signal crayfish to a size of eight centimetres. Electrofishing in River Suså showed perch up to about 15 centimetres but no trout. However local fishermen report catching trout every once in a while, suggesting that trout are present in the river system although not in high quantities.

To examine whether or not the attempt at reducing the population of signal crayfish is effect, quantitative data on the size and population density in River Suså is needed. Chadwick et al. (2020) tested a method of draining part of a stream and emptying it of any form of cover such as logs and rocks. Any crayfish found is removed and the water is returned to the area. Any crayfish hiding in the banks or elsewhere might move out following the return of the water and can be removed. Afterwards the water is drained again and the method is repeated at least three times or till no more crayfish is seen. This method showed a population density of up to 110.4 crayfish per square meter. Compared to previous reported densities of 3-20 per m^2 this result is extremely high (Chadwick et al., 2020). The high density is partly due to a more thorough search than net fishing, electrofishing or trapping and partly due to a removal of bias. The draining method includes all sizes while other methods often focus on the larger crayfish while ignoring the juvenile. Mask size of nets and traps are often too big to catch the juvenile crayfish. Chadwick et al. (2020) found that 36%-72% of the populations examined were juveniles meaning that a large part of the population will be ignored by conventional methods.

Morphological comparison

The bullheads did not show much morphological variation between the different populations. Even though the bullheads from Basel and Băile Herculane are recorded as *C. gobio* in the Zoological Museum database they could potentially be a different species as mentioned before. According to Kottelat and Freyhof (2007) some of the other species can only be distinguished from *C. gobio* by one or two characters, their distribution or by molecular characters. Since the bullheads from Băile Herculane were caught in 1963 and the bullhead from Basel in 1882 it is possible that their distribution have changed since then. Most likely it will be smaller today for instance due to pollution. Several other populations, such as the one in River Suså, have been reduced or exterminated due to human influence.

The same argument can be used when comparing the Danish bullheads with the bullhead from Stockholm. They are highly similar in morphology and the colours and patterns still visible on the old Danish specimens seem to be the same as on the Swedish one (Figure 8). In this case both populations are *C. gobio*.

Figure 8. Top: Preserved European bullhead from Stockholm, 2008. Bottom: Preserved European bullhead from River Suså, 1881.

Only two species of bullhead live in Denmark, *C. gobio* and *C. poecilopus*, and they do not share any habitat. *C. gobio* is only found on Sjælland and *C. poecilopus* is only found in Jylland (Carl & Møller, 2012). Furthermore both species are relatively easy to distinguish. The bullhead from Stockholm comes from an area where the same two species are found but as mention, they are relatively easy to distinguish. Even though the specimen from Stockholm is not from the same population as the one released in River Suså, the morphology should be the same. The fish released in River Suså are from the south and southwest of Sweden and are geographically closer to River Suså. The climate in southern Sweden is similar to the climate at Sjælland and the bullheads should be accustomed to it. Even if the populations have slightly different adaptations, according to Kottelat & Freyhof (2007) many of the European species of bullhead have the same or similar biology to *C. gobio*. The biggest difference is the age of maturity, the adaptation to brackish water and the temperature of spawning. Some species and populations spawn at higher or lower temperatures compared to *C. gobio* and some are adapted to spend part of or the whole year in brackish water. In some areas where several species share a habitat, hybrids have been found between the species. This occurs both in the Baltic Sea, the Gulf of Bothnia and in some rivers such as the Rhine (Kottelat & Freyhof, 2007). The highly similar morphology and biology, the hybridisation and the difficulty distinguishing different species without the use of molecular methods suggest that all the different species fill the same role in the ecosystem and are potentially interchangeable if adapted to the environment. If that is the case, then even with small differences between the original Danish population of *C. gobio* and the reintroduced population their function in and effect on the ecosystem should be the same.

Strengths of various methods

During the surveys various methods showed different strengths. Electrofishing especially presented at problem. The method is widely used for official population surveys but it is not very effective for catching fish hiding in mud, in dense macroalgae cover or beneath rocks. During electrofishing at one of the sites an attempt were made at catching the fish with nets at the stretch of the stream were shortly before we had done electrofishing. Following approximately 15 meters behind the people electrofishing nets were used to catch fish in the mud and vegetation. The method showed not only that large amount of fish were not caught by the electrofishing but also that some specific species were difficult to catch using electrofishing. Especially spined loach (*Cobitis taenia*) were not caught with electrofishing but was caught using a net. Spined loach hide buried in the mud which could make them more resistant to the electric current especially if the power of the electric current is weak. Even if the fish were stunned they were difficult to spot hiding in the mud.

The same problem can potentially occur with the bullhead. During daytime they hide in caves and cavities beneath the rocks. If they are stunned by the electric current they could remain in hiding beneath the rocks. Since many bullheads prefer flowing water (Kottelat & Freyhof, 2007), back currents behind the rocks could also help keeping the fish from flowing away while stunned.

