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Detection of an invasive, semi-aquatic mammal: development of an environmental DNA assay and comparison to a conventional method

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A UK perspective on detecting and managing invasive American mink

Abstract

Invasive species, particularly mammalian predators cause serious negative ecological and economic impacts across the globe. This thesis focuses on the American mink *(Neovison vison*), which is one of the most invasive vertebrate species in Europe; having negatively impacted on the populations of at least 47 native species in Europe alone. Although elimination of an invasive species is the most efficient way to protect native wildlife, it is often not possible and population control projects are frequently employed instead. In the UK, although a previous attempt to eliminate mink failed in the 1960's, subsequent control projects across the country have succeeded.

However, traditional methods of detection are often imperfect and can have poor detection rates, both of which can ultimately lead to wasted effort and resources. The predominant method of detecting and controlling mink in the UK, by using rafts, is no exception in its imperfections. Raft monitoring requires substantial physical effort, native mustelid signs on rafts can be confused with American mink and the rafts can be easily damaged or lost. Detection of environmental DNA may provide a suitable alternative means of monitoring mink as its use has repeatedly proven to be a more sensitive and effective means of detection compared to traditional methods. Here, we provide an overview on the impact of mink, past mink control projects and the potential of eDNA in invasive species detection.

Introduction

Humans have been introducing non-native species throughout the world for hundreds of years, both deliberately and accidentally (Manchester & Bullock, 2000). Consequently, non-native species have helped shape ecosystems into their current states, with approximately 3.9% of the globe's vascular plants having already become naturalised outside their native ranges due to anthropogenic activities (Doherty et al., 2015, Pagad et al., 2018). Globally it has been estimated around 20-30% of non-native species cause adverse impacts (Pimentel

et al., 2001), which leads to these species being defined as “invasive” (Doherty et al., 2015). Invasive species, described as “immense, insidious and usually irreversible” by the IUCN are considered the second foremost contributors to the endangerment and recent extinction of native species (Bellard et al., 2016, Bremner & Park., 2007**,** Pejchar & Mooney., 2009). Furthermore, analysis by Bellard et al., (2016) revealed invasive species were the most common threat connected to vertebrate extinctions overall. Prior estimates of the economic impact of invasive species, although varying greatly, have been immense. Indeed, the cost to the EU alone has been estimated as 12.5 billion EUR per annum at a minimum, but it may well exceed 20 billion EUR (Keller et al., 2011). If current trends in international trade and travel continue, more introductions will inevitably occur and the environmental challenges presented by these species continue to grow (Boltovskoy, Sylvester & Paolucci., 2018). Invasive species create new trophic links, which impacts the ecosystem either directly or indirectly though several mechanisms: competition, predation, hosting disease, hybridisation, herbivory or even through habitat alteration (David et al., 2017). Whatever the mechanism, this additional link to the food web can result in a reduction in species diversity, altered community interactions and decreased abundance of native species abundances (Gallardo et al., 2016). For example, predation by the red fox (*Vulpes vulpes)* and feral cats (*Felis catus)* in Australia have driven the decline or extinction of approximately 66% of native digging mammal species (Doherty et al., 2016). This has had to cascading ecological effects, with a reduction in topsoil disturbance leading to impoverished environments where seed germination is low and little organic matter accumulates (Doherty et al., 2016).

Invasive predators, especially mammals, are particularly damaging to native populations and are implicated in 58% of all bird, mammal and reptile extinctions (Bellard et al., 2016b, Veitch & Clout 2002; Doherty et al., 2016). Indeed, of the top ten invasive species threatening the most vertebrate species, six are mammals (Ballard et al., 2016b). Furthermore, invasive predators are thought to target native prey more swiftly and efficiently than indigenous predators - thus reducing populations more severely (Clavero et al., 2009). Rats (*Rattus spp.)*, cats and dogs (*Canis familiarise)* are theh species that have had profound impacts on biodiversity; cats alone have been linked to the extinction of 63 species globally (Doherty et al., 2015). The exact reason invasive predatory mammals have been so

damaging remains unclear, however it does appear that they have a higher establishment and spread success rate than other taxa. Additionally, the most problematic or "successful" invasive species are highly mobile and adaptable generalists (David et al., 2017; Melero et al., 2018; Courchamp, Chapuis & Pascal., 2003). Finally, it should also be noted that humans are very effective dispersal agents and also provide the means (through husbandry) by which populations of non-native species can grow to a viable number needed to establish a population (Courchamp, Chapuis & Pascal., 2003). Mammals have often been purposefully introduced by humans, including those either originally brought as pets (cats or dogs) or as livestock (goats or pigs) (Medina et al., 2011). Rats, although introduced accidentally, have a commensal relationship with humans and so, have been transported around the globe alongside humans (Jones et al., 2008).

Invasive species are not often the sole cause of species extinction, but commonly occur with other stressors such as habitat loss, pollution, overhunting and human disturbance (Bellard et al., 2016, Denley, Metaxas & Fennel., 2019). In these Instances while the true contribution of each stressor cannot be known, invasive species can be pivotal in driving population declines (Denley, Metaxas & Fennel., 2019). For instance, although the Nile perch (*Lates niloticus*) caused the extinction of several hundred haplochromine fish species in lake Victoria through predation, the stocks of these species had already declined due to overfishing and pollution (David et al., 2017). Multiple stressors interacting can result in compounded and synergistic consequences, which can make determining the specific role of a particular invasive species complex (Garrick et al., 2013; Brook 2008). Additionally, Invasive species can also modify ecosystem structure, which further complicates interactions and even facilitates additional invasion (Denley, Metaxas & Fennel., 2019). For example, invasive rhesus macaques (*Macaca mulatta*) on the Desecheo Island, Puerto Rico, were implicated in the modification of vegetation structure through several extinctions of local populations of animal and plant species (Hanson et al., 2019).

American mink (Neovison vison)

The American mink (hereafter mink), as its name suggests, is native to North America, specifically Canada and the USA, where their predominant prey species are muskrat

(*Ondatra zibethicus)* and hares (*Leporidae)* (Macdonald & Harrington., 2003). Mink are semi-aquatic mammals and their home ranges tend to be linear to waterbodies, particularly riparian corridors with dense riverbank vegetation (Zabala-Albizua et al., 2007). They can adjust to practically any waterbody, including rivers, marshland, fjords, coastlines, offshore islands and even small ephemeral streams and ditches (Macdonald et al., 2015). Mink are adaptable, intelligent, highly mobile, generalist predators that can change diet and prey base depending on conditions, such as habitat, prey availability or concentration (Stefansson, von Schmalensee & Skorupski., 2016). Mink diet analysis indicates that predominate prey species are terrestrial birds and mammals, but reported prey items also include crustaceans, invertebrates, fish, amphibians and reptiles (Harper et al., 2020, Zschille et al., 2013). Macdonald et al., 2015 described them as voracious predators, "Mink eat almost anything they can catch, whether it be covered in scales, carapace, feathers or fur, in water or on land. Food items range from relatively small invertebrates to a 1-2kg rabbit". They will also surplus kill, that is, hunt beyond immediate needs and store the excess food for later. It is this behaviour in mink that has been linked to complete breeding failure in tern (*Sterna spp*.) and gull (*Larus spp.)* colonies (Bonesi & Palazón., 2007). To illustrate this point, in Iceland over 200 dead guillemot (*Urias spp.)* chicks were discovered in a single mink den (Macdonald & Harrington., 2003).

Following numerous escapes and releases from fur farms, and contrary to population decline in its native range, mink are now established in 28 countries; throughout Russia, South America and Europe, including the UK (Melero et al., 2010). Within Northern Europe, mink are considered to be one of the most invasive mammal species and are a considerable threat to biodiversity (Zuberogoitia et al., 2010). Mink were first introduced to the UK in 1929 for fur farming. Subsequently they have been found from the Northern Scottish Highlands to Cornwall in the southwest of England, despite the ban of UK mink farming in 2002 through the Fur Farming (Prohibition) Act 2000 (Martin & Lea., 2020). As a highly mobile mammal, mink will progressively colonise an area if left unchecked. For example, a study monitoring 161 sites in the upper Thames Valley from 1975 to 1995, found that sites colonised by mink had increased from 7% to 46%, (Macdonald et al., 2015).

Ecological Impacts

Mink have often caused conservation problems when they have established outside their natural range (Macdonald & Harrington., 2003). This has included predation of the threatened, endemic Magellanic woodpecker (*Campephilus magellanicus)* in Southern Chile, competition with the Japanese weasels (*Mustela itatsi)* in Japan and contributed to the extinction of the water rail (*Rallus aquaticus)* in Iceland (Jiménez et al., 2014, Uraguchi et al., 1987, Magnusdottir et al., 2014). In Europe at least 47 native species populations have been negatively affected by mink, with the water vole (*Arvicola amphibious*) being one of the most seriously impacted species, particularly in the UK (Harrington et al., 2020). The water vole is the UK's fastest declining mammal, which in no small part is due to mink predation. There have been multiple instances where the arrival of mink has been followed by the extinction of water vole in the area (Macdonald & Harrington., 2003). However, while the water vole is a rodent, the majority of species impacted are birds, particularly those that nest on the ground. Ground nesters are distinctly vulnerable to mink, who take eggs, chicks and even adult birds (Ratcliffe et al., 2008). Mink can also cause complete colony abandonment and repeated "up flights", both of which can lead to nest failure (Nordström et al., 2004). In Scotland, mink have caused widespread breeding failures at tern colonies, while nest predation has had significant impacts on the blackheaded gull (*Chroicocephalus ridibundus*) and common gull (*Larus canus)*, who's populations declined by 52% and 30% respectively (Macdonald & Harrington., 2003).

Europe is home to a number of native mustelids, including the pine martin (*Martes martes)*, badger (*Meles meles)*, weasel *(Mustela nivalis)*, stoat (*Mustela erminea)*, European polecats *(Mustela putorius)*, European mink (*Mustela lutreola)* and European otter *(Lutra lutra).* The latter three species are in direct competition with mink as they have similar diets and can occupy the same habitats (Harrington et al., 2020). This competition can be especially problematic when native species are smaller and less aggressive than mink. Indeed, a study radiotracking both mink species over a 4-year period demonstrated that American mink drove European mink, a critically endangered species, away from rivers (Sidorovich & MacDonald., 2001).

Mink became widely established in the UK during the 1960's, while otter and polecat were largely absent due to persecution and pollution. However, these populations are now

recovering and are more widespread (Bonesi and Macdonald., 2004b, Harrington et al., 2020). Several studies have demonstrated that the presence of otter affects mink behaviour, with mink becoming more diurnal and their diet more terrestrial, consuming less fish (Bonesi et al., 2006, Macdonald & Harrington., 2003, Macdonald et al., 2015). A study by Harrington et al., (2009) also recorded reduced body weight and body condition of mink in the presence of otters. Conversely, otter do not appear to change behaviour in the presence of mink. This is most likely due to them being approximately seven times larger than mink and therefore a superior competitor. Otters have even been observed taking food from mink yet there is little evidence that the return of otter has caused an actual decline in mink populations across the UK (Bonesi et al., 2006, Bonesi, Dunstone & O'Connell., 2000, Harrington et al., 2009). The situation regarding pole cats is less clear; a study in Belarus reported that female polecats were outcompeted by American mink and expelled from riparian habitats (Sidorovich & MacDonalds., 2001). However, there has been no evidence of this occurring in the United Kingdom, perhaps because polecats are slightly larger, and so more comparable in size to mink (Harrington et al., 2020).

Mink do not only impact individual species but can affect entire ecosystems, inducing a tropic cascade (Fig 1). On small islands in the Baltic sea it was reported that areas under long term mink removal had higher plant diversity and greater equability between the abundances of plant species, compared to areas not under management (Fey et al., 2009). This was because mink directly impacted on vole populations through predation, and indirectly by driving alterations in vole behaviour which reduced grazing pressure in certain areas. Reduced grazing pressure and disturbance on patches normally provided by voles (*Microtus agrestis* and *Myodes glareolus)*, allowed fewer, more competitive plant species to dominate (Fey et al., 2009).

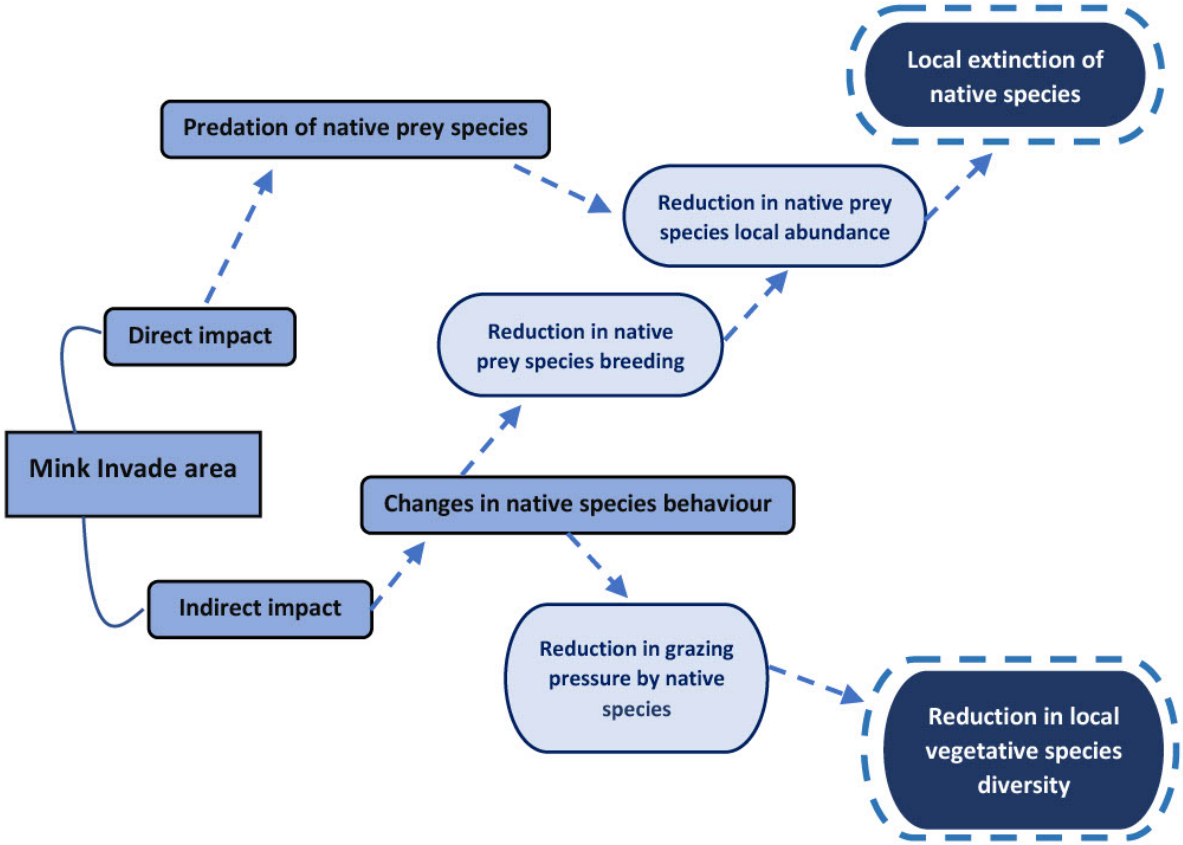

Fig 1. Impacts of American mink outside range on non-native wildlife

COVID 19

Mink have been identified as a potential source of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); the virus responsible for coronavirus diseases 19 (COVID-19). SARS-CoV-2 has been detected within farmed mink populations in at least eleven countries, with 17 million farmed mink culled in Demark due to human health concerns (Dyer., 2020, Fenollar et al., 2021, Opriessnig & Huang., 2020). The virus was first introduced to farmed mink via humans, and mink have since acted as a vector and passed it back to humans (Oude Munnink et al., 2020). Infected farmed mink from the United States have experienced widespread mortality with post-mortem examinations of culled mink showing signs of pneumonia (Sharun et al., 2020). Other mustelids are also susceptible to COVID 19; European mink infected with SARS-CoV-2 have also been detected, while domestic ferrets (*Mustela putorius furo*) have demonstrated extremely low resistance to COVID-19 (Manes, Gollakner & Capua., 2020, Sharun et al., 2020). While the exact effects of COVID-19 on native populations of wild animals is still unknown, any impact to fitness would have far

reaching consequences for both individual species, especially for highly endangered species such as European mink, and the ecosystem.

Uncontrolled infection in wild populations would also allow the virus more opportunities to mutate. In comparison to humans, the virus in mink seems to mutate faster; around 170 mutations were detected in SARS-CoV-2 samples collected from only 40 mink farms (Fenollar et al., 2021). Mink specific SARS-CoV-2 mutations have also been detected in humans, including the Cluster-5 variant, which may be less responsive to vaccinations due to an alteration in its spike protein (Fenollar et al., 2021). Furthermore, a wild mink trapped just outside a mink farm in Utah, USA was confirmed to have tested positive for SARS-CoV-2 in December 2020, the first case in a free-ranging animal. SARS-CoV-2 within wild mustelid populations could be particularly problematic as humans would be unable to control their movement. Thus, the spread of SARS-CoV-2 within wild populations would be very challenging to manage, which could ultimately result in a permanent reservoir of the virus, as in bovine tuberculosis in badger populations (Manes, Gollakner & Capua., 2020).

Population management of mink in the UK

The UK government supports the management of around 20 invasive species, including mink (DEFRA., 2015). Key legislation associated with UK invasive species management includes the EC Habitats Directive and the Convention on Biological Diversity, both of which require the control of non-native populations. Further legislation includes the Bern Convention that gives legal protection to native species and habitats, and The Wildlife and Countryside Act 1981 which, likewise, gives legal protection to native species while also prohibiting the release and spread of certain invasive species (Manchester & Bullock., 2000).

The options available for dealing with invasive species such as mink are eradication, control, mitigation or no action (Bremner & Park., 2007). Active management of invasive species can employ a variety of methods, including snares, shooting, toxic baits, fencing, food removal, judas animals, detection dogs and live or kill traps (Gary et al., 2007, Orueta & Ramos., 2001). However, it should be noted that certain methods are illegal to use depending on individual countries' regulations (Gary et al., 2007). For example, an effective mink eradication project in Estonia on Hiiumaa Island used leg-hold traps, while hunting dogs were used in the Archipelago National Park, South-west Finland (Melero et al., 2010). In the

UK however there are strict guidelines on what methods of capture and dispatch can be adopted, due to risk of capturing and/or killing of non-target protected schedule six species (otter, polecat, pine martin, badger), kill-traps, self-locking snares, poison and leg hold traps are illegal. Hunting wild animals with dogs was also made illegal under the Hunting Act, 2004. Such methods are also controversial and disliked by the public, whose support is critical for a successful control project.

Long-term eradication of invasive species is the most effective and cost-efficient method of management (Martin & Lea., 2020). However, this would require large scale cooperation between landowners and stakeholder organisations across the UK mainland (Martin & Lea., 2020). Such operations require extremely good planning and a large investment of resources over a relatively short time period, which few eradication operations have successfully managed (Mehta et al., 2007). The UK attempted to eliminate mink in the 1960s but failed - despite the culling of 5000 mink - partly due to insufficient effort and planning (Macdonald & Harrington., 2003). Furthermore, several invasive species control projects have been implemented without full scientific understanding, evaluation or consideration of pre-existing scientific advice, leading to the waste of resources and failing to gain desired results (e.g. protecting native wildlife or eradication of invasive species). For example, in Australia, millions of dollars and thousands of man hours had been expended on cane toad (*Rhinella marina)* management before the scheme's failure to control toads was discovered. The project was not evaluated beforehand and focussed on the number of individuals removed instead of density changes, a much more reliable indication of control effectiveness for cane toads. (Shine & Doody., 2011).