An alternative method for detection of the presence of fish, either specific species or all the present species, is eDNA. The method is less invasive and requires less time in the field. By using the DNA present in the water from all present organisms it is possible to get a complete picture of the species composition of the tested area. Even species with very few individuals or species always in hiding releases DNA to the environment. The chance of obtaining the DNA in a water sample depends on the concentration in the water and the amount of samples taken (Goldberg et al., 2016; Jane et al., 2015). Goutte et al. (2020) used a combination of electrofishing and eDNA to monitor the complete species composition of fish in the Marne River and Seine River in and around Paris. The rivers had been surveyed using electrofishing since 1990 in accordance with the European Water Framework Directive. They wanted to compare the results of consistent electrofishing surveys with short term eDNA sampling. In 2017 and 2018 both electrofishing and eDNA sampling were done at nine different sampling sites and the results compared. The study showed that for short term studies the two years using eDNA gave a better picture of the species composition than the electrofishing surveys done over the last three to six years. However the electrofishing over the last 14 to 19 years showed four more species than eDNA. The complete 29 years of electrofishing showed six to seven more species than eDNA. However some species where only caught occasionally and always in low numbers suggesting that they are not established in the river and could be present due to random migration from other connecting rivers. The eDNA survey also detected six species that could not be present in the river, for instance iridescent shark (*Pangasianodon hypophthalmus*), European bass (*Dicentrarchus labrax*) and Atlantic salmon (*Salmo salar*). The six species of fish are all used for human consumption and were most likely detected due to contamination from trash and sewage released into the river. The study show some of the strengths of eDNA compared to electrofishing. In streams where electrofishing is difficult to carry out effectively, for instance due to high water levels or murky water as it was during spring in River Suså, eDNA can be used as an alternative method. McDevitt et al. (2019) did a similar study in an English canal and got the same result. eDNA showed more species than electrofishing but also species that might only be detected due to contamination from humans, such as brown trout and salmon. They also noted that small, solitary or nocturnal species were difficult to catch using electrofishing but were registered using eDNA.

An issue with electrofishing that can be avoided using eDNA is the invasive and stressful nature of the method. During electrofishing the fish are stunned, caught in nets and often contained in some way for a time. Not only does this stress the fish but at times they do not survive the treatment (Shaw et al., 2016; McDevitt et al., 2019). This happened during electrofishing in River Suså as well where a few smaller perch and roach and one of the larger perch caught took severe damage and potentially did not survive after being released again. Actions can be taken to reduce the amount of damage done to the fish but it might compromise the effectiveness of the survey. For instance by keeping the fish contained in properly oxygenated water for as short a time as possible or reducing the power of the gear. The last will however affect the catchment rate since some fish might not be affected by the lower power. If the species affected are common or unwanted species the loss of a few fish will not present a problem but if the affected species are rare or threatened it becomes problematic. In a situation such as the one in River Suså every bullhead and minnow released count towards a reestablishment of the species. In this case a less invasive method would be preferable when during population surveys. Especially considering that during the restoration project electrofishing is done at least once a year resulting in a high probability that some of the released fish will eventually die because of electrofishing. In this case the less invasive eDNA method would be preferred. Shaw et al. (2016) found that five 1L water samples were enough to detect 100% of the species in a river when using two complementary genetic loci. If only detection of species is the goal then eDNA is a far more effective method when tested thoroughly. The decreasing cost of sequencing also makes the method ever cheaper. Goutte et al. (2020) calculated the cost of electrofishing versus eDNA and found eDNA to be the cheaper of the two methods. However eDNA does require some equipment and knowledge that smaller government organisations, municipalities or similar might not have. They will instead be relying on outsourcing which could increase the price.

A drawback with eDNA is the lack of a way to quantify the results. It is sometimes possible to obtain a relative abundance of the different species based on the counts of matches in the samples but a more accurate population density is difficult to measure (Evans & Lamberti, 2018). Too many factors affect the concentration of eDNA. While there is a positive relationship between the biomass of a species and the amount of DNA in the surrounding environment other factors can distort the result. Fish are rarely spread homogenous in the environment and the DNA will therefore not be found in the same concentrations everywhere (Goldberg et al., 2016; Hempel et al., 2020; Jane et al., 2015). In running water such as a stream or river, the DNA can quickly spread downstream but in lower and lower concentrations. If an attempt were made to quantify the population of a species different results would occur depending on where the samples were taken as shown by Jane et al. (2015). This along with other factors such as degradation speed and settling makes it very difficult to obtain an accurate estimate of a population. Rare species with low abundance or species with a very low concentration can be difficult to detect both by electrofishing and eDNA. At one site Goutte et al. (2020) caught seven species in very low numbers using electrofishing but did not register them using eDNA. The opposite was the case for Hempel et al. (2020). They registered Rhine sculpin (*Cottus rheanus*) upstream of a barrier using eDNA but could not find them using electrofishing. This could either be due to false positive results for instance caused by contamination, a very low concentration of Rhine sculpin in the area, or in this case it could be caused by a predator moving a dead sculpin to the area upstream the barrier leaving DNA in the water.