The best method to reduce the negative impact of mink is to remove them from the wild - catching and humanely euthanising them (Martin & Lea., 2020). The benefit of removing mink has been highlighted by a number of studies (Bodey et al., 2009). For instance, a mink control experiment on a Baltic Island demonstrated a subsequent increase in the breeding of many bird species (Nordström et al., 2003). In one removal area, two species that had become locally extinct, the razorbill (*Alca torda)* and black guillemot (*Cepphus grille)*, returned (Nordström et al., 2003). Mink removal from islands in the UK has been followed

by the increased abundance and/or breeding success of native prey species (Harrington et al., 2009).

Mink have already been widely trapped and lethally controlled in the UK for decades. Projects have varied in size, from individual initiatives on a voluntary basis to large-scale schemes employing professionals. Millions have been spent on this mink control, for example, one successful mink control project in the Hebrides cost £5.25 million alone, over 16 years (2001-2017) (Macleod et al., 2019). However, conservation and environmental management are chronically underfunded globally; cost being the major restriction of most control projects (Roberts et al., 2018). Conservation organisations must prioritise their limited resources and are often only able to implement management on localised scales (Byers et al., 2002). There are obvious incentives for the development of new, effective methods for mink detection to assess whether localised control is effective. It has also been argued that there is a fundamental ethical requirement to ensuring that lethal population control is as efficient as possible (Harrington et al., 2009).

The Raft Method

Rafts are the most commonly used means for detecting mink in the UK (Martin & Lea., 2020). The raft is used to detect signs of mink, and then to house a live trap once mink presence is confirmed. The raft method (developed by the Game and Wildlife Conservation Trust) has proved to be a much more effective means of detecting mink presence compared to that of field signs surveys that were previously relied upon (Reynolds et al., 2004). Rafts are deployed at a recommended interval of 1km to increase chances of detecting mink (Harrington et al., 2009). The floating raft itself is made of polystyrene encased in plastic or wood with a polystyrene filled and clay topped basket in the centre, the raft is then covered with a plastic or wooden housing (Fig 2). When a trap is set it is placed inside the plastic housing (GWCT., 2015, Reynolds et al., 2004). To reduce the likelihood of trapping non-target species the entrance of this plastic housing restricts species bigger than mink from being able to enter (Reynolds et al., 2004). The clay reliably records mink footprints and allows for comparison between rafts, no matter the substrate of the river (Reynolds et al., 2010). Some projects use lures; bait (food, normally fish) and commercially available mink glands – the scent lures attracting mink 50% more successfully compared to fish bait (Moore

et al., 2003). Rafts should be checked approximately every two weeks for signs of mink (footprints or scats) and if a mink is detected then a trap should be set. The trap should then be checked every day until removed or a mink caught. If a mink is not caught within 10 days; the trap should be removed (GWCT., 2015). A trained individual should dispatch trapped mink humanely.

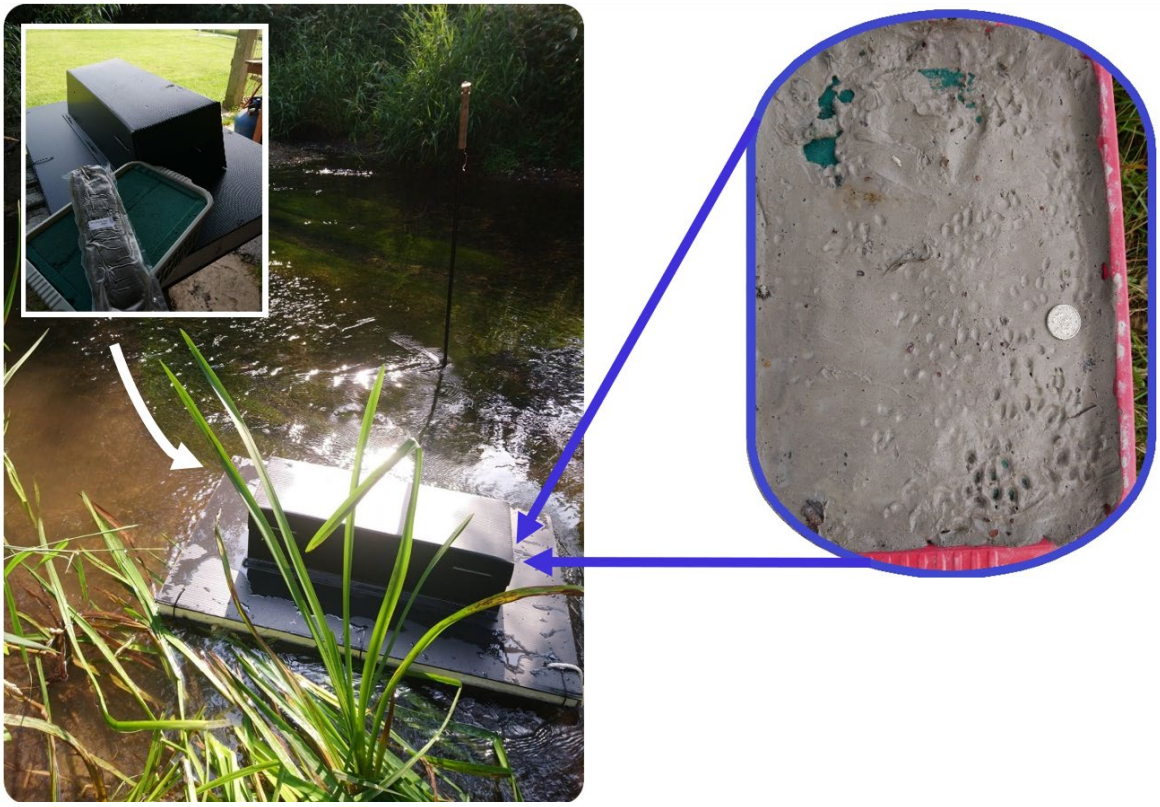

Fig 2. Mink raft method: components of mink raft including oasis basket and clay top (image top left corner), mink raft in situ, and basket with mink prints

Mink control projects

Several mink-control projects in the UK demonstrated that the rafting method does work, provided they have enough resources and support (Bryce et al., 2011, Harrington et al., 2020, Roy., 2012, Zuberogoitia et al., 2006). The first notable example includes four successive schemes centred on the Cairngorms National Park. Mink control started in 2004 at 30 km^2 and scaled-up to 29,000 km^2 in 2010 - the largest mainland invasive species eradication project globally (Fig 3, Lambin, Horrill & Raynor., 2019, Harrington et al., 2020). The project deployed a “rolling carpet” of mink rafts (which were mainly surveyed by

volunteers) and it successfully reduced mink presence to absent or scarce, highlighting how valuable the help of volunteers is in conservation (Lambin, Horrill & Raynor., 2019).

A second large-scale project started in 2001 and was run by the Eastern Region Mink and Water Vole Project, which covered 26,000km^2 across nine counties in England (Fig 3). Not all the counties recorded mink declines, but Norfolk recorded 90% declines in annual mink trapping numbers. It was proposed that while Norfolk trapping efforts had been consistent for over 10 years, counties with high mink abundance often underwent discontinuous trapping efforts which may have impacted on its efficiency to control mink. A lack of data however, means it is not possible to fully assess project efficacy - a common challenge in population management (Harrington et al., 2020).

A third project in the River Monnow catchment area, UK over 203km demonstrated that continuous trapping can successfully reduce mink presence (Fig 3). The 13-year project (2006-2010) recorded an initial rapid decline in detection during trapping followed by increasing periods where no mink were seen (Reynolds et al., 2013). Lastly, a study was conducted on the Upper Thames catchment and included a 20 km stretch of river, the smallest stretch assessed in the academic literature (Fig 3, Harrington et al., 2009). It concluded that mink removal using rafts could effectively reduce mink population through trapping during only four months per year; although, on smaller sites the approach had to be more flexible and adaptive. Because of the constant risk of immigration, constant monitoring and a reactive removal strategy would still be required to protect water vole populations. In combination, these projects demonstrate with continuous monitoring and consistent, strategic effort, it is possible to control mink over large areas of the UK, and perhaps would be feasible to eliminate them entirely from the UK in the future.

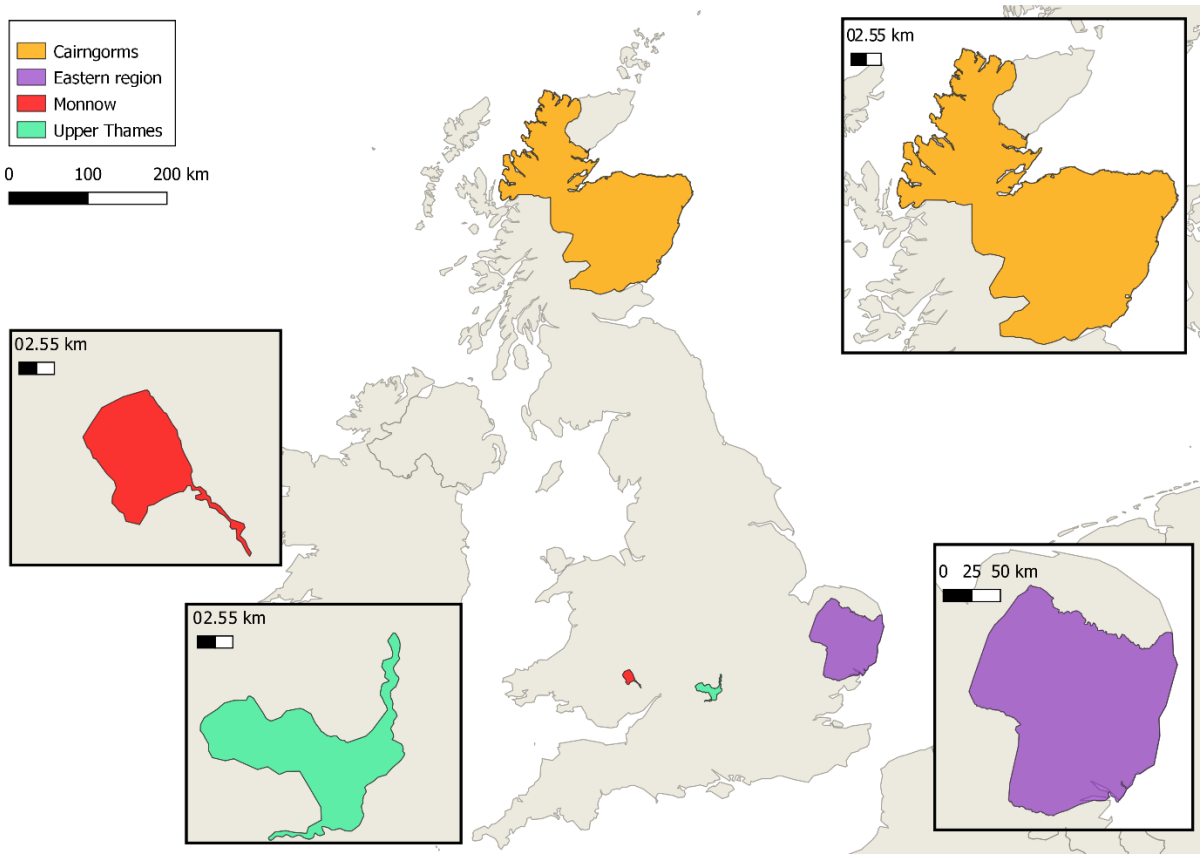

Fig 3. Areas in which mink control projects were undertaken in the UK. Based on maps provided by Lambin, Horrill & Raynor, 2019 (Cairngorms), Tansley, 2018 (Eastern regions), Reynolds et al., 2013 (Monnow), Harrington et al., 2009 (Upper Thames)

Challenges with the raft method

However, the rafts are not without problems. Manually checking rafts and traps, especially when mink population is at low density, is time consuming and thus relatively expensive (Martin & Lea., 2020; Zabala-Albizua et al., 2010; King et al., 2009). As rafts need to be left in situ for a period of time, they can become damaged or lost due to the weather or human interference. Further increasing project costs in replacing lost equipment and wasted effort. Moreover, in areas where native mustelids are present, it is possible to confuse signs (scats and footprints) with mink. Polecats are of a similar size to mink and footprints can be difficult to differentiate. For example, an otter survey in the Wye valley reported a suspicious “200% increase in mink” most likely due to an increased abundance of polecats in the area, rather than mink (Harrington et al., 2020). When mustelids have similar diets, their

scats or spraints can also look visually similar. The Harrington et al., (2009) study reported that experts misidentified 60% of scats as mink. Although another study analysed 198 "mink" scats and deemed only 12 not to be mink, a low error rate of only 6% (Harrington et al., 2008). The likelihood of sign misidentification will vary depending on location, presence and abundance of native mustelids within an area. Falsely detecting mink would result in wasted effort attempting to trap (and check traps every 24 hours in accordance with animal welfare standards).

Furthermore, the raft method may not detect all mink as some individuals could be "trap/raft shy". So far, the current evidence regarding avoidance is rather limited and conflicting. A study by Harrington et al., 2009 recorded similar abundance pattens between sign and raft surveys (indicating that the raft method reliably detected general mink presence). However, sign surveys are neither an effective means of detection and tend to underestimate relative abundance nor an independent means of detecting mink, thus not an appropriate method to evaluate raft efficiency. It has also been found that some ferrets - closely related to mink - actively avoid traps (King et al., 2008). Yet, the only other methods of detecting mink besides using rafts are to rely on field sign surveys, camera traps and reported sightings; all of which are generally less successful at detecting mink than the raft method (Harrington et al., 2008, Macleod et al., 2019, Reynolds et al., 2004, Reynolds et al., 2010). The lack of independent and effective alternative methods of detection mean it is difficult to determine the efficiency of rafts/traps and, if individual mink remain undetected (Reynolds et al., 2013). Detection of all mink in an area is crucial as even a single individual missed by control efforts could still have significant negative impacts and allow for recolonisation of the area.

Genetic approaches for detection

Environmental DNA (thereafter eDNA) may have potential as a new method to detect and monitor mink. The environment retains physical imprints of species that have and may still inhabit it (Evans & Lamberti., 2017). Organisms shed DNA into their environment as they occupy it, including skin, hair, metabolic waste, blood, saliva or even decaying corpses (Williams et al., 2017). eDNA analysis involves the isolation and detection of this shed DNA, which indicates presence of the target species (Rees et al., 2014). Mitochondrial DNA is

often targeted for detection due to greater copy number per cell compared to DNA or rDNA, thus increasing chance of detection in a sample (Evans & Lamberti., 2017). The choice of sequence detected must also be typically 80-250 base pairs, as DNA starts to deteriorate as soon as it enters the environment, fragmenting into shorter strands (Evans & Lamberti., 2017). Indeed, eDNA can persist from just hours to thousands of years, the speed of degradation depending on environmental conditions, such as; temperature, salinity, pH, moisture content, flow rates, UVB radiation (Barnes & Turner., 2015, Buxton, Groombridge & Griffiths., 2017).

The concept of detecting degraded wildlife DNA was first applied to ancient sediment, and then in 2008 to detect an invasive species in French wetlands, the American bullfrog *Rana catesbeiana* (Dejean et al., 2012). Following this study, the use of eDNA has increased considerably in the field of conservation. eDNA detection has been investigated in many studies to infer presences, diversity and abundance of species (Darling & Mahon., 2011). Species that have been detected by eDNA include: mammals, amphibians, insects, reptiles, crustaceans, birds and fish (Jane et al., 2014; Ushio et al., 2018). Most eDNA studies have focused on the detection of eDNA suspended within aquatic systems, both marine, freshwater, lentic and lotic waterbodies (Sales et al., 2019). Legally and politically DNA evidence is widely accepted in society and eDNA evidence is also becoming more commonly used, for example as evidence that great crested newts (*Triturus cristatus)* are present in consulting ecology (Barnes & Turner., 2015). However, using eDNA as a method of detection is a developing field and there are further knowledge gaps to be filled. For example, how abiotic and biotic conditions affect eDNA degradation and transport is still being investigated (Lance et al., 2017). Several studies have investigated eDNA persistence for different species and environmental conditions. A study using metabarcoding for mammal eDNA found the signal was lost within only 1-2 days (Harper et al., 2019). Another study detecting Idaho giant salamanders (*Dicamptodon aterimus),* showed that 94-98% eDNA degraded over the first two days, with detectable eDNA concentration lost after 11 days (Pilliod et al., 2013). Despite differences in persistence, most eDNA studies show the general trend that after organism removal, the eDNA signal rapidly declines and then more gradually approaches zero (Lance et al., 2017).

Abundance

eDNA can detect a single species by using quantitative polymerase chain reaction (qPCR) or a community using metabarcoding. While metabarcoding is becoming increasingly popular, qPCR may be particularly useful when monitoring invasive species as it holds the possibility to indicate abundance of the target species (Pont et al., 2018). Several studies have found a positive relationship between eDNA concentration and species abundance/biomass (Jane et al., 2014). Doi et al., (2017) found a significant correlation between the eDNA concentration and abundance/biomass of the ayu (*Plecoglossus altivelis)*, Saba River, Japan. Another study also found a positive relationship for Lake Trout (*Salvelinus namaycush)* relative abundance against eDNA in 12 natural lakes in Quebec (Lacoursière-Roussel et al., 2016). Estimation of species abundance/ site occupancy would be partially useful for invasive species. Potentially this would allow for estimation of effort needed to trap all individuals, determine immigration/migration in the area and to ensure all individuals have been trapped. However, not all studies have found a positive relationship between eDNA and species abundance/biomass (Evans & Lamberti., 2017). For example, researchers looking to improve hellbender (*Cryptobranchus alleganiensis)* detection in North Carolina did not find a correlation between eDNA and field survey counts (Spear et al., 2015). The lack of relationship between eDNA and abundance/biomass found in several studies may be due to a number of factors, including site and seasonal environmental conditions affecting eDNA presence. Greater understanding of eDNA persistence and transport, as well as how and what environmental factors affect eDNA is required if an accurate estimation of species abundance is to be inferred (Evans & Lamberti., 2017). For instance a study on sockeye salmon (Oncorhynchus nerka), found eDNA rate reflected fish abundance more accurately when stream flow was taken into account (Levi et al., 2019).

Traditional methods versus eDNA

There is a widespread acknowledgement that traditional methods of detection, such as trapping, netting or acoustics surveys, can have poor detection rates. This is particularly true for rare or cryptic species, thus making detection impractical and/or costly (Darling & Mahon., 2011; Lawson Handley., 2015). However, eDNA has often been found to be more sensitive to rare or elusive species found within an aquatic environment than traditional techniques (Lugg et al., 2017). A study in Australia detected platypus, a semi aquatic

mammal, eDNA in water samples at all thirteen of their sites but only successfully trapped individuals at eight (Lugg et al., 2017). Another study that focussed on terrestrial and semi aquatic mammals, compared detection using metabarcoding against latrine surveys and camera trapping. For the same detection results, six eDNA sample occasions would be needed for every 14 weeks of camera trapping or five latrine surveys (Sales et al., 2019). This demonstrates that in many cases the traditional survey methods require increased effort in the field compared to eDNA, in order to achieve high detection probability for rare species (Jerde et al., 2011). In the UK, detection rates for great crested newt eDNA have shown occupancy at 99.3% whereas the traditional methods were significantly lower: bottle trapping (76%), torch count (75%) and egg search (44%) (Lawson Handley., 2015). Indeed, there have also been studies when species presence (initially detected by eDNA) has only been confirmed by traditional methods after intensive effort. (Jane et al., 2014). The detection of Asian carp eDNA in Brandon Road Pool, Chicago prompted a search using traditional survey methods, with a single carp discovered after 93 days of effort (Jerde et al., 2011). In instances where eDNA indicates mink are not currently present, this could allow for time and resources to be saved - particularly in remote, challenging locations. In areas where mink are present, eDNA may initially pick up on mink presence faster and with less effort than using rafts. eDNA could also be used as an alternative, independent detection method to the raft method. Having a second, reliable method of detection to complement the traditional method can also increase level of confidence when determining whether a species is absent or present.

Further advantages

Collection of eDNA samples in the field requires considerably less experience than most traditional surveys and, can thus also be less labour inattensive - especially if the traditional method can require repeated visits to confirm presence, as mink rafts do. (Evans & Lamberti., 2017). The high sensitivity of eDNA could prove particularly useful for detection when population numbers are low. This is a critical element for a successful control project when only a few individuals remain and are more difficult to detect, especially in aquatic environments (Jerde et al., 2011). On occasions where eDNA indicates mink are not currently present, this could allow for a saving of time and resources. Moreover, eDNA detection should also be species specific and avoid misidentification, unlike reliance on signs. Because

eDNA requires more experience in the lab and less in the field, it is a method that can still give reliable results when samples are collected by volunteers, who are often critical in mink control projects. One project surveying for GCN in the UK had 80 volunteers who surveyed 239 ponds and detected GCN eDNA in 91% of ponds, demonstrating that eDNA can allow much larger and more rapid surveys to be conducted than was previously possible (Lawson Handley., 2015). Furthermore, there have been multiple instances where eDNA has been more cost-effective than the traditional techniques. Detection of Bullfrogs eDNA was 2.5 times cheaper and less time consuming than the traditional survey (Dejean et al., 2012). Using eDNA to detect and quantify the Yangtze finless porpoise population (*Neophocaena asiaeorientalis*) was on average 1.65 times more cost-effective per month than visual surveys (Qu and Stewart., 2019).

Occupancy modelling

Although eDNA has been found to be more sensitive than traditional detection methods for numerous species, it can still be imperfect in its detection (missing individuals that are present). An occupancy modelling approach uses location specific detection histories from repeated survey occasions to account for imperfect detection and to provide a more reliable estimate of species occupancy. Depending on weather models assumptions are met, it can also be used as a proxy for abundance or habitat use (Smith & Goldberg., 2020). There has been an increasing incorporation of occupancy modelling in eDNA studies with the growing realisation that detection probability needs be taken into account. Studies incorporating occupancy modelling have ranged from monitoring of Burmese pythons (*Python bitittatus)* to the Chinook Salmon (*Oncorhynchus tshawytscha)* (López et al., 2017, Piaggio et al., 2014, Sales et al., 2019).

Furthermore, multi-level models allow for estimation of detection probability at different levels associated with eDNA sampling: occupancy within the site, eDNA within the water sample and detection of eDNA by qPCR r (Schmelzle & Kinziger., 2015). This approach can also be used to compare detection probability of multiple methods, as different survey techniques have different detection probabilities, occupancy modelling makes methods comparable. Sales et al., (2019) revealed that eDNA outperformed camera trapping in efficiency when comparing eDNA and conventional survey methods utilising a single season

occupancy model. To reach a detection probability of ≥0.95 for water vole; 3 latrine surveys, 4 eDNA water samples or 5 weeks of single camera deployment would be required respectively.

Challenges with eDNA for detecting mink

It should be noted that previous studies have indicated that the detection of carnivores using eDNA may be more difficult than for other mammals. While detection of mink using qPCR has not been reported in the academic literature, the use of metabarcoding to detect mammal communities has been documented (Harper et al., 2019, Sales et al., 2019, Thomsen et al., 2011). These studies have often reported lower detectability of carnivores compared to other mammals, or no detection at all. A study by Sales et al., (2019) failed to detect otter or weasel eDNA, despite camera traps confirming presence. Also, stoats and mink were not recorded by any of the methods employed, despite likely presence. Another study found that detection rates of otter (a mustelid with a similar lifestyle to mink) had a weaker eDNA signal compared to other semi aquatic mammals (Harper et al., 2019). A third metabarcoding study detected mink at one site on the river Colne, UK (Broadhurst et al., 2021). However, despite detection at only one site, detection probability was relatively high at 0.8. High detection probability could have been due to sample collection, date and mink ecology, as by late July any kits (with litter size varying from five to eleven) will have been hunting for themselves but remained in the same territory of their mother. This means the site could have had a relatively high abundance of mink at the site, thus allowing for a high detection probability (Pagh et al., 2021). However, this study did not employ any other detection methods or undertake sampling at a time when kits would have dispersed, so this cannot be confirmed (Broadhurst et al., 2021). Finally, a study by Lyet et al., (2021) found that carnivores were much less detectable than omnivores or herbivores. This lower detection probability is likely due to top predator ecology and behaviour, leading to a low presence of DNA within water bodies. For example, otters tend to spraint on obvious features outside of water, can have large home ranges, are territorial and solitary - characteristics shared with mink (Harper et al., 2019).

Metabarcoding may be less sensitive than single species detection via PCR, qPCR or digital droplet PCR (ddPCR). During metabarcoding eDNA from low abundance species can be masked by high abundance species eDNA, due to competition for metabarcoding primers and amplification bias (Harper et al., 2018). This is where common templates are more likely to be amplified than rarer sequences, using up finite metabarcoding primers. Quantitative PCR detected great crested newt (*Triturus cristatus)* in 50% of ponds, whereas metabarcoding detected GCN in only 34% when no threshold was applied (Harper et al., 2018). Another study, on Mediterranean fanworm (*Sabella spallanzanii*), found ddPCR and qPCR detection probabilities were nearly double that of metabarcoding (Wood et al., 2019). A reduced sensitivity could partly explain the difficulty detecting carnivores previous metabarcoding studies have encountered.

However, a PCR assay to detect river otter (*Lontra canadensis*) has been successfully developed and tested in the field by Padgett-stewart et al., (2015). This may indicate that species specific assays could still successfully be used as a method to detect top predators such as mink. Nevertheless, It is important to remember that it is possible to falsely detect a species that is not present using the eDNA method, due to: the presence of a carcass within the tested waterbody, preservation of eDNA after an individual had left the area or contamination of samples during sampling and testing (Roussel et al., 2015).

Conclusion

Invasive species have been repeatedly shown to cause negative effects on native species and ecosystems, which necessitates their active management. However, elimination projects are often difficult and expensive, with conservation organisations frequently limited by resources (Martin & Lea., 2020). Therefore, new means of detection should be developed to improve efficiency and reduce cost. Mink are an invasive species that have significant negative impacts on populations of native species, particularly water voles and ground nesting birds In the UK (Bonesi et al., 2007, Harrington et al., 2009). They may also act as a potential reservoir of SARS-CoV-2 (Harrington et al., 2021). Mink are managed to protect native wildlife in the UK. However, current mink detection methods (rafts, camera trapping and sign surveys) are imperfect and have various shortcomings that may make mink population control inefficient and costly to run (Harrington et al., 2020). eDNA has the

potential to provide an alternative, non-invasive, cost effective and more sensitive way to detect and monitor invasive species, including mink (Martin & Lea., 2020). This would provide the absence/presence data critical for successful management programmes and reduce the inefficiency of control. The continued development of species specific eDNA qPCR assays may play an important role in the detection of invasive species for future control projects and have far reaching benefits for the conservation sector.

Chapter 2: Detection of an invasive, semi-aquatic mammal – development of an environmental DNA assay and comparison to a conventional method

Abstract

The American mink (*Neovision vision*, hereafter mink) is an invasive predator that seriously negatively impacts native wildlife and needs managing. However, the conventional method of detection, using rafts, is effort-intensive and control projects are expensive. Environmental DNA (eDNA) has been found to be more sensitive than traditional detection methods in numerous instances and could provide an alternative, effective and efficient means of detection. This study developed a species-specific qPCR assay to detect mink eDNA. Mink was monitored on two rivers in North Wales using the conventional raft method paired with eDNA sampling. Occupancy modelling was used to account for imperfect detection and provide detection probability for both methods, in addition to probability of false and true positive eDNA capture and detection. eDNA detection probability was significantly lower than that of the raft method and eDNA capture was far lower than eDNA detection using the qPCR assay. This indicates that it is likely that a very low concentration ofmink eDNA was present within the river, and therefore eDNA is an unsuitable method to detect mink. This study on American mink, a territorial, highly mobile and semi-aquatic mammal, is an example of an instance in which eDNA was outperformed by a conventional method.

Introduction

Invasive species have been a contributing factor to the decline and subsequent extinction of native species around the world (Clavero et al., 2009). Invasive species are separated from other non-natives by the harm they cause; the IUCN defining invasive species as, “Species

that are introduced, accidentally or intentionally, outside of their natural geographic range and that become problematic". They are widely regarded as a primary cause of biodiversity loss, second only to habitat destruction (Dueñas et al., 2018). The damage invasive species cause, and the cost of controlling them can be immense; the cost in Great Britain of controlling only freshwater Invasive species is estimated to total between £26.5 - £43.5 million annually (Oreska & Aldridge., 2011).

The American mink (*Neovision vision*, hereafter mink) is an invasive mammalian predator that causes numerous problems globally (Macdonald & Harrington., 2003). Within Europe, mink is one of the most publicised invasive species and ranks as the 19th worst invasive species (Martin & Lea., 2020, Nentwig et al., 2018, Zuberogoitia et al., 2010). Mink were first introduced to Europe, Russia and South America in the early twentieth century for fur farming (Melero et al., 2010). Today they are well established throughout the UK, found from most southern to northern counties (Macdonald et al., 2015, Martin & Lea., 2020).

Mink are a semi-aquatic, intelligent, mobile, fast moving carnivore that exhibit high levels of plasticity in behaviour so that they can thrive in almost any waterbody (Macdonald et al., 2015, Stefansson et al., 2016). Mink will eat almost anything they can catch, and prey species range from birds to crustaceans, small mammals and more (Zschille et al., 2013). This predation has had serious negative effects on native wildlife. In Western Bohemi, Czech Republic mink prey heavily on the endangered stone crayfish *Austropotamobius torrentium*, whereas in Sweden they have eliminated several small seabird colonies (Moore et al., 2003, Padyšáková et al., 2009). Furthermore, In the UK one of the mink's favourite prey species, the water vole *Arvicola amphibius*, has been driven to local extinctions on the rivers where mink have established (Harrington et al., 2009).

More recently, it has become apparent that mink are highly susceptible to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and wild populations could become a reservoir for COVID19 (Manes, Gollakner & Capua., 2020). Multiple instances of humans contracting COVID from farmed mink, and vice versa have been documented (Kiros et al., 2020). Mink-associated variants already exist and have been prevalent within human

populations. It was estimated 25-30 percent of all COVID cases in Northern Denmark between 10 August–29 November 2020 were mink variant strains (Larsen et al., 2021). Mink should be controlled to protect native wildlife. However, management has to be effective if being used to protect vulnerable native populations. Localised control must also be ongoing due to the threat of reinvasion (Byers et al., 2002). In UK sites, populations of reintroduced water voles have disappeared due to insufficient control measures against mink (Harrington et al., 2009). Previous attempts to eliminate invasive species have frequently been unsuccessful, often due to their complicated nature (both in management terms and biologically) as well as lack of resources (Dana et al., 2019). An attempt to eliminate mink in Spain from 2002-2006 ceased due to the lack of impact on mink population despite the capture of over 1,300 individuals (Melero et al., 2010). Nevertheless, a number of successful mink control projects have been undertaken (Bryce et al., 2011, Harrington et al., 2020, Reynolds et al., 2013, Roy., 2012). Control in the Baltic Finish islands was followed by increased breeding density of nine bird species, including ringed plover *Charadrius hiaticula*, arctic skua *Stercorarius parasiticus* and Northern wheatear *Oenanthe oenanthe* (Nordström et al., 2003).

The characteristics of mink: high mobility, speed, and wide-ranging, mean that detection of mink can be difficult and rafts are currently the most effective detection method (King et al., 2009). However, use of rafts is susceptible to species misidentification and labour intensive, making it costly (Harrington et al., 2020, Martin & Lea., 2020; Zabala-Albizua et al., 2010). The employment and advancement of new techniques could be the key to potentially reducing cost and difficulty of mink management, while increasing efficiency.

Detection of environmental DNA (hereafter eDNA) is a potential alternative means of mink detection. The method works on the principle that animals release DNA into the environment. This genetic material can then be extracted from environmental samples (water, soil or air) and analysed for species presence (Barnes & Turner., 2015). Detection of species through their eDNA provides a promising, species specific, non-invasive method of detection that, in many cases, has proven to be more sensitive to species presence than conventional detection methods (Evans & Lamberti., 2017). For example, in Japan, invasive bluegill sunfish *Lepomis macrochirus*, eDNA were detected in 11 ponds that traditional

methods failed to identify presence (Takahara et al., 2013). Furthermore, eDNA could be particularly useful for population control projects where detecting the remaining individuals requires increasingly more resources when using conventional methods - a crucial element needed to make mainland elimination possible (Martin & Lea., 2020).

A quantitative PCR assay to successfully detect mink eDNA has not yet been published if it exists, and previous attempts to use metabarcoding for mammal communities have performed weakly regarding mustelid species. For example, a metabarcoding study failed to detect both otter and weasel in eDNA samples from Assynt UK, despite confirmed presence, while mink and stoat were also not detected despite likely presence (Sales et al., 2020). Though, Padgett-Stewart et al., (2015) successfully detected river otter eDNA with a species-specific PCR assay they had developed. This demonstrates that development of a successful species-specific assay to detect mustelid is possible.

This study aims to develop and test a qPCR assay to detect environmental mink DNA. Mink presence was monitored on two rivers in North Wales using rafts that were deployed over an 18-week period. Paired eDNA sampling was conducted at these rafts' sites on three occasions. Furthermore, the use of multi-method occupancy modelling will allow for comparison of the detection probability of these different approaches using paired field data (raft and eDNA). The multi-scale occupancy model will also be used to calculate true and false positive detection probability at multiple levels of eDNA; capture of mink eDNA in water samples, and detection of mink eDNA by qPCR assay. Accounting for detection probability will allow for more accurate inferences on effectiveness of mink eDNA detection compared to using the conventional raft method.

Methods

Raft method

The raft method was developed by the Game and Wildlife Conservation Trust (GWCT) and has previously been used successfully in larger mink control projects (Reynolds et al., 2010, Reynolds et al., 2013). Rafts in this study were made of plastic encasing polystyrene, with a

clay topped polystyrene filled basket fitted in the centre, covered by a plastic housing. The clay reliably records mink footprints when mink visit rafts and allows for comparison between rafts, no matter the substrate of the river (Reynolds et al., 2010, Reynolds et al., 2004). Rafts are placed 1km apart to ensure at least one raft being present in even the smallest of mink home ranges, thus increasing the chance of detection (Harrington et al., 2009).

Field work and study area

Mink presence was monitored by one researcher at two rivers in North Wales, the Clywedog, Wrexham, and Llifon, Caernarfon. Rafts were first placed on the Llifon on the 12/08/20 and Clywedog on the 26/08/20. However, on the Clywedog two rafts had to be repositioned on the 09/09/2 due to land access issues. The Llifon and some surrounding waterbodies underwent trapping and control during 2019-2020 but did not undergo any mink control five months prior to, or during data collection for this project (August-December 2020). The monitored length of the river is predominantly surrounded by agricultural land and woodland, but also flows through a residential campus.

Fig 1. Raft placement on the Llifon

The Clywedog had never been monitored but previously underwent inconsistent mink control in 2019, over 12 months prior to this study. The site is predominantly surrounded by agricultural land and small amounts of woodland, with Wrexham Industrial Estate immediately Northwest to the site.

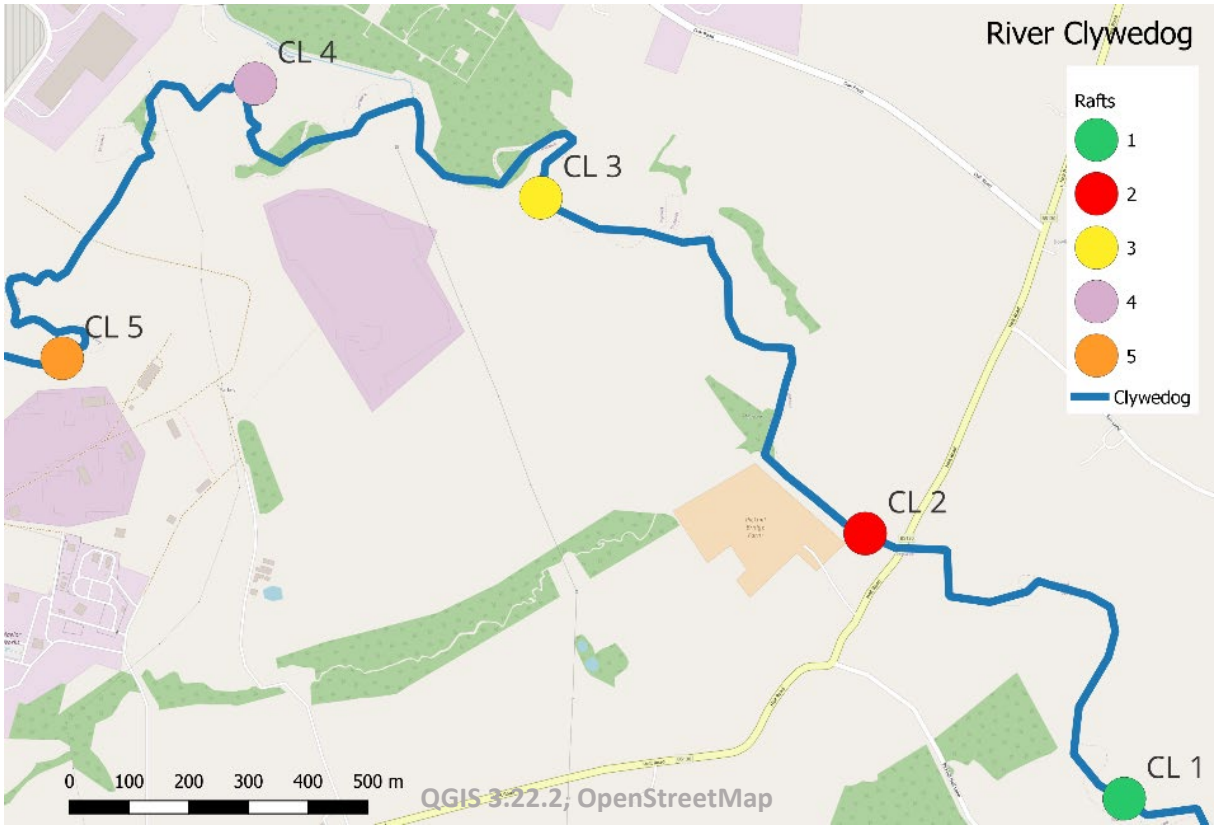

Fig 2. Raft placement on the Clywedog

Five rafts were placed on both rivers as near to 1 km apart as land access and physical accessibility constraints allowed. Rafts were visually checked for signs (footprints or scats) approximately every two weeks for nine visits, commencing two weeks after original raft placement for both sites (Appendix A, Table A-1, Table A-2). Raft checks were not undertaken in unsafe conditions (high wind, high rainfall) due to unacceptable risk to the researcher. Due to this, some visits took place a few days later or earlier than scheduled (Appendix A, Table A-3). Clay within inner baskets was smoothed over after every visit and replaced when necessary. Signs of any non-target species and abiotic factors (temperature, rainfall, wind on Beaufort scale etc.) were also recorded (Appendix A, Table A-3). Due to

adverse weather the baskets of Gllynllifon raft 2 were lost on two occasions (27/08/20 and 09/10/20), and had to be replaced, leading to a loss of data on these occasions.