The studies by Goutte et al. (2020) and Hempel et al. (2020) show that both electrofishing and eDNA have strengths and weaknesses and the best result is often achieved using a combination of methods. The studies by Goutte et al. (2020) and McDevitt et al. (2019) also shows that a cautionary approach is necessary when using eDNA, especially in close proximity to human activity. The samples can easily be contaminated by human sources and provide a false positive for species not present in the examined water body. False positives can also be mitigated slightly when using a combination of methods. If eDNA shows a high amount of DNA from an unlikely species and electrofishing does not result in any being caught it could indicate a false positive from human contamination.

A different approach using eDNA for monitoring of the reintroduction success of a fish was attempted by Riaz et al. (2020). They used a combination of eDNA and species distribution models (SDMs) to locate suitable habitats for the reintroduced fish followed by confirming the results with electrofishing. The SDM use available data on the relevant species and the environment it is introduced to, to predict suitable locations for reintroduction or to suggest site to survey after reintroduction. The method takes time because the SDM has to be calibrated to work properly but each new data point increases the accuracy of the model. While it is time consuming in the beginning SDMs could be more effective long term than for instance electrofishing or mark-recapture methods and require less fieldwork to monitor the reintroduction success. Especially if combined with a fast method such as eDNA (Riaz et al., 2020). The drawback of the SDMs is the requirements of data for the area where the reintroduction takes place and data on the habitat requirements for the species in question. The spatial scale of the data is also relevant with small scale data being far more useful. However if a suitable model is created it could also help locating undetected populations as was the case with Riaz et al. (2020). This application might not be too useful in Denmark where every body of water is thoroughly searched by fishermen but in countries with more wild areas with little to no human contact SDMs could provide some useful data.

A different method of recording the presence of certain species is through observations. This can for instance be from the shore, from boats or snorkelling. Both observations from the shore and snorkelling were used to survey River Suså. Observations of bullheads by local fishermen prompted a more thorough search at some of the survey site. The fishermen reported seeing bullheads at sites where electrofishing had not provided any. The help of locals and volunteers can often provide results that professionals perhaps don't have the time to get. In relation to the project in River Suså some fishermen had spent large amount of time at night during the summer along the stretch of the river where the bullheads had been released. The municipality did not have the time and resources to survey during the night and the fishermen were therefore the first to report the migration of the bullheads far upstream. They were also the first to report seeing juvenile bullheads and further examination showed their observations were correct. While not all reports from volunteers are correct, especially when it comes to less known and rare species, they are a valuable resource.

The methods of snorkelling and observations from the shore were used because they could be done by one person and did not involve any specialised equipment. Methods such as electrofishing are always done by at least two people, both for practical reasons but also for safety reasons. Furthermore it requires a course and a licence. In relation to this survey only the people from the municipality had the equipment and necessary license. Since they were limited by time and funding alternative methods had to be used to increase the chance of finding migrating bullheads and minnows. However snorkelling is time consuming and the observations are difficult to quantify. They also require a bit of experience in identifying the different species to avoid wrong identifications in the bad light conditions during night surveys.

The snorkelling were made difficult during the summer due to very low water levels. This was even more the case in areas with rocky bottom and fast flowing water. This however is one of the places bullheads prefer. That being said the method did prove effective in finding bullheads if only at the initial release site. Here several bullheads were found including juvenile. The bullheads often stayed at the bottom when found without fleeing when left alone. The made it easier to observe them and estimate their size and through that, their age. Even though the method was effective it should not be used during bullhead spawning. Many of the bullheads were found hiding beneath rocks and moving the rocks would destroy the eggs if there were any. The method also requires relatively clear water to be effective. During most of the late fall, the winter and most of the spring the water was too murky and the current too strong for snorkelling.

Conclusion

The restoration project in River Suså seems to be a success when it comes to the European bullhead. It has expanded from the initial release site and they are breeding. However the same cannot be said for the European minnow. Despite more than 15.000 minnows released over the last four years, few remain. This result is most likely due to a lack of suitable habitat for the minnow. Work on improvements has begun but whether or not they will be enough is impossible to tell yet. Neither the populations of minnows or the bullhead have so far spread to the site containing the thick-shelled river mussel but hopefully an increase in the population will eventually lead to an expansion into the site.

The morphological comparison between the old preserved specimen of bullhead from the museum and the specimen from Sweden showed no clear morphological. It can therefore be assumed that introduced population of bullhead should be able to establish themselves in the River Suså as long as the habitat is of sufficient quality.

Further work in the attempt to restore the River Suså could include improvements for the minnows in the form of creating pools and slow moving backwaters the minnows can use as resting sites. For the bullheads creation of shelters might help but mainly a reduction in the amount and size of the signal crayfish if possible would improve conditions.

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