Environmental DNA

eDNA filed sample collection

Water samples were taken from the Clywedog and Llifon on three occasions: visit seven (18-19/11/20), visit eight (2-3/12/20) and visit nine (14-15/12/20). Three replicates of 1L water samples (a standard measure for eDNA sampling) were taken from physically accessible locations 0-10 metres downstream of all rafts (Rees et al., 2014). Sampling collection started downstream and moved upstream to limit sediment and eDNA disturbance. The samples were collected by hand using eDNA clean 1L water collection bottles and gloves. The bottles were rinsed in stream water (both with and without the lid) before collection of the sample. The samples were then filtered onsite using a GeoTech peristaltic field pump in combination with 0.22 µm Sterivex filters. Samples were filtered until all water had been filtered, or for a maximum of 15 minutes due to time constraints. Filter time and filtered volume (ml) were recorded (Appendix A, Table A-4). Filters were then fixed with 1mL of ATL buffer and sealed with combi-lock caps on site, then stored in a fridge (3°C) as soon as possible.

To check for contamination, a blank 1L (negative control) sample of double distilled water underwent the same treatment at both sites during all survey occasions except Clywedog visit seven. Cleaned sample collection equipment was used for every sample to reduce risk of contamination. Abiotic factors were also recorded (temperature, rainfall, past weather, filtered sample volume, sample filtering time) (Appendix A, Table A-4). Equipment was cleaned after each use by soaking in 10% diluted bleach for a minimum of 4 hours.

DNA extraction

Sample DNA extractions were processed in a clean, eDNA-specific lab to reduce risk of contamination. eDNA was extracted from filters using the Qiagen DNeasy Blood & Tissue extraction kit following a modified version of the capsule Methodology developed by Spens and Evans et al., (2017) (Appendix B). Samples were extracted in batches ranging from 3-9

samples over three weeks, each batch of extractions included a laboratory negative. Samples were then stored at -20°C until qPCR testing.

qPCR assay development

Species specific primers and probes were designed using Geneious Pro R10 Software and sequences obtained from the National Centre for Biotechnology Information (NCBI Reference Sequence: NC_020641.1), for which the complete American mink mitochondrial genome was available. Primers- probe sets were designed to individually target all 13 mitochondrial proteins, D-loop, 12s and 16s regions. Primer-probe sets were designed with these specifications in mind:

- Product size of 70-200 base pairs (pb)
- Primer melting temperature between 59-65, with a maximum difference of 2 degrees between forward and reverse primers
- Avoiding hairpins and primer dimers
- GC content of 50-60%
- Avoiding repetition of bases (no more than 4)
- Probe melting temperature 6-8 degrees higher than primer melting temperature
- Probe CG content of 35-65%
- Primers and probes 20-30 bp long
- Most mismatches in primers and probes against non-target organisms
- Mismatches at 3 prime end

Probes were included in the assay as they enhance target specificity (Pabinger et al., 2014). All primers were then tested *in-silico,* initially against all UK native mustelids. Promising sets were then tested against 26 mammals likely to be present, all with complete mitochondrial sequences available in NCBI (Appendix A, Table A-5). Three primers-probe sets that best fit the criteria were chosen for in-vitro qPCR testing. Primers-probe sets Neov1&2 targeted Cytochrome c oxidase I (COX1) and Neov3 targeted NADH dehydrogenase 2 (ND2). Of the primer-probe sets chosen, only the Neov1 probe had any chance of forming primer dimers or hairpins, with a hairpin Tm of 36.4 and primer dimer Tm of 23.5.

Name	Target region		Sequence	Tm	Length	CG%	Total product size
		Primer Forward (5'-3')	TGCACGGAGGGAACATCAAA	59.9	20	50	
Neov1	COX1	Probe (5'-3')	TCCAGCTATGCTATGGGCCTTAGGGT	66.8	26	53.8	88
		Primer Reverse (5'-3')	ATACCCGTTAAGCCACCCAC	59.7	20	55	
		Primer Forward (5'-3')	CACCATATGTTCACTGTAGGCC	58.8	22	50	
Neov2	COX1	Probe (5'-3')	GCTGCACGGAGGGAACATCAAATGA	65.8	25	52	200
		Primer Reverse (5'-3')	ATACCCGTTAAGCCACCCAC	59.7	20	55	
		Primer Forward (5'-3')	TCGGAGGATGAGGAGGACTG	60.1	20	60	
Neov3	ND2	Probe (5'-3')	GGCCTACTCATCAATCGCACACATAGG	65.3	27	51.9	117
		Primer Reverse (5'-3')	GCAGTGTAAGGGTTGGGTTG	59	20	55	

Table 1. Specifications of primer-prob sets Neov1, Neov2 & Neov4

All sets were also checked against all available genomic sequences for mink in NCBI, to ensure there was no variation at target regions. The three sets matched all available sequences exactly. All probes were labelled with 56-FAM, double quenchers ZEN and 3IABkFQ (for example, 5'-/56-FAM/GCTGCACGG/ZEN/AGGGAACATCAAATGA/3IABkFQ/-3'), which have previously been shown to lower background noise and increase signal detection in qPCR experiments. All custom PrimeTime Assay Std Probes and PrimeTime Std DNA Primer sets were ordered from Integrated DNA Technologies (IDT). Primer-probe sets were resuspended with nuclease-free water to a final stock concentration of 500nM for primers and 250nM for probes.

Primer-probe sets were tested in triplicate with tissue extracted genomic DNA of mink and all British mustelids (Otter, stoat, pine martin, badger, weasel and pole cat) to test

specificity, alongside triplicate qPCR lab blanks. All genomic DNA concentrations were checked using a Qubit™ dsDNA HS Assay Kit (Thermofisher Scientific, Massachusetts, USA) and standardised to a concentration of 6 ng µl for qPCR. Negative controls for each primer were also tested in triplicate. Each 20 µl reaction contained 10 µl PrimeTime® Gene Expression Master Mix (IDT), 3 µl of extracted DNA, 6 µl nuclease-free water and 1 µl primer-probe mix. Reactions were run on QuantStudio® Flex 6 (Applied bioscience) under an absence/presence experiment with the following standard thermocycling conditions; 3 min at 95 °C followed by 40 cycles of 95°C for 15 s and 60 °C for 60 s.

Tissue extraction

Genomic mink DNA was extracted from mink tissue using Qiagen DNeasy Blood & Tissue Kits, according to the manufacturer's protocol. Mink tissue was sourced from an individual trapped locally in Anglesey by the Menter Môn control project. Before use in qPCR experiments extractions were tested using a Qubit 1X dsDNA High Sensitivity Assay kit, with included protocol. DNA was standardised to desired quantity with nuclease free water.

Standard curves

An initial qPCR standard using Neov1 was produced with a dilution series of Genomic DNA to test for preliminary efficiency. Genomic DNA was used in a seven step 10x dilution series starting at 10ng/ul, in triplicate, to generate a standard curve, alongside triplicate qPCR lab blanks. Each reaction contained 20 µL PrimeTime® Gene Expression Master Mix, 3 µl of extracted DNA, 1 µl primer-probe mix and enough nuclease-free water to bring the reaction total up to 20 µl. Reactions were run on QuantStudio® Flex 6 with QuantStudio® Real-time PCR software v1.7.1, under the following standard cycling conditions; 3 min at 95 °C followed by 40 cycles of 95°C for 15 s and 60 °C for 60 s.

A final standard curve was generated using synergised double stranded DNA of 291 base pairs long, including target amplicon (gBlocks Gene Fragments 125-500, IDT). A 10x step dilution series of 15 dilutions starting at copy number of 3.35 x 10^^{9} / µl produced a standard curve to quantify the lowest concentration amplified and environmental samples copy number. Reactions contained the same quantities as initial genomic DNA and were run on

QuantStudio® Flex 6 under the following standard cycling conditions; 3 min at 95 °C followed by 45 cycles of 95°C for 15 s and 60 °C for 60 s. Cycle number was increased to 45 to give lowest dilutions a chance to amplify (D'haene et al., 2010).

NanoDrop and cleaning

Samples from both sites were initially tested on a NanoDrop™ Spectrophotometer ND-1000 to check for approximate DNA concentration and contamination. A selection of samples aliquots of 20 µl were cleaned using a DNeasy PowerClean Pro Cleanup Kit according to the manufacturer's protocol. Cleaned aliquots were then also checked on a NanoDrop™ Spectrophotometer ND-1000 with recommended protocol.

Gel electrophoresis

Gel electrophoresis was performed with 2/% agarose gel run for 50 minutes at 60 volts. Wells were loaded with 100 bp DNA Ladder (New England biolab), samples of mink genomic DNA amplified with primer set Neov1, Neov2 or Neov3, and an environmental sample amplified with Neov1 (5 µl sample, 1 µl dye). A subsequent gel was also run under the same methodology. This gel was performed with Neov1 amplified sample qPCR samples containing a; positive control of genomic mink DNA, negative extraction control, negative qPCR plate control, negative environmental sample, and positive environmental samples (in which mink eDNA successfully amplified).

Inhibition testing

Qiagen QuantiFast master mix was used with a seven step 10x dilution series of genomic mink DNA starting at 1ng/ µl, and a selection of 22 environmental extraction samples. Environmental extraction samples were also tested in quantities of both 1 and 5 µl to test effect of varying DNA concentration in reactions. Bovine serum albumin (BSA) was added to half of these samples. Reactions contained; 10 µl Qiagen QuantiFast master mix, 1 µl primer-probe mix, 3 or 5 µl of extracted DNA, 1 or 0 µl BSA, and enough nuclease-free water to

bring reaction total up to 20 µl. Reactions were run using the same set up as the genomic DNA standard curve.

Additionally, a three step 10x dilution series of synthesised target mink DNA (gBlocks Gene Fragments 125-500, IDT) starting at a copy number of 3.35 x 10^^{9} µl, were spiked with equal parts (1.5 µl) of nuclease-free water or environmental samples, to test if environmental samples (a selection from both sites) inhibited amplification. A selection of environmental samples were also diluted with equal parts of nuclease-free water (1.5 µl of each), to see if dilution of any present inhibiting contaminates would allow amplification to occur.

Primer-probe quantity

IDT PrimeTime gene expression master mix protocol recommends use of 1 µl of primer-probe mix. But to test the effect of varying Neov1 primer-probe mix, quantities of 1, 0.8 or 0.6 µl were used. in reactions. Reactions contained 10 µl PrimeTime® Gene Expression Master Mix (IDT), 3 µl of extracted genomic DNA at 1 ng/ µl, 1, 0.8 or 0.6 µl primer-probe mix and enough nuclease-free water to bring total reaction volume up to 20 µl. Reactions were run using the same set up as the genomic DNA standard curve.

Storage time effect

In order to assess whether time between sample collection and DNA extraction may have had a negative effect on DNA, a further two replicate samples were taken on the 20/04/21 from Llifon rafts 3 & 4, with eDNA from samples also extracted on the same day. Both rafts confirmed mink presence within two weeks prior to sample collection. 20 µl reactions included the same quantities as standard curves and were run using the same set up.

Environmental samples qPCR protocol

Environmental samples were then tested in 20 µl reactions containing; 10 µL PrimeTime® Gene Expression Master Mix (IDT), 3 µl of extracted DNA, 6 µl nuclease-free water and 1 µl primer-probe mix. Reactions were then run using the same set up as the synthesised DNA

standard curve. Samples from each field visit were tested on a single plate with 3 qPCR replicates tested for every field sample. Blanks for the field (except Clywedog visit seven), DNA extraction and qPCR stages were used for each plate and each sample run in triplicate. A positive control of genomic DNA at a concentration of 1ng/µl was tested in triplicate on all plates. Any amplification after Cycle threshold (Ct) of 40 was discounted as genuine amplification or presence, and eliminated from data (Williams et al., 2020). This threshold cut-off was set by the standard curves lowest successful amplified concentration (Ct 39.79).

Contamination

Samples from the Llifon visit nine and Clywedog visit seven had to be retested due to contamination within negative controls. Two trouble shooting plates were undertaken to confirm the source of contamination before sample reruns. Upon confirmation of source further steps were employed to eliminate the possibility of future contamination. Subsequently, all racks were soaked with 10% bleach solution after every use, as well as pipettes being subject to UV and thoroughly cleaning surfaces with 10% bleach solution.

Despite elimination of contamination at the qPCR step, results from Clwyedog visit seven could not be used due to contamination in extraction controls.

Occupancy modelling

Subsequently, occupancy models were used to give more accurate inferences regarding mink use and detection of eDNA. Hierarchical occupancy models also allow for comparison of detection probabilites; both between methods or levels of eDNA methodology, without imperfect detection (false negatives) bias influencing results.

However, it should be noted that mink control using the standard raft method breaks assumptions of occupancy modelling as raft positioning under GWCT guidance is not random. This means rafts are not spatially independent of each other due to their linear arrangement, and rafts neighbouring those with confirmed presence have a higher chance of mink presence (Reynolds et al., 2010). Due to this assumption break, only inferences of use, rather than occupancy can be gained.

Multi-scale occupancy analysis: comparison of detection probability between methods

Using the likelihood-based occupancy modelling program PRESENCE v2.13.6 (https://www.mbr-wrc.usgs.gov/software/presence.html) a single species, single season, multi-scale (also known as a multi-method) model was run to compare mink occupancy (ψ) and detection probability (*p*) given when using eDNA or the raft method. Paired data from both methods can be used within this model (Appendix A, Table A-6).

The three levels of occupancy parameters within the multi-method model are:

ψ (occupancy); probability of detecting mink at a site

θj (species presence); probability that the target species will be present at the sampling location during survey occasion (conditional on the site being occupied by mink)

p (detection); probability of detecting mink with the survey method (conditional on the mink being present at sampling location and site at the time of survey)

The detection history was comprised of data from nine visits (meeting the requirement for repeat visits with temporal replication) at 10 sites/rafts (Appendix A, Table A-6). Species detection information from eDNA water sample and qPCR replicates were collapsed into 1 (detection) or 0 (non-detection) in detection histories in order to be comparable to detection history given from the raft technique. Raft detection data covered all nine visits, however eDNA only covered three. Missing detections were denoted as (-). For example, 1-00, meaning presence on the first occasion, missing data on the second, and no detection on the third and fourth. Missing observations in PRESENCE do not contribute to the model likelihood as corresponding detection probabilities are set to zero. Due to the small data set only one covariate (site locations on the river Llifon or Clywedog) was included in some of the models to limit overfitting, as site location is likely to be one of the most important variables to the data (Long et al., 2011).

Overall, 12 models were run with combinations of detection probability differing or remaining constant for survey occasion/methods/site (Llifon/Clwyedog), as well as a null model (with a constant detection/occupancy probability). Candidate models were ranked according to Akaike Information Criterion (AIC) weights with models of the highest weight

considered to be the models of best fit for the detection history given (Table 2). It should be noted the highest scoring model is not necessarily the correct model, it is just the best model to fit the data of those proposed. The highest scoring model may also not reveal all covariates that significantly affect occupancy or detection probability, especially with a small data set.

Multi-stage occupancy: detection of eDNA capture and qPCR detection

However, the models within in PRESENCE do not separate probability of capturing eDNA in water samples from probability of detecting any captured eDNA. This is important because failure to detect eDNA could either be due to failure of the detection method or a lack of eDNA within samples.

To separate these probabilities, a freely available single -species, single-season, multi-stage occupancy model specifically developed for eDNA data by Griffin et al., (2020) was used, run in the R Shiny application (https://seak.shiny apps.io/eDNA/).

Occupancy parameters within the model are as followed:

ψ (occupancy); probability site is occupied by mink

θ (capture): probability that water samples contain mink eDNA

P (detectability); probability of eDNA detection by qPCR.

The model calculates both true positives and false positives in addition to the posterior mean and 95% credible interval for all model parameters.

Species detection history was composed of eDNA data from 3 occasions, with replicate water samples from rafts not being collapsed into one result but considered as different "sites" (Appendix A, Table A-7). This allowed for separation of probabilities for each replicate water sample and demonstrated true effectiveness of the qPCR assay. Only one covariate, site location (Llifon or Clwywdog) was included, along with confirmation of presence (data from the raft method). Furthermore, occupancy probability was modelled as a function of covariates and the number of qPCR replicates (3) were included. Prior distribution and advanced setting were left as those specified by Griffin et al., (2020).

Sample volume and filter time

Logistic regression analysis was performed in R to determine if there was a relationship between sample filter time and filter volume on mink eDNA detection success (presence or absence).

Estimated cost comparison

An estimated cost comparison of paired mink detection with eDNA or rafts over three occasions was undertaken.

Estimates included:

- Cost of consumables and reagents, including unused consumables and reagents due to small sample size.
- Likely increased time for laboratory processes due to inexperience.
- Time for filtering samples; significantly increased due to high sediment connection.
- Mileage - calculated at 40p per mile as per government regulation.
- Cost of rafts - despite being preowned– the cheapest raft available online was used as base cost.
- Cost of overall time – for ease of comparison, cost of time was calculated for all activities at the flat minimum living wage for over 23s rate (£8.93). The true cost of professionals would almost certainly be higher (for lab technicians and consulting ecologists).

Estimates did not include:

- Cost of equipment preowned by the university (Including filter pump or lab equipment).

Results

qPCR assay development

Of the three primer-probe sets tested, all three amplified mink DNA and failed to detect any amplification in any non-target species DNA or controls. Primer Neov1 produced the strongest amplification curves with a mean Ct value of 21.13 and final florescent intensity of 3.55 Δ**Rn**. Primer- probe sets Neov2 & 3, while successfully amplifying mink DNA, had higher Ct values of 25.01 and 22.8 respectively, producing far lower levels of fluorescence (ranging from 0.53- 0.47Δ**Rn**) than Neov1. Levels of fluorescence were so low in two replicates of Neov2 and all three of Neov3, that they failed to reach above intensity threshold to confirm "presence". Primer-probe Neov1 was therefore chosen to be taken forward for further testing.

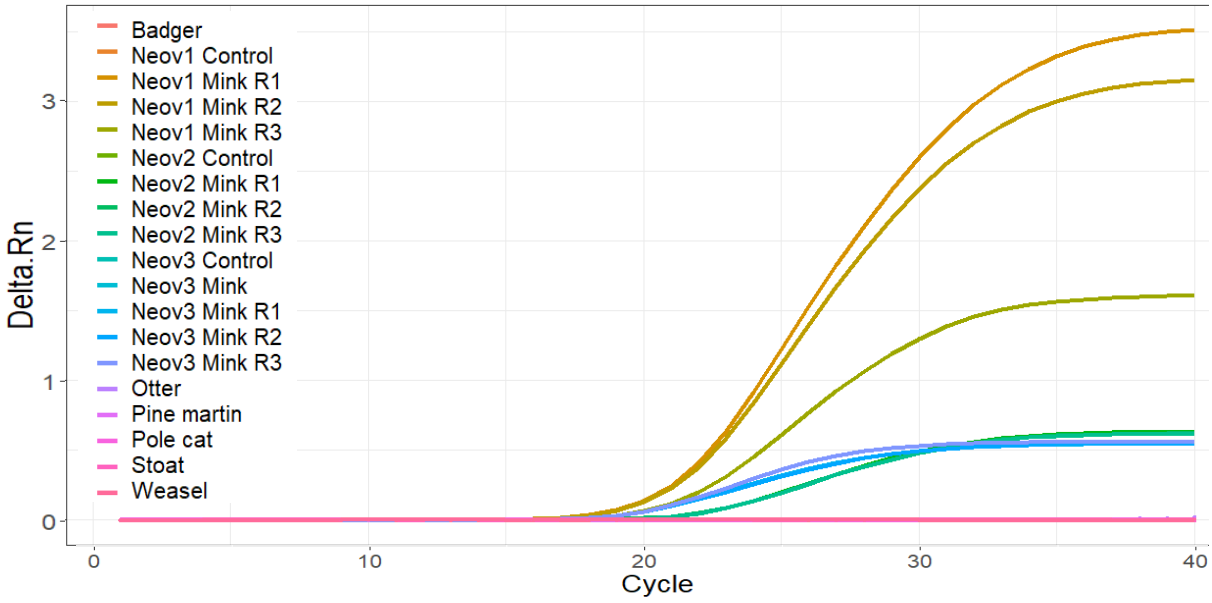

Fig 3. Specificity test of primer-prob sets Neov1, 2 & 3
Amplification curves of American mink and all native British mustelids amplified using primer-probe sets produced (R1 = replicate 1)

Standard curve

The preliminary standard curve was produced using a dilution series of Genomic DNA and gave an initial efficiency of 98.6% (R^2 = 1, slope = -3.356). However, measurement of mink DNA extracted from tissue, by NanoDrop or Qubit, does not give the precise quantity of target DNA, only total quantity of all DNA present (both genomic and mitochondrial). Synthetic target DNA of a known quantity was therefore used to produce a final standard curve to quantify eDNA in environmental samples (To 2020).

Primer Neov1 and 10-fold dilution series of synthesised target mink DNA (gBlocks) generated a standard curve with a robust amplification efficiency of 101.23%. The assay also proved to be highly sensitive, with the lowest concentration of DNA detected at a copy number of 0.003 copies per reaction.

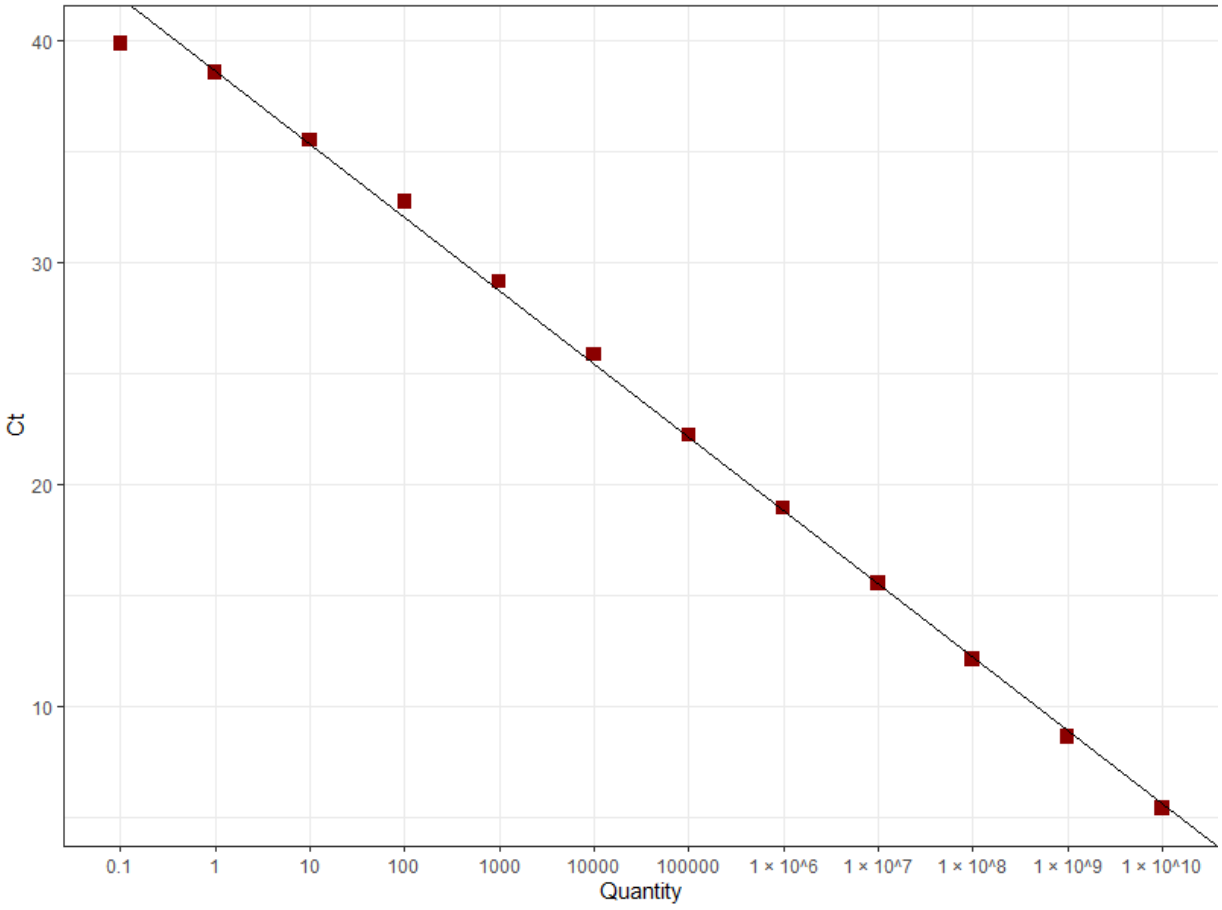

Fig 4. gBlock standard curve with Neov1

R = 0.997, Efficiency %= 101.234, Slope = -3.3, Y-intercept = 45.25.

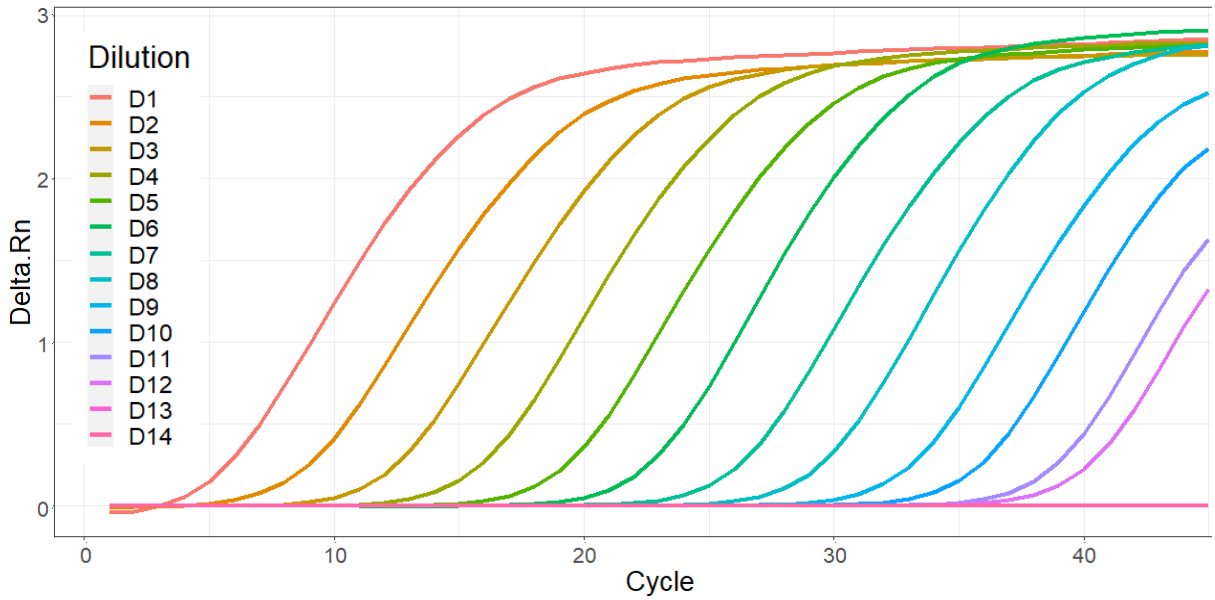

Fig 5. Amplification curves from gBlock standard curve

A 15 step x10 dilution series, starting at copy number 3.35 x 10^^{9} / µl.

Environmental samples check

NanoDrop

Tested samples contained between 306- 81 ng/µl of DNA, with an average of 161.07ng/µl. Samples produced spectrums indicating nucleic acid presence (peak at 260) and contaminating compounds (peaks at 230 and 245, Fig 6). Field and qPCR blanks contained only small amounts of DNA (13-0.60 ng/ µl) and no contaminats (Appendix A, Table A-8).

As contamination could potentially inhibit qPCR amplification (a frequent problem with environmental samples), a small selection of five sample aliquots were initially cleaned (Amberg et al., 2015). Cleaning successfully removed contamination, with only a single peak at the expected wavelength. However, it also removed most previously present DNA, producing an average of 27.84 ng/ µl (7-44ng/ µl, Fig 6). This loss of DNA could potentially include removal of any mink eDNA previously present. Cleaned samples were subsequently run in qPCR reactions but failed to see any amplification.

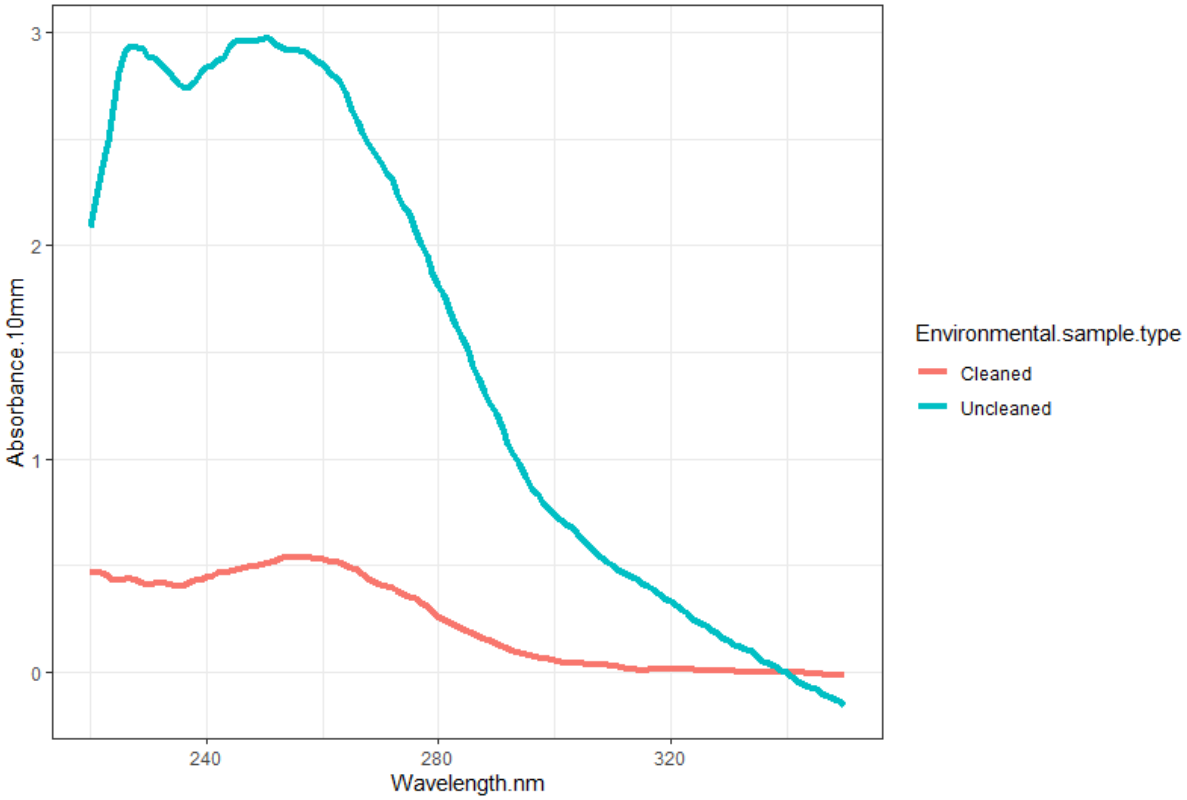

Fig 6. Spectrogram of cleaned and uncleaned environmental samples

Gel electrophoresis

Samples of genomic DNA amplified with primer-probe sets Neov 1, 2 & 3, showing a strong single band of the expected amplicon length when run through agarose gel. Negative (both extraction and qPCR) controls produced no bands (Fig 7).

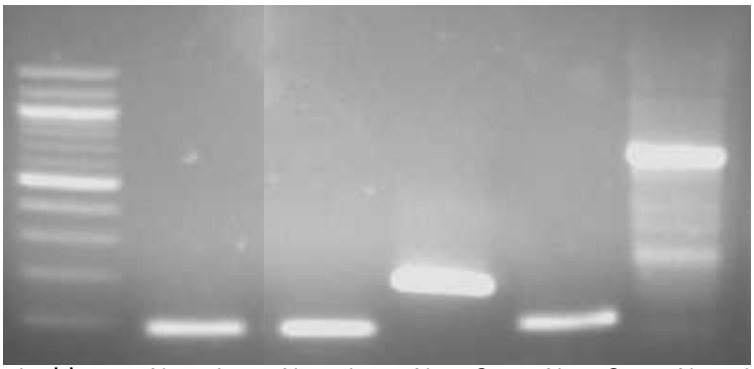

Fig 7. Gel Electrophoresis results of genomic Mink DNA and a positive environmental sample amplified with primer-probe sets

However, environmental samples amplified with Neov1 produced multiple bands at non-target amplicon length due to amplification of non-target DNA.

Inhibition testing

Bovine serum albumin (BSA) has been used widely to reduce inhibition in PCR and, Qiagen QuantiFast master mix has been found to produce the most consistent positive results across numerous qPCRs compared to other master mixes (Buzard, et al., 2012, Kreader 1996). As cleaning of the samples reduced the amount of DNA present, a selection of uncleaned samples were tested with BSA and Qiagen QuantiFast master mix to examine if inhibition in environmental samples impacted amplification. Genomic mink DNA successfully amplified for all but the last two dilutions, which is to be expected with only 3×10^{-6} and 3×10^{-7}ng/ul of genomic DNA present (so likely did not include any target DNA). However, no amplification occurred in any of the environmental samples, including those containing BSA, higher or standard quantity of DNA.

Furthermore, spiking of genomic DNA with environmental samples did not negatively affect amplification at any dilution. Amplification of genomic DNA spiked with water or extracted environmental samples differed very little with a mean difference in Ct of 0.07. Additionally, no amplification occurred in environmental samples, including those diluted with water. From the results of the experiments combined, it would appear neither inhibitors nor non-specific amplification within environmental samples are responsible for lack of amplification or detection of mink eDNA.

Primer-probe quantity

Of the three primer-probe quantities tested, reactions containing the recommended and greatest quantity of primer-probe mix (1 µl) produced the most successful amplification curves; with a Ct of 22.06. Reactions containing 0.8 or 0.6 of primer-probe mix amplified copy numbers averaging at a Ct of 22.37 and 22.45 respectively. 1 µl of primer-probe mix was therefore used when testing environmental samples. In retrospect, testing higher quantities of primer-probe mix would have been advantageous to see if effectiveness could have been further improved.

Storage time effect

No amplification occurred in any of the samples immediately extracted after collection despite positive confirmation of mink presence. It is therefore likely that the time between collection and DNA extraction was not responsible for lack of mink eDNA presence or detection in qPCR.

Presence / absence data

Naïve field data; raft and eDNA data

Over all nine survey occasions, mink signs were detected at all rafts on at least one occasion (Fig 8 & 10). On average, mink were detected using rafts on 57% of occasions on the Llifon and 70% on the Clwyedog. Mink were detected on multiple occasions for most rafts, averaging five detection occasions per raft. The only exception was Lliffon raft 5 as mink signs were detected on only a single occasion.

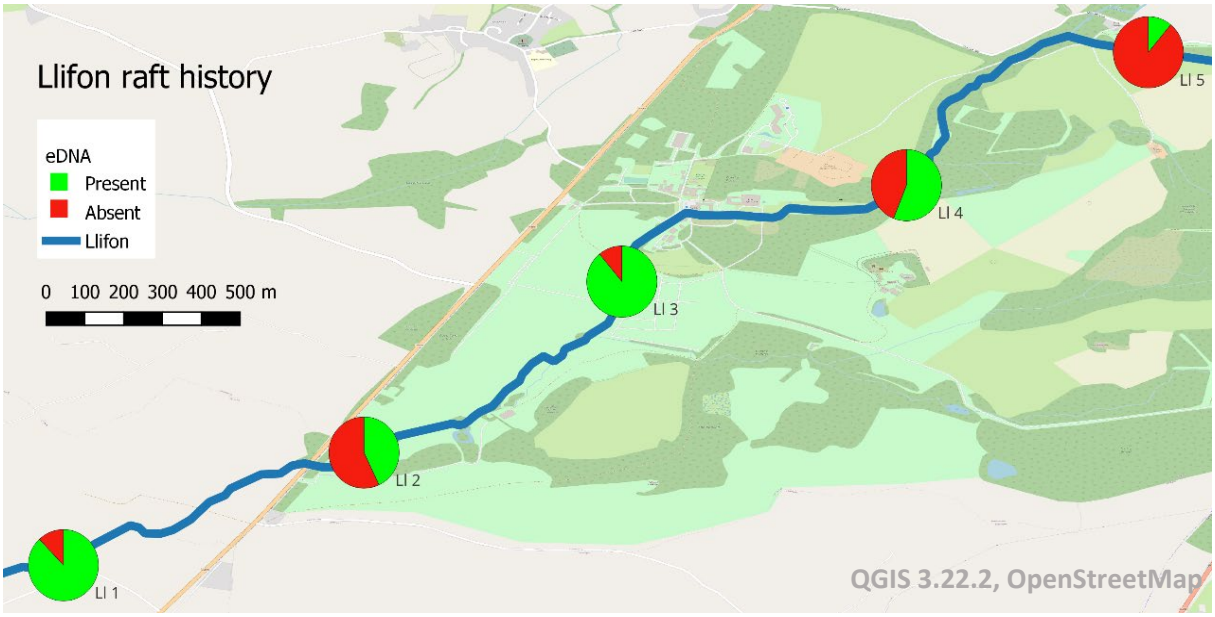

Fig 10. Naïve occupancy history of mink signs detected on the Llifon using the rafts.

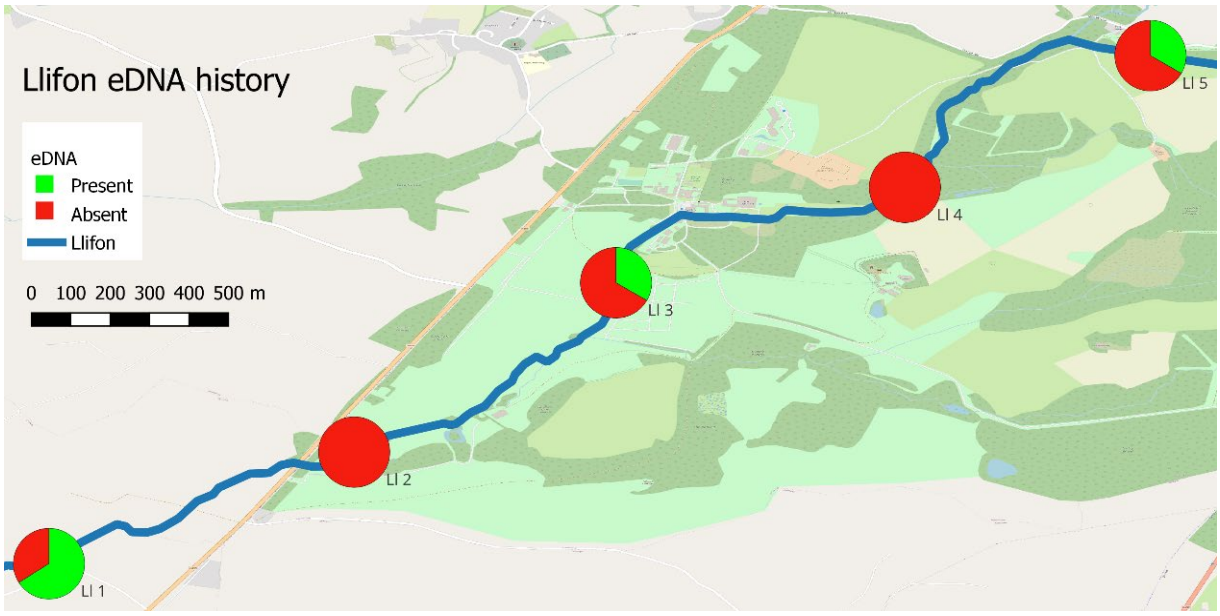

Fig 11. Naïve occupancy history of mink eDNA detected on the Llifon

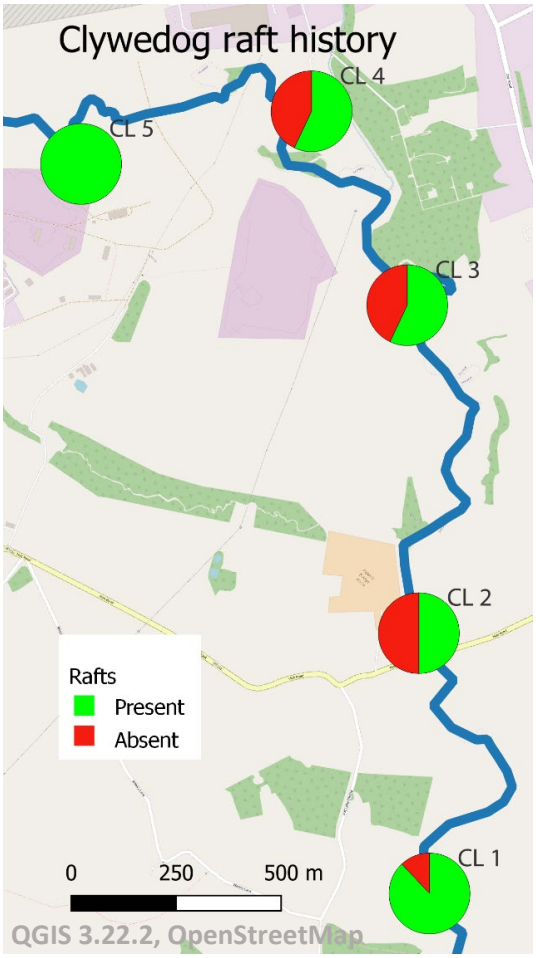

Fig 8. Naïve occupancy history of mink signs detected on the Clywedog using the rafts.

Fig 9. Naïve occupancy history of mink eDNA detected on the Clywedog.

Over the three eDNA collection occasions, mink signs were detected on 50 % of occasions, and on nine rafts. In comparison, mink eDNA was detected on 26.7% of occasions and only on six rafts (Fig 9 &11). eDNA detection was also not consistent over survey occasions, with most detections occurring from visit eight. eDNA concentration in positive environmental samples was very low with an average of 1.57 copies per reaction (0.55-4.65 copies), and mean Ct of 38.21 +/- SD 0.67. Most positive samples had multiple positive qPCR replicates (either in qPCR replicates or water sample replicates). Only results from Lliffon visit eight had single positive results. However, this is not surprising with an average sample copy number of 0.72 per reaction (0.24 per µL) in positive samples, indicating not all replicate samples would contain DNA. All positive controls successfully amplified.

Occupancy modelling

Multi-scale occupancy analysis: comparison of detection probability between methods

The two models most supported by that data had almost equal AIC weights, and together accounted for 93% of AIC weight. In both these models, methods (eDNA and raft) detection probability were modelled to be different. Both models produced higher detection probabilities for the raft method (0.65 +/-0.06), than eDNA (0.34 +/- 0.1, Fig 12).

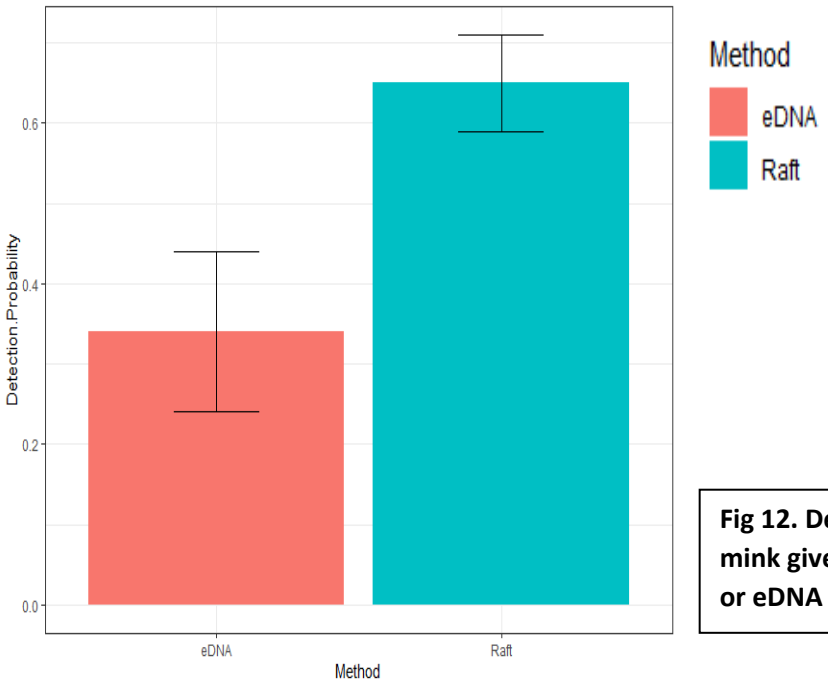

Fig 12. Detection probability of mink given use of the raft method or eDNA

The two top scoring models differed only in one aspect; whether occupancy probability was different or consistent between the Llifon and Clwydog. The top scoring model psi,theta(.),p(m), modelled occupancy probability of 1 at both sites. Whereas the second scoring model psi,theta(Llifon),p(m), modelled occupancy probability to be different between the sites; 0.82 at the Llifon, and 1 at the Clwydog. However, models' AIC weights only differ by 0.012, so there is insufficient evidence whether site location affected occupancy probability.

Survey occasion, however, did not affect detection or occupancy probability. Models in which detection probability or occupancy varied with survey occasion had low AIC weights (0.0025-0).

Model	AIC	AIC wgh	-2*LogLike'
psi,theta(.),p(m)	145.19	0.4687	137.19
psi,theta(Llifon),p(m)	145.24	0.4571	135.24
psi,theta(Llifon),p(.)	150.34	0.0357	142.34
psi,theta(.),p(.)	150.41	0.0345	144.41
psi,theta(survey),p(m)	155.69	0.0025	131.69
psi,theta(Llifon, survey),p(m)	157.13	0.0012	131.13
psi,theta(survey),p(.)	161.43	0.0001	139.43
psi,theta(.),p(survey.m)	161.68	0.0001	121.68
psi,theta(Llifon, survey),p(.)	162.32	0.0001	138.32
psi,theta(Llifon)	163.68	0	121.68
psi,theta(.)	176.84	0	136.84
psi,theta(Llifon),p(survey)	178.84	0	136.84

Table 2. multi-method AIC scores as given by program PRESENCE
(.) content probability, (Llifon) different occupancy probability between Llifon/Clywedog, (m) different detection probability between methods, (survey) different occupancy/detection probability.

Multi-stage occupancy: detection of eDNA capture and qPCR detection

Mink occupancy probability was high, at 0.93 (0.81-0.99), whereas probability of true positive eDNA capture was very low at 0.067 (θ11 = 0-0.15). However, true positive eDNA detection was still relatively high at 0.87 (P11 = 0.35- 1, Fig 13). This indicates that while probability of capturing eDNA in water samples was very low despite presence of mink, probability of amplifying captured eDNA was relatively high.

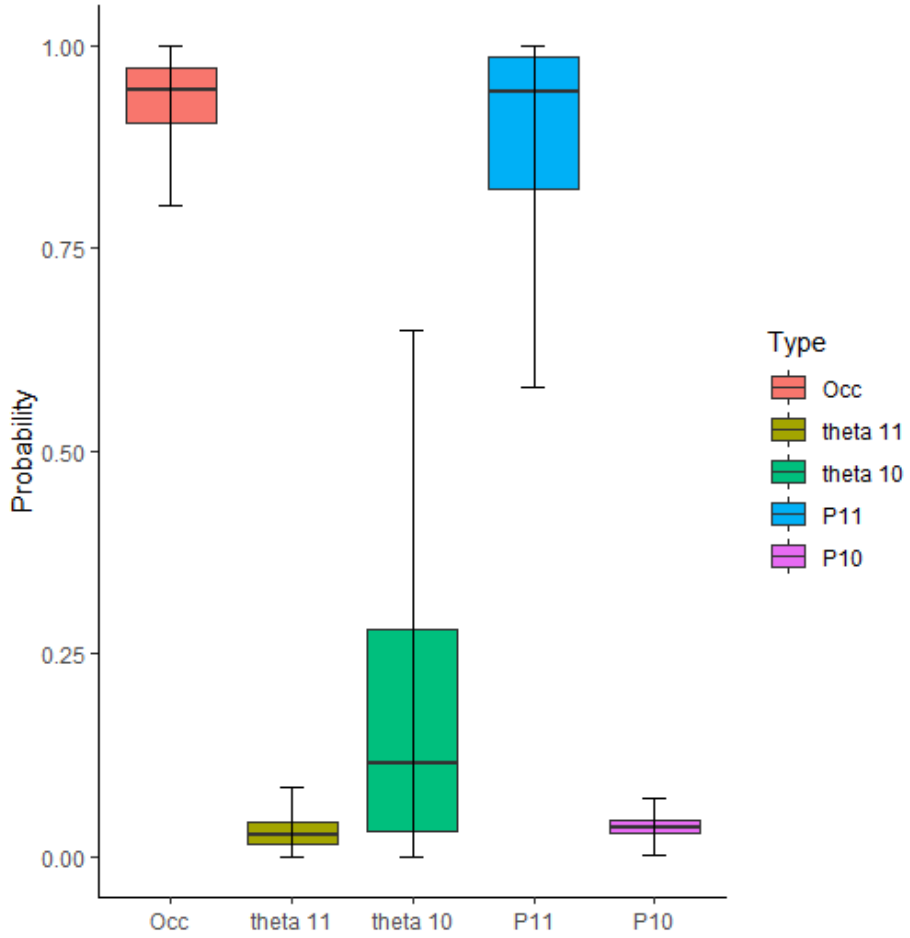

Fig 13. Multi scale occupancy and detection probability

(Occ) Occupancy, (theta11) true positive eDNA capture, (theta10) false positive eDNA capture, (P11) true positive eDNA detection, (P10) false positive eDNA detection and 95% posterior credible intervals

Moreover, the model calculated probability of false positive eDNA capture as 0.2 (θ10 = 0-0.9) and false positive detection as 0.037 (P10 = 0.01-0.06). A low false positive detection probability is consistent with previous specificity testing results; any non-target amplification that may be occurring does not appear to show up in qPCR results.

Sample volume and filter time

Logistic regression analysis did not detect a statistically significant relationship between water sample filter time or sample filter volume and eDNA detection. P-values of sample filter time and volume were calculated to be 0.622 and 0.464 respectively, well above 0.05.

Estimate cost comparison

Over the three survey occasions the eDNA method estimate cost totalled £3085, whereas the raft method estimate cost was significantly less at £945.6, which is 326% less than the cost of eDNA.

Raft method

Source of expense	**Cost (£)**
Raft checks time - 20h - 2.5h per site (4 visit- including putting rafts out)	178.6
Mileage - £ 68 for 1 visit both sites (4 visits)	272
Mink rafts - 11 rafts including cost of replacing lost raft, based on cheapest online rafts at £45	495
Total	**945.6**

Table 3. Raft methods cost estimate

Environmental DNA

Source of expense	Cost (£)
1 mL PrimeTime® Gene Expression Master Mix (x5)	224.4
Fast 96well resection plate 0.1Ml (x10) &Optical Adhesive covers (x25)	88
PrimeTime® Std qPCR Assay	81.6
Nuclease Free Water (10 x 2ml)	9.7
Eppendorf DNA LoBind tubes 1.5ml PCR clean (250 x2)	25.84
1xBD Plastipak Polypropylene Disposable Syringe Luer-Lok Concentric Tip Sterile 5mL (x125)	19.4
Pipette tips (5 boxes varying sizes - 60.48 each)	302
0.45 µm Sterivex filter units (50x2)	600
Syringe Luer-Lok Concentric Tip Sterile 309658 3mL (200)	20.56
DNeasy Blood & Tissue Kit (50 x2)/ Buffer AL (264ml)/ Buffer ATL (4 x 50 ml)/ Qiagen Proteinase K (2ml)	574
Collection samples time - 15h - (2.5h per site)	134
Filtering time - 15h - (5 hours both sites x3 occasions)	107
Extraction time - 66h - (6h per 10 samples, 11 sessions)	588
qPCR time -12h - (6 plates, 2 hours per plate)	107
Mileage: £ 68 for 1 visit both sites (x3 visits)	204
Total	**3085.5**

Table 4. eDNA detection cost estimate

Discussion

This study successfully developed a qPCR assay to detect mink DNA; one which is both specific to mink and successfully amplified mink DNA at low concentrations (0.003 copies per reaction). However, in the field mink eDNA detection occurred far less often than detection of mink signs using rafts. Occupancy modelling estimated probability of mink detection via eDNA was 0.34, far lower than that of the conventional raft method at 0.65. Further occupancy modelling estimated probability of true eDNA capture (in water samples) at a mean of only 0.067, whereas true positive eDNA detection (using qPCR assay) had a mean of 0.87. This indicates that the low probability of detecting mink using eDNA is not due to a failure to detect captured eDNA with the developed qPCR assay, but due to a lack of eDNA being captured in water samples – the rivers themselves appeared to contain little mink eDNA. This would also be consistent with the low copy number of mink eDNA present within positive environmental samples, which on average contained 1.57 copies per reaction.

False negatives

Despite eDNA having often been found to be a more reliable and sensitive method of detection compared to conventional methods, this is not always true (Beng & Corlett., 2020). In this study it would appear eDNA was not detected because it was not captured within water samples. The lack of mink eDNA, despite confirmation of mink presence, could be explained by several factors: environmental, ecological or methodology.

Ecological

Mink ecology directly influences the concentrations of eDNA within water. Mink are a semi-aquatic carnivore that can exhibit increased use of terrestrial habitats in the presence of otter (Harrington et al., 2009). While the raft method does not provide information regarding mink behaviour, signs of otter were detected in this study at both sites. Mink are also highly mobile and territorial; generally scatting outside the water to territory mark and, are solitary within sizable territories. Mean territory size of mink in the UK has varied from

2.7-6.8 km along waterbodies and densities of mink have ranged from 0.2-2.3 individuals per km in the UK (Harrington et al., 2009, Medina-Vogel et al., 2015). As the raft placement (every 1km) is purposely designed to increase the chances of detecting mink, this could potentially mean that only a single mink was present at either of the 4km survey sites. Previous studies have demonstrated that relative abundance positively correlates with eDNA concentration in a number of species, including common carp *Cyprinus carpio*, common spadefoot toads *Pelobates fuscus* and American bullfrog *Lithobates catesbeianus (*Everts et al., 2021 Pilliod et al., 2013). Thus, fewer mink present would also mean less eDNA. Mink ecology resulting in low detection probability is supported by previous metabarcoding studies which have also experienced difficulty detecting carnivores in comparison to omnivores or herbivores. For example, in one metabarcoding investigation, detection probabilities of carnivores were on average 0.27 lower than small mammals, and on average 0.25 lower than lagomorphs (Lyet., 2018).

Furthermore, mink life cycle and timing of water sample collection could also explain low probability of eDNA capture. Life cycles and reproductive timing have been found to significantly affect the likelihood of detection. This is illustrated in the detection of eDNA of the Black Warrior Waterdog *Necturus alabamensis*, and the Flattened Musk Turtle *Sternotherus depressus*; two species with contrasting seasonal activity that are more detectable during the season in which they are most active (de Souza et al., 2016). Mink mate between January to March and females raise their young from April until they disperse in August or September (Harrington et al., 2009). October to December, during which water samples for this study were collected, are the few months when mink are truly solitary and in their lowest local abundance. The lifecycle of mink also impacts on likelihood of detecting signs. Bonesi and Macdonald., (2004) detected most mink signs during mating and kit rearing season (January - June) with least during December. Considering mink ecology, it would be no surprise if relatively little mink eDNA was present within waterbodies, particularly compared to fully aquatic species found in relative abundance for whom eDNA detection has been highly successful (Jane et al., 2014, Schmelzle & Kinziger., 2015). This is supported by Thomsen et al., (2011) study, in which otter, a semi aquatic and territorial mustelid with a similar lifestyle to mink were detected at only 27% of sites, a much lower eDNA detectability

via qPCR compared to fish and shrimp species, which were detected at 100% of sites with known occurrence.

Environmental

Complex environmental processes, both chemical and physical, influence eDNA transport and decomposition (Evans & Lamberti., 2017). Conditions constantly change within a habitat and a wide range of factors have been considered to affect eDNA degradation, including pH, temperature, salinity and microbial community composition (Barnes et al., 2014). There is still a lack of understanding regarding the effects of complex interacting environmental processes on eDNA, combined with eDNA state (fragment size and target region) (Jo & Minamoto., 2021). However there have been a growing number of studies investigating eDNA and increasing understanding as to how environmental factors could cause false positives (Beng & Corlett., 2020).

Lotic systems (rivers and streams) present another level of complexity compared to lentic systems (ponds and lakes); with differences in channel morphology, flow, and discharge rate (Hinlo et al., 2017). A recent study by Curtis et al., (2020) observed that higher stream flow led to decreased concentrations of eDNA and detectability of their target species, suggesting that during high stream flow eDNA would be transported further and diluted within the river. The researchers recorded occasions where eDNA was not detected at all in high stream flow despite relative high abundance of target species at site. Water samples from the Llifon and Clwyedog in this study were collected during November and December during periods of rainfall. Although sampling itself was not undertaken while rivers were in spate due to high risk, all sample occasions occurred soon after periods of rainfall. It Is therefore likely that high stream flow may have had a negative effect on eDNA concentrations in this study.

Sediments have also been suggested as a source of false negatives – through increasing PCR inhibition or binding of DNA to sediments reducing concentration of eDNA within water (Barnes et al., 2014). Clay and soil may particularly be problematic in comparison to sand due to a higher number of binding sites. Indeed, it has been reported that samples

containing soil or clay had lower starting probability of eDNA detection and a faster reduction in detectability over time, in comparison to control samples or those containing sand (Buxton et al., 2017). Wet weather (such as that which occurred before sampling occasions in this study) washes sediment into the watercourses and increases turbulence, increasing sediment transport within the river (Bever & Harris., 2013). Although testing of environmental samples from this study would indicate PCR results were not hindered by inhibitors, it is possible eDNA binding to sediment may have reduced eDNA concentrations within the Llifon and Clywedog. This possibility is made more likely as rafts use clay to detect mink. Clay will have been washed into the rivers during heavy rainfall and mink will have carried clay into the river after visiting rafts. Sediment was evident in water samples as it blocked filters and caused a need for a maximum filtering time. Reduced quantities of filtered water due to blocked filters may also have further reduced probability of capturing eDNA (Hunter et al., 2019). However, logistic regression analysis did not detect a statistically significant relationship between water sample filter volume and eDNA detection. Equally, it should be remembered that the effect of sample volume and filter time on detection success was not specifically examined in this study and these logistic regression results will be biased. In order to maximise chance of capturing eDNA, the majority of samples had high quantities of water filtered, with 96% of samples' filter volume between 800 and maximum 1000ml.

Methodology

The moment eDNA leaves its source (the target species), it begins degrading. Shorter fragments (<100 base pairs) remain detectable for a greater period compared to longer fragments (>500bp) (Shogren et al., 2018). As such, a short target amplicon (88 base pairs) was purposefully chosen when designing the Neov1 primer-probe set. However, even short fragments will degrade eventually; the time eDNA takes to become undetectable has varied from an hour to a month (dependent on environmental conditions) (Jo et al., 2017). For example, eDNA of Idaho giant salamander *Dicamptodon aterrimus* was only detectable in water samples from controlled conditions for 8 - 11 days due to variations in ultraviolet (UV) radiation (Pilliod et al., 2013). Furthermore, a metabarcoding study reported that mammal

eDNA in ponds often disappeared within 1-2 days after initial detection, whereas amphibian eDNA could be detected for multiple days and even had increases in quantity (Harper et al., 2012). The raft method, which was used as confirmation of mink presence, only confirmed presence anytime within two weeks prior. The exact time since mink last visited sites before water sample collection cannot be known using rafts. Thus, eDNA results may include false negatives due to any shed mink eDNA having degraded before water samples were taken.

Additionally, it is possible amplification of non-specific DNA may have caused false negatives. Whilst limited production of non-specific amplification can be tolerated, large quantities of non-target DNA amplifying in samples introduces competition for primers and bases (To 2020). Competition can reduce efficiency and overall sensitivity, particularly if quantity of target DNA is very low (To 2020). This is supported by efficiency testing results of Neov1, which demonstrated that the greatest quantity of primer-probe mix (1ul) performed best by amplifying at lower Ct's than lower quantities of the primer-probe mix (0.8 & 0.6ul).

False positives

False positives are highly problematic as they can lead to wasted time and effort due to incorrect inferences. Within this study, occupancy modelling calculated eDNA detection had a low probability of false positives (0.037), which is consistent with previous specificity testing and gel electrophoresis results. Any non-target amplification that may have occurred does not appear to have caused false positives in qPCR results.

However, probability of false positives eDNA capture (within water samples) was relatively high (0.2). As mentioned previously, the effect complex environmental processes have on eDNA still lacks understanding. The distance eDNA has been detected from its source in flowing water has varied massively, from 5m to 12.3 km (Evans & Lamberti., 2017, Pilliod et al., 2014). It is therefore entirely possible that, as rafts were positioned in a linear arrangement, mink eDNA may have travelled downstream to rafts mink did not visit, causing a false positive detection in water samples.

Another likely cause of false positives is contaminations. eDNA results from Clywedog visit seven had to be excluded from analysis due to amplification in extraction blanks. It is possible other environmental samples were contaminated despite a lack of contamination in blanks. High sensitivity of qPCR assays to eDNA low copy numbers is one of the largest benefits of eDNA detection. However, this sensitivity also makes contamination one of its most serious vulnerabilities; contamination by even a single copy of target DNA within samples can cause a false positive (Darling & Mahon., 2011). Contamination can occur in the field between raft sites, or within the lab, including cross contamination from previous experiments. eDNA studies often lack negative controls in the field; Sepulveda et al., (2020) report 49% of targeted (e.g., qPCR/PCR) and 80% of metabarcoding studies did not include field collection negative controls. Yet amplification in field negative controls due to contaminations occurs equally or more frequently than lab controls. Contamination within this study did not consistently occur, different samples contaminated either during eDNA extraction or qPCR plate set up, but never both. This highlights the need for negative controls for all sampling occasions and processes as it allows more reliable detection of when cross contamination occurs. Best practices were used in this study to minimise contamination risk included: cleaning of equipment with 10% bleach solution, single use of plastics and use of a separate eDNA extraction lab. Nevertheless, elimination of contamination can be very difficult as most plates also included samples of highly concentrated target DNA as positive controls (Sepulveda et al., 2020). This difficulty means that amplification in blanks is not an uncommon problem; a review of 695 eDNA detection papers reported amplification in negative controls in nearly 9% of studies despite best use practices (Sepulveda et al., 2020). The true number of studies that experience contamination issues is likely higher due as studies that experienced these issues are less likely to be published.

Non-specific amplification can also cause false positives; the presence of which was revealed in gel electrophoresis of qPCR samples. Due to the long length of off-target products, non-specific amplifications in this instance were likely due to the amplification of non-target species DNA, not primer dimers or hairpins (Ruiz et al., 2017). This is supported by the presence of only one band of expected length in gels of amplified mink DNA. However, non-

specific amplification does not appear to have caused false positives in qPCR results in this instance. Environmental samples that tested either negative or positive for mink presence in qPCR analysis both produced multiple bands in gels. The non-specific amplification will not have resulted in fluorescence in the qPCR reaction due to inclusion of a probe (To., 2020). The inclusion of a probe means florescence will only occur if the probe is displaced from target DNA by primers extending target DNA (To., 2020).

Occupancy

Mink use of the sites at both sites was extremely high, with mink detected at every raft on the Llifon and Clwyedog. The occupancy model with the highest AIC weight modelled an occupancy probability of 1 on the Llifon and Clwyedog. There is some evidence that occupancy could have been slightly lower on the Llifon as the second highest scored occupancy model (in which occupancy probability differed between the Llifon and Clwyedog) had an almost equal weight. A difference in occupancy probability could have been due to the more recent and sustained trapping effort that took place on the Llifon five months prior to initial survey occasion, whereas the Clywedog underwent ad hoc control over 12 months prior to initial surveying. However, as there are almost equal weights for both models, with a difference of only 0.012, and therefore insufficient evidence whether site location affected occupancy probability. A high level of occupancy by mink at both sites further supports that lack of detection was due a failure of the eDNA method, not a lack of mink at the sites.

Cost effectiveness

Increasing replicate numbers (both sample and qPCR) is one way to increase probability of eDNA capture and detection (Schultz & Lance., 2015). However, to do so also increases time needed to filter, extract eDNA and set up qPCR plates - dramatically increasing effort, cost and reducing cost-efficiency. Contamination further reduces cost-efficiency as it causes the need to re-test samples, or even means results are unusable.

Detection of eDNA has often found to be more cost-efficient than detection using conventional methods, yet this is not always the case. For example, detection of the smooth newt (*Lissotriton vulgaris)* via eDNA was only more cost efficient than bottle trapping if assay development and sampling costs were low (Smart et al., 2016). The raft method is labour intensive and rafts themselves can be easily lost or damaged in bad weather (Reynolds et al., 2004). Equally, equipment and reagents needed for qPCR are not inexpensive. For this study, estimated cost of eDNA was 326 % more than the estimated cost of the raft method over three survey occasions. While it should be noted that costs for this study are just estimates, including leftover consumables due to small sample size, increased filtering time from high sediment concentration, increased time for laboratory process and 20 µl qPCR reaction volumes due to inexperience. It should also be considered that use of rafts are needed for trapping anyway, and can allow volunteers to take on the majority of the work, with successful control projects having done exactly that (Lambin, Horrill & Raynor., 2019). Furthermore, raft losses could be reduced by removing rafts before extreme weather and placing them out of the reach of members of the public. Conversely, detection of eDNA currently requires laboratory testing in almost all instances, which are not currently freely available for use and molecular experiments cannot easily be undertaken by volunteers. Though the cost of eDNA detection is decreasing as the method becomes more widely used, the low detection probability of mink eDNA would likely still make it far less cost efficient than detection using rafts (Thomsen et al., 2011). In this study three survey occasions were all that were required to detect mink at 90% of the rafts, whereas mink eDNA was only detected at 60% of the rafts during these occasions.

Conclusion

This study successfully developed a specific and sensitive qPCR assay to detect mink eDNA. However, detection probability of mink eDNA in the field was far lower than detection probability of mink signs using the conventional raft method. The low probability of eDNA capture and high probability of eDNA detection indicate that samples from the Llifon and

Clwyedog contained little mink, despite confirmation of mink presence. Retaking water samples during summer - in dry periods and when mink are most active - might increase likelihood of eDNA capture. However, during winter detection probability of mink eDNA would still likely be lower than detection probability of mink signs using rafts. Moreover, there are still large gaps in knowledge concerning the effect of environmental and ecological components on eDNA persistence, transport and detection. False positive eDNA capture is a significant concern that reduces both reliability of results and useability of eDNA data for management. The use of negative controls throughout all steps to detect contamination is highly advisable.

Alternatively, use of digital droplet PCR (ddPCR) may be a more suitable and effective method to detect the eDNA of carnivores, as it has been found to be more sensitive than qPCR. In a qPCR vs ddPCR comparison for detection of the critically endangered and nationally rare *Isogenus nubecula*, this Perlodidae was detected by ddPCR in four or five locations with known presence, whereas it was not detected at any sites with qPCR (Mauvisseau et al., 2019). ddPCR is more effective at detection as it divides each sample into thousands of droplets and measures fluorescence of each droplet. This partitioning reduces inhibition and non-specific amplification within droplets, allows for increased detectability of small changes in florescence, and increases ratio of target DNA to PCR reagents substantially compared to qPCR, thereby increasing likelihood of interaction between reagents and target DNA (McDermott et al., 2013, Yang et al., 2014). However, ddPCR is also much more expensive and less available than qPCR (Park et al., 2021). Doi et al., (2015) reported ddPCR costs of over three time as much per well compared to qPCR, excluding cost of needed ddPCR apparatus, which is also comparatively much more expensive.

eDNA could potentially still be used as an independent method of confirming mink presence, especially if cost of real time PCR decreased. Currently however, use of eDNA to detect mink would have limited use in conservation management, as detection with qPCR or ddPCR does not currently provide additional information compared to rafts and is more expensive. Alternative methods such as camera trapping provides additional information that eDNA cannot, including time of visits and behaviour. Considering problems faced in this study, combined with low-cost efficiency compared to rafts and low detection probability of mink

eDNA in winter, eDNA may be an unsuitable method to detect mink for conservation management.

Currently, the perception that eDNA is more effective than conventional surveys is common and true for many species. However, successful eDNA projects are more prone to be published, which is likely to bias perception of the field (Curtis et al., 2021, Sepulveda et al., 2020). It should be remembered that not all species release DNA into the environment equally, and detection methods are not universally suited to detect all species. Indeed, recently an increasing number of studies have been published in which conventional methods of detection outperformed eDNA detection (Beng & Corlett., 2020). In conclusion eDNA will not be suitable for the detection of all species or in all circumstances and, conventional methods will sometimes be more effective and informative. This study on American mink, a territorial, highly mobile and semi-aquatic mammal, is one such example in which eDNA was outperformed by a conventional method.

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Chapter 1

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Appendix A

Table A-1: Raft detection history

Raft	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9
LI1	1	1	1	1	1	1	0	0	1
LI2	-	1	1	-	0	0	0	0	1
LI3	1	0	1	1	1	1	1	1	1

LI4	0	1	0	0	1	1	1	0	1
LI5	0	0	0	0	1	0	0	0	0
CI1	-	1	0	1	1	1	1	1	1
CI2	-	1	1	0	0	0	0	1	1
CI3	-	0	0	1	0	1	0	1	1
CI4	-	-	0	1	1	1	0	0	1
CI5	-	-	1	1	1	1	1	1	1

Table A-2: Raft check information

Location	Date	Raft and notes
Clyw	27/08/2020	Raft 1: mink footprints and individual spotted. Individual looked young, probably Juvenile
Clyw	27/08/2020	Raft 2: raft turned by channel current due to recent heavy rainfall. Clay washed away. Clay reapplied and raft left on bank till next check
Clyw	27/08/2020	Raft 3: mink footprints, raft left on back till next check
Clyw	10/09/2020	Raft 1: mink footprints, looks like mink has tried to eat oasis/clay. Backet had to be replaces. Raft moved a few meters into better position
Clyw	10/09/2020	Raft 2: few, quite light mink footprints. Put back into water
Clyw	10/09/2020	Raft 3: put back into water
Clyw	10/09/2020	Raft 4: mink footprints
Clyw	24/09/2020	Raft 1: mink few prints, fairly small, probably young individual
Clyw	24/09/2020	Raft 2: lots of mink prints and activity
Clyw	24/09/2020	Raft 3 lots of mink prints. Fairly small- young individual
Clyw	09/10/2020	Raft 2 basket missing: basket replaced, and raft left on bank
Clyw	09/10/2020	Raft 1: mink prints and scat
Clyw	09/10/2020	Raft 3: lots of small mink prints
Clyw	22/10/2020	Raft 1: mink and otter prints on raft
Clyw	22/10/2020	Raft 2: still on bank
Clyw	22/10/2020	Raft 3: lots of mink prints
Clyw	22/10/2020	Rafts 4: a few mink prints

Location	Date	Raft and notes
Clyw	22/10/2020	Rafts 5: medium mink prints
Clyw	05/11/2020	Raft 1: at a funny angle so edge in water, had washed some clay away. Still had footprints (fairly fresh too)
Clyw	05/11/2020	Raft 2: still out of water
Clyw	05/11/2020	Raft 3: mink footprints and small scat
Clyw	05/11/2020	Raft 4: mink footprints
Clyw	19/11/2020	Raft 1: no signs but they may have been washed away
Clyw	19/11/2020	Raft 2: no prints, raft washed into river from bank
Clyw	19/11/2020	Raft 3: Prints on raft
Clyw	19/11/2020	Raft 4: Prints on raft
Clyw	19/11/2020	Raft 5: sediment on clay (water flown over top of clay at some point)
Clyw	03/12/2020	Raft 3: footprints on raft, clay very wet
Clyw	03/12/2020	Raft 2: put back in water
Clyw	14/12/2020	Raft 1: mink prints
Clyw	14/12/2020	Raft 2: mink prints
Clyw	14/12/2020	Raft 3: mink prints
Clyw	14/12/2020	Raft 4: mink prints
Llifon	26/08/20	Raft 2b: Otter footprint
Llifon	09/09/20	Raft 1: moved due to land access issues
Llifon	09/09/20	Raft 2: moved due to land access issues
Llifon	09/09/20	Raft 2b: destroyed by the public
Llifon	09/09/20	Raft 1 (3): mink footprints (lots), looks like mink has tried to eat clay/oasis
Llifon	09/09/20	Raft 2 (4): mink footprints (lots), looks like mink has tried to eat clay/oasis. Probably same mink as raft 3
Llifon	23/09/20	Raft 2 (4): muddy mink footprints on actual raft, basket gone (but replaced)
Llifon	23/09/20	Raft 3 (5): raft gone and needed to be replaced
Llifon	23/09/20	Raft 5 (7): slight mink footprints on clay

Location	Date	Raft and notes
Llifon	09/10/20	Raft 1 (3): lots of mink print. Look to be two different sizes (probably two individuals: large adult male and juvenile)
Llifon	09/10/20	Raft 3 (5): Mink prints on raft and bank
Llifon	09/10/20	Raft 4 (6): prints on raft and bank
Llifon	09/10/20	Raft 5 (7): lots of small prints on raft
Llifon	21/10/2020	Raft 1 (3): mink and otter footprints
Llifon	21/10/2020	Raft2 (4): EWR have been doing work and leaving floodlight on at night
Llifon	21/10/2020	Raft 3 (5): nothing on raft, otter footprints on bank
Llifon	21/10/2020	Raft 4 (6): lots of mink prints
Llifon	21/10/2020	Raft 5 (7): possibly 2 individuals (prints on raft)
Llifon	06/11/2020	Raft 1 (3): mink footprints on raft. Probably otter footprints on bank
Llifon	06/11/2020	Raft 2 (4): nothing but had to retie raft to make it easier to access
Llifon	06/11/2020	Raft 3 (5): one print on rafts, otter and mink prints also on bank
Llifon	06/11/2020	Raft 4 (6): prints on raft
Llifon	06/11/2020	Raft 5 (7): lots of prints on raft
Llifon	18/11/2020	Raft 1 (3) lots of prints
Llifon	18/11/2020	Raft 5 (7): lots of prints
Llifon	18/11/2020	Raft 2 (4): no prints but light from pump station may have been on
Llifon	02/12/2020	Raft 1 (3): mink prints on raft, and possibly cat
Llifon	02/12/2020	Raft 2 (4): mink prints on raft
Llifon	02/12/2020	Raft 3 (5): mink prints on raft, otter on bank
Llifon	02/12/2020	Raft 4 (6): nothing on raft, rat prints on bank
Llifon	02/12/2020	Raft 5 (7): mink prints on raft
Llifon	15/12/2020	Raft 1 (3): mink prints
Llifon	15/12/2020	Raft 2 (4): mink prints
Llifon	15/12/2020	Raft 3 (5): mink prints

Location	Date	Raft and notes
Llifon	15/12/2020	Raft 4 (6): mink prints
Llifon	15/12/2020	Raft 5 (7): mink prints

Table A-3: Site visit weather

Weather	Rain	Breeze	Temp (C°)	Cloud cover (%)	Recent weather
12/08/2020	0	1	29	10	Dry and hot
13/08/2020	0	2	26	20	Dry and hot
26/08/2020	0	4	16	80	Heavy rainfall in past 24H
27/08/2020	3	2	13	100	Heavy rainfall in past week
09/09/2020	0	3	12	50	Some rain earlier in the week but dry now
10/09/2020	0	2	11	100	Rained earlier in the day/ heavy rain in night
23/09/2020	3	0	12	100	light/medium rain.
24/09/2020	3	2	9	70	Rained heavily earlier in the day/ showers day before/ relatively dry weeks beforehand
09/10/2020	0	1	9	70	Relatively dry last two days, extremely wet 4 days before (rivers have been quite high- fieldwork proposed till Friday)
09/10/2020	1 (6)	2	10	90	One heavy downpour during fieldwork
21/10/2020	1	1	13	100	bit drizzly but not too bad the past week, rained day before
22/10/2020	3	1	10	100	Drizzling, rained 2 days before but fairly dry recently
05/11/2020	0	2	5	100	heavy rain earlier in the week
06/11/2020	0	2	6	20	heavy rain earlier in the week, mostly dry past couple of days
18/11/2020	2	4	11	100	some light showered during day. Continuous rain week before, river been in flood few days previously (fairly fast flowing/turbulent)
19/11/2020	4	3	8	80	Heavy rain earlier in morning, some downpours during day. Continuous rain week before (river fast flowing/turbulent)
02/12/2020	1	0	8	100	Rained in the morning, was drizzly the past few days but not too bad
03/12/2020	3	0	5	100	Rained heavily in morning (rivers turbulent: sediment, drizzled a bit then rained again for raft 5
14/12/2020	1	4	8	100	Rained heavily in morning and day before,
15/12/2020	0	1	6	10	Rain heavily in morning and previous coupled of days

Table A-4: eDNA samples information

Sample	Raft	Date	Filter time (m, s)	Vol (ml)	Date eDNA extracted	Extracted amount (ml)	Time in/out incubator
1	CL1	18/11/20	3.5	1000	20/21.01.21	1.2	15:30- 9:45
2	CL1	18/11/20	3.4	1000	25/26.01.21	0.8	15:15-9:45
3	CL1	18/11/20	3.07	1000	25/26.01.21	0.9	15:15-9:45
4	CL2	18/11/20	3.2	1000	25/26.01.21	1	15:15-9:45
5	CL2	18/11/20	3.1	1000	25/26.01.21	1.2	15:15-9:45
6	CL2	18/11/20	3.15	1000	25/26.01.21	1.3	15:15-9:45
7	CL3	18/11/20	2.4	1000	27/28.01.21	1.2	14:50-9:40
8	CL3	18/11/20	3.3	1000	20/21.01.21	1.3	15:30- 9:45
9	CL3	18/11/20	3.15	1000	20/21.01.21	1.2	15:30- 9:45
10	CL4	18/11/20	3.2	1000	27/28.01.21	1.1	14:50-9:40
11	CL4	18/11/20	3.1	1000	27/28.01.21	1	14:50-9:40
12	CL4	18/11/20	3.25	1000	27/28.01.21	1.4	14:50-9:40
13	CL5	18/11/20	3.5	1000	27/28.01.21	1	14:50-9:40
14	CL5	18/11/20	3	1000	27/28.01.21	1.4	14:50-9:40
15	CL5	18/11/20	N/A	1000	27/28.01.21	1.2	14:50-9:40
16	LL1	19/11/20	3	1000	27/28.01.21	1.4	14:50-9:40
17	LL1	19/11/20	4	1000	27/28.01.21	1.3	14:50-9:40
18	LL1	19/11/20	4.3	1000	02/03.02.21	1.3	14:40-9:40
19	LL2	19/11/20	3	1000	02/03.02.21	1.3	14:40-9:40
20	LL2	19/11/20	3.15	1000	02/03.02.21	1.1	14:40-9:40
21	LL2	19/11/20	3.3	1000	02/03.02.21	1.2	14:40-9:40
22	LL3	19/11/20	3.2	1000	02/03.02.21	1	14:40-9:40
23	LL3	19/11/20	3.2	1000	02/03.02.21	1.2	14:40-9:40
24	LL3	19/11/20	N/A	1000	02/03.02.21	1.3	14:40-9:40
25	LL4	19/11/20	3.3	1000	02/03.02.21	1.2	14:40-9:40
26	LL4	19/11/20	3.3	1000	02/03.02.21	1.3	14:40-9:40
27	LL4	19/11/20	3.3	1000	03/04.02.21	1.4	13:50-9:50
28	LL5	19/11/20	3.1	1000	03/04.02.21	1.4	13:50-9:50
29	LL5	19/11/20	2.3	1000	03/04.02.21	1.3	13:50-9:50
30	LL5	19/11/20	3.2	1000	03/04.02.21	1.5	13:50-9:50
31	Field blank	19/11/20	3	1000	03/04.02.21	1.1	13:50-9:50
32	CL1	02/12/20	2.5	1000	04/05.02.21	1.3	13:30-9:50
33	CL1	02/12/20	3.07	1000	04/05.02.21	0.9	13:30-9:50
34	CL1	02/12/20	2.5	1000	04/05.02.21	0.9	13:30-9:50
35	CL2	02/12/20	2.5	1000	04/05.02.21	1.2	13:30-9:50
36	CL2	02/12/20	3.4	1000	04/05.02.21	1.3	13:30-9:50
37	CL2	02/12/20	3.45	1000	04/05.02.21	1.3	13:30-9:50
38	CL3	02/12/20	3.15	1000	04/05.02.21	1.3	13:30-9:50
39	CL3	02/12/20	3.1	1000	04/05.02.21	1.2	13:30-9:50
40	CL3	02/12/20	3	1000	04/05.02.21	1.4	13:30-9:50

Sample	Raft	Date	Filter time (m, s)	Vol (ml)	Date eDNA extracted	Extracted amount (ml)	Time in/out incubator
41	CL4	02/12/20	3.2	1000	09/10.02.21	1	13:00-9:45
42	CL4	02/12/20	2.45	1000	09/10.02.21	1.4	13:00-9:45
43	CL4	02/12/20	3	1000	09/10.02.21	1.3	13:00-9:45
44	CL5	02/12/20	2.4	1000	09/10.02.21	1.2	13:00-9:45
45	CL5	02/12/20	2.45	1000	09/10.02.21	1.3	13:00-9:45
46	CL5	02/12/20	2.45	1000	09/10.02.21	1.4	13:00-9:45
47	Field blank	02/12/20	2.45	1000	09/10.02.21	0.9	13:00-9:45
63	LL5	03/12/20	14	850	11/12.02.21	1.1	13:50-9:50
48	LL5	03/12/20	13.5	800	09/10.02.21	1.4	13:00-9:45
49	LL5	03/12/20	14	800	09/10.02.21	1.4	13:00-9:45
50	LL4	03/12/20	7	1000	10/11.02.21	1.3	14:00-9:45
51	LL4	03/12/20	8.5	1000	10/11.02.21	1.4	14:00-9:45
52	LL4	03/12/20	9.5	1000	10/11.02.21	1.2	14:00-9:45
53	LL3	03/12/20	12.3	900	10/11.02.21	1.2	14:00-9:45
54	LL3	03/12/20	13.1	900	10/11.02.21	1.4	14:00-9:45
55	LL3	03/12/20	12.5	950	10/11.02.21	1.4	14:00-9:45
56	LL3	03/12/20	3.1	1000	10/11.02.21	1.3	14:00-9:45
57	LL1	03/12/20	5.3	1000	10/11.02.21	1	14:00-9:45
58	LL1	03/12/20	4.4	1000	10/11.02.21	1.3	14:00-9:45
59	LL1	03/12/20	3.15	1000	11/12.02.21	1.2	13:50-9:50
60	LL2	03/12/20	3.1	1000	11/12.02.21	1.3	13:50-9:50
61	LL2	03/12/20	4	1000	11/12.02.21	1.2	13:50-9:50
62	Field blank	03/12/20	3	1000	11/12.02.21	1.2	13:50-9:50
64	LL1	14/12/20	14.22	850	11/12.02.21	0.9	13:50-9:50
65	LL1	14/12/20	14.2	700	11/12.02.21	0.9	13:50-9:50
66	LL1	14/12/20	14.05	950	11/12.02.21	1.3	13:50-9:50
67	LL2	14/12/20	N/A	N/A	N/A- filter cracked	N/A	N/A
68	LL2	14/12/20	11.4	1000	11/12.02.21	1.3	13:50-9:50
69	LL2	14/12/20	14	950	15/16.02.21	1.3	16:30-10:45
70	LL3	14/12/20	4.25	1000	15/16.02.21	1	16:30-10:45
71	LL3	14/12/20	8.3	1000	15/16.02.21	1.4	16:30-10:45
72	LL3	14/12/20	4.2	1000	15/16.02.21	1.3	16:30-10:45
73	LL4	14/12/20	2.45	1000	15/16.02.21	1.2	16:30-10:45
74	LL4	14/12/20	3	1000	15/16.02.21	1.2	16:30-10:45
75	LL4	14/12/20	3	1000	15/16.02.21	1.2	16:30-10:45

Sample	Raft	Date	Filter time (m, s)	Vol (ml)	Date eDNA extracted	Extracted amount (ml)	Time in/out incubator
76	LL5	14/12/20	2.5	1000	15/16.02.21	1.1	16:30-10:45
77	LL5	14/12/20	2.44	1000	15/16.02.21	1.3	16:30-10:45
78	LL5	14/12/20	3.2	1000	18/19.02.20	1.3	16:00-9:45
79	Field blank	14/12/20	2	1000	18/19.02.21	1	16:00-9:45
80	CL1	15/12/20	14.3	550	18/19.02.21	1.2	16:00-9:45
81	CL1	15/12/20	14	825	18/19.02.21	1.4	16:00-9:45
82	CL1	15/12/20	14	1000	18/19.02.21	1.3	16:00-9:45
83	CL2	15/12/20	14.1	600	18/19.02.21	1.1	16:00-9:45
84	Cl2	15/12/20	14.15	875	18/19.02.21	1.4	16:00-9:45
85	CL2	15/12/20	14.5	875	18/19.02.21	1.4	16:00-9:45
86	CL3	15/12/20	11.5	1000	18/19.02.21	0.9	16:00-9:45
87	CL3	15/12/20	5.4	1000	26/27.02.21	1.3	15:30-10:05
88	CL3	15/12/20	8.3	1000	26/27.02.21	1.2	15:30-10:05
89	CL4	15/12/20	6.5	1000	26/27.02.21	1	15:30-10:05
90	CL4	15/12/20	5	1000	26/27.02.21	1.1	15:30-10:05
91	CL4	15/12/20	3.16	1000	26/27.02.21	1.4	15:30-10:05
92	CL5	15/12/20	5.44	1000	26/27.02.21	1.2	15:30-10:05
93	CL5	15/12/20	5.55	1000	26/27.02.21	1.2	15:30-10:05
94	CL5	15/12/20	5.2	1000	26/27.02.21	1.2	15:30-10:05
95	Field blank	15/12/20	3.15	1000	26/27.02.21	1	15:30-10:05
96/ Lb	Lab blank	N/A	N/A	N/A	20/21.01.21	0.8	15:30- 9:45
97/ LB	Lab blank	N/A	N/A	N/A	25/26.01.21	0.65	15:15-9:45
98/ LB	Lab blank	N/A	N/A	N/A	27/28.01.21	0.8	14:50-9:40
99/ LB	Lab blank	N/A	N/A	N/A	02/03.02.21	0.7	14:40-9:40
100/ LB	Lab blank	N/A	N/A	N/A	03/04.02.21	0.7	13:50-9:50
101/ LB	Lab blank	N/A	N/A	N/A	04/05.02.21	0.7	13:30-9:50
102/ LB	Lab blank	N/A	N/A	N/A	09/10.02.21	0.7	13:00-9:45

Sample	Raft	Date	Filter time (m, s)	Vol (ml)	Date eDNA extracted	Extracted amount (ml)	Time in/out incubator
103/ LB	Lab blank	N/A	N/A	N/A	10/11.02.21	0.8	14:00-9:45
104/ LB	Lab blank	N/A	N/A	N/A	11/12.02.21	0.9	13:50-9:50
105/ LB	Lab blank	N/A	N/A	N/A	15/16.02.21	0.8	16:30-10:45
106/ LB	Lab blank	N/A	N/A	N/A	18/19.02.21	0.8	16:00-9:45
107/ LB	Lab blank	N/A	N/A	N/A	26/27.02.21	0.8	15:30-10:05

Table A-5: NCBI sequence references

Common name	Latin name	RefSeq
American Mink	*Neovison vison*	NC_020641.17
Otter	*Lutra lutra*	NC_011358.1
Weasel	*Mustela nivalis*	NC_020639.1
Stoat	*Mustela erminea*	NC_025516.1
Polecat	*Mustela putorius*	NC_020638.1
Badger	*Meles meles*	NC_011125.1
Pine martin	*Martes martes*	NC_021749.1
Hedgehog	*Erinaceus europaeus*	NC_002080.2
Common shrew	*Sorex araneus*	NC_027963.1
Water shrew	*Neomys fodiens*	NC_025559.1
Rabbit	*Oryctolagus cuniculus*	NC_001913.1
Brown hare	*Lepus europaeus*	NC_004028.1
Red squirrel	*Sciurus vulgaris*	NC_002369.1
Grey squirrel	*Sciurus carolinensis*	NC_050012.1.
Beaver	*Castor fiber*	NC_028625.1
Bank vole	*Myodes glareolus*	NC_024538.1
Field vole	*Microtus agrestis*	NC_041250.1
Common vole	*Microtus arvalis*	NC_038176.1
Water vole	*Arvicola amphibius*	NC_049220.1.
Brown rat	*Rattus norvegicus*	NC_001665.2
House mouse	*Mus musculus*	NC_005089.1
Fox	*Vulpes vulpes*	NC_008434.1
Boar	*Sus scrofa*	NC_000845.1
Red deer	*Cervus elaphus*	NC_007704.2
Fallow deer	*Dama dama*	NC_020700.1
Roe deer	*Capreolus capreolus*	NC_020684.1
Goat	*Capra aegagrus hircus*	NC_005044.2

Common name	Latin name	RefSeq
Sheep	*Ovis aries*	NC_001941.1
Cattle	*Bos taurus*	NC_013996.1
Bechstein's bat	*Myotis bechsteinii*	NC_034227.1
Noctule bat	*Nyctalus noctula*	NC_027237.1
Brown long eared bat	*Plecotus auritus*	NC_015484.1
Human	*Homo sapiens*	NC_012920.1.

Table A-6: eDNA detection history

Location	Raft	Visit 7.1	Visit 8.1	Visit 9.1
Llifon	Ll1	0	1	1
Llifon	Ll2	0	0	0
Llifon	Ll3	0	1	0
Llifon	Ll4	0	0	0
Llifon	Ll5	0	1	0
Clywedog	Cl1	-	1	0
Clywedog	Cl2	-	0	0
Clywedog	Cl3	-	1	0
Clywedog	Cl4	-	1	0
Clywedog	Cl5	-	0	0

Table A-7: eDNA positive replicate data (Shiny detection history)

Location. Raft. Sample	Visit 7	Visit 8	Visit 9
Ll 1.1	0	3	2
Ll 1.2	0	1	1
Ll 1.3	0	1	0
Ll 2.1	0	0	0
Ll 2.2	0	0	0
Ll 2.3	0	0	0
Ll 3.1	0	1	0
Ll 3.2	0	0	0
Ll 3.3	0	1	0
Ll 4.1	0	0	0
Ll 4.2	0	0	0
Ll 4.3	0	0	0
Ll 5.1	0	1	0
Ll 5.2	0	0	0
Ll 5.3	0	3	0
Cl 1.1	-	1	0
Cl 1.2	-	1	0

Location. Raft. Sample	Visit 7	Visit 8	Visit 9
Cl 1.3	-	0	0
Cl 2.1	-	0	0
Cl 2.2	-	0	0
Cl 2.3	-	0	0
Cl 3.1	-	0	0
Cl 3.2	-	0	0
Cl 3.3	-	1	0
Cl 4.1	-	0	0
Cl 4.2	-	1	0
Cl 4.3	-	0	0
Cl 5.1	-	0	0
Cl 5.2	-	0	0
Cl 5.3	-	0	0

Table A-8: NanoDrop™ results

Sample ID	Date	ng/ul	260/280	260/230
33 cleaned	23/04/2021	12.51	1.69	-6.25
36 cleaned	23/04/2021	43.74	2.41	0.42
39 cleaned	23/04/2021	14.76	1.54	3.89
36 cleaned	23/04/2021	19.04	1.6	1.59
42 cleaned	23/04/2021	14.32	1.47	2.09
45 cleaned	23/04/2021	19.95	1.6	1.66
48 cleaned	23/04/2021	20.87	1.53	1.62
51 cleaned	23/04/2021	24.96	1.58	1.92
54 cleaned	23/04/2021	23.99	1.58	1.42
57 cleaned	23/04/2021	18.04	1.52	2.57
60 cleaned	23/04/2021	7.88	1.5	-0.86
29	15/04/2021	102.25	1.57	0.72

33	15/04/2021	181.64	1.49	0.87
36	15/04/2021	88.53	1.58	0.93
38	15/04/2021	119.46	1.66	1.1
42	15/04/2021	81.34	1.53	0.77
42	15/04/2021	99.52	1.61	0.89
45	15/04/2021	89.55	1.63	0.84
49	15/04/2021	161.11	1.58	0.87
54	15/04/2021	129.39	1.6	0.84
57	15/04/2021	149.67	1.53	0.79
60	15/04/2021	137.87	1.57	0.93
65	15/04/2021	142.54	1.57	0.99
68	15/04/2021	231.61	1.61	1.08
70	15/04/2021	158.02	1.58	0.83
74	15/04/2021	161.21	1.6	0.95
77	15/04/2021	142.18	1.56	0.77
80	15/04/2021	247.95	1.51	0.97
85	15/04/2021	220.01	1.5	0.88
87	15/04/2021	231.35	1.54	0.98
90	15/04/2021	200.64	1.6	1.03
93	15/04/2021	306.63	1.57	1.08
Extraction blank 10.02	15/04/2021	10.87	1.49	0.36
Extraction blank 11.02	15/04/2021	-0.6	0.48	-0.04
Extraction blank 26.02	15/04/2021	4.89	1.76	0.24

Extraction blank 10.02	15/04/2021	13.28	1.59	0.45
Field blank 79	15/04/2021	-3	1.41	-0.22
Field blank 62	15/04/2021	-0.3	0.26	-0.04

Appendix B

Capsule Methodology

- Original Spens and Evans et al., (2017) Appendix S1, modifications by Seymour, M. (*pers. comm.*) added 30.05.18.

Protocol

1. **1ml of ATL added in field**
2. **Before extraction:**
   Carefully wipe the outer surfaces of all the collection tubes and filter capsules with 5% bleach using clean tissue paper. Dry and wipe with 70% Ethanol using tissue paper.
3. ***4 b SX$_{CAPSULE}$***
   Keep the outlet end closed with the outlet cap. Carefully add 50 µL proteinase K (per 1ml buffer) per sample using a 100 or 200 µL pipet and sterile filter tips (step 4). Pipet the proteinase K between the outside of the filter and the capsule walls. Close with an inlet cap.
   Shake SX filter vigorously for a few seconds.
4. **Snip off**, the long-luer (inlet) cap using scissors.
5. **Incubate**, while rotating, at 56^{o}C for 24 hrs *(Not more than 24 hrs).*
6. **Handshake** SX filter capsules vigorously 5 times.
7. **Transfer:** Remove ALL the liquid from inlet end of capsule by using a Luer Lock syringe.
   Measure the volume, transfer to 5 mL LoBind tube.
   Invert 30 times.
   Spin down for 2 seconds to seed out excess debris. Go to step
8. **Add** *Add 800 µL of Buffer AL and 800 µL of* ice cold molecular grade 99% Ethanol *to tubes regardless of volume (assumes 1 ml of buffer)*. AL and ethanol can be premixed
9. **Vortex** vigorously.
10. **Label all tubes will need (including final 2 tubes)**
11. **Pipet** the mixture (max 650 uL at a time) into a DNeasy MiniSpin column in a 2 mL collection tube provided in the kit.
12. **Spin** in micro-centrifuge preferably at 4^{o}C at 6000 * g (8000 rpm for rotor max capacity 24 * 1.5-2 mL tubes) 1 min.
13. **Discard** flow through.
14. **Repeat** steps 10-12 until all sample is filtered through DNA Mini spin column.
15. Place the **DNeasy Mini Spin Column** in a new 2 ml collection tube (provided),
    add 500 µl Buffer AW1,
    and centrifuge for 1 min at 6000 * g (8,000 rpm). Discard flow-through and collection tube. (QiaGen protocol).
16. Place the **DNeasy Mini Spin Column** in a new 2 ml collection tube (provided),
    add 500 µl Buffer AW2,

and centrifuge for 3 min at 20,000 * g (14,000 rpm) to dry the DNeasy membrane.
Discard flow-through and collection tube.
Place spin column in a new collection tube, centrifuge at 1 min at 17,000 * g (13,000 rpm).

17. **Transfer** spin column to a new 1.5 or 2 mL DNA LoBind tube with caps removed.
18. add 70 µl Buffer AE (pH 8.0) to the spin column membrane,
    immediately transfer spin column with filter to room temperature.
19. **Incubate** at room temperature for 10 min.
20. **Centrifuge** for 1 min at 6,000 * g (8, 000 rpm).
21. **Re-elute DNA** from DNA LoBind tube. (Apply elute back on spin column on heating plate).
22. **Incubate** at room temperature for 10 min.
23. **Centrifuge** for 1 min 6,000 * g (8, 000 rpm).
24. **Discard** the spin column.
25. **Transfer** DNA to pre-marked DNA LoBind tube with lid intact.
26. **Aliquot** 10 µL in a separate tube for DNA measurement.
27. **Store** at -20°C