Environmental DNA metabarcoding and citizen science as a cost-effective and rapid tool for monitoring terrestrial mammalian species.

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2. Abstract

The use of environmental DNA (eDNA) has become increasingly important as a surveying technique for monitoring rare, elusive and/or invasive species. eDNA metabarcoding (which targets multiple species simultaneously) has been frequently used to monitor aquatic species but more recently, has been applied to detect semi-aquatic and terrestrial mammals. eDNA, with the help of citizen science, enables large, geographical areas to be sampled with minimal effort and is completely non-invasive, an important feature when monitoring species of conservation significance. The suitability of citizen science collated eDNA samples has not been fully tested to date. The Essex Wildlife Trust (EWT) have been working with citizen scientists to carryout eDNA sampling along two rivers (Colne and Blackwater) in Essex, in the southeast of England. In this study, we replicate the eDNA sampling from a previous study that was carried out in July 2019 by professionals. Here, eDNA samples were collected by the EWT volunteers between December 2019 and January 2020 and have been sent to Salford for eDNA metabarcoding analyses. With the focus on mammalian ecology along the Rivers Colne and Blackwater, we investigated patterns of species presence/absence, distribution, species richness and β-diversity between rivers and sampling periods. A t-test was performed to compare the mean proportion of human contamination per sample in both sampling seasons, resulting in similar levels of human contamination between professionals and trained volunteers collecting the samples, demonstrating the effectiveness of citizen science in this project. In total 21 individual mammalian species were detected in the winter sampling (20 - Colne, 19 – Blackwater) compared to 25 in the summer (23 – Colne, 12 – Blackwater). However, the overall mean species richness was higher in December versus July. This study

demonstrates that citizen science can be used as an excellent source for sample collection for eDNA analysis to evaluate species diversity for a particular area. This can then be projected to areas larger in scale and make a huge contribution to the monitoring and conservation of species. The use of citizen science in these types of projects really gets the community involved, along with educating and spreading knowledge which is key to community-based conservation.

3. Introduction

3.1. Why monitor and conserve

The earth's ecosystems and its biodiversity are facing unparalleled threats, with extinction rates increasing exponentially on a global scale (Anderson, 2018; Ceballos et al., 2017; Wood, et al., 2020). According to the World Wildlife Fund (WWF, 2020) the 2020 global Living Planet Index shows that there has been a 68% decline of all monitored vertebrate species from 1970 – 2016 and a 26% decline in mammals that were assessed by the IUCN (ICUN, 2021). The main threats to biodiversity today are habitat loss and degradation, species overexploitation, invasive species, disease, climate change and pollution (Anderson, 2018; Cardinale et al., 2012). The functioning of our ecosystems has already been affected by these threats meaning that extinction rates will increase, the resources we rely on will be no longer available and the earth will become less inhabitable (Cardinale et al., 2012). In Great Britain, 47 native or formally native terrestrial mammal species have been assessed; resulting in 1 in 4 being categorized as threatened with extinction (Mathews & Harrower, 2020). Mammals seem to have a higher extinction rate than other groups due to their size and unique ecological needs. Ceballos and Ehrlich (2002) found that worldwide mammalian habitat ranges have declined by 50% over the last 100 years meaning their populations have subsequently declined. The UK Biodiversity Action Plan (BAP) was established in 1994 in order to support the species and habitats most under threat in the UK. This enables individual action plans to be made for the conservation of species or habitat in need of critical action.

Biomonitoring techniques are used to gather information about a population's conservation status (population size and distribution) to enable the correct management

strategies to be applied. It is important to use suitable monitoring technique/s for specific biodiversity assessments or single/multi-species studies, as using inappropriate methods can skew results or create biases (Garden et al., 2007). Scientists can then use this information to help protect species from potential threats e.g., make a habitat a protected area, translocate a species to prevent extinction or prevent invasive species from becoming established in an area. Monitoring techniques will vary depending on which species is being surveyed or whether there are multiple species being survey at once. There are many different techniques that can be used to monitor mammals and should be carefully selected according to the target species and their conservation status.

Live trapping is a suitable method to monitor small mammals due to their size and elusiveness (McDevitt et al., 2014) and has also been used to monitor larger mammals e.g., badgers (Harrison, 2015). This method is effective to carry out precise species identification, which is difficult to do with other monitoring techniques, as close observation is not always possible (Krebs, 2006). Capture and Mark methods, which are generally less invasive, can be carried out using live trapping to estimate species abundance or to monitor population changes (Southwood & Henderson, 2000). Capture and mark methods can be very time consuming as considerable sampling effort must be applied to capture a good proportion of the target population and will require multiple sampling periods (Krebs, 2006). Live trapping methods are also used to capture species for genetic or diet analysis which requires the animal to be euthanised (Browett et al., 2021). Although live trapping is invasive, it is deemed a thorough way to sample small mammals. When trapping just for observation and the animal is released, mortality rates can still be high for both target and non-target species (Barros et

al., 2015; Do et al., 2013). Some species, for example shrews, often die after just a few hours in a live trap due to their high metabolic requirements (Do et al., 2013). Trapping can be made more selective by using modified traps with shrew escape exits to prevent mortality (Eccard & Klemme, 2013). Many species are nocturnal which makes them difficult to monitor using visual surveys. Leaving traps out over a 24-hour period ensures to target both diurnal and nocturnal species. However, the mortality rate of live trapping is relatively higher compared to other non-invasive methods where the mortality rate would be close to zero (Do et al., 2013). This method could therefore be unsuitable for monitoring rare or endangered species. Pitfall trapping is a similar method which can be used to monitor small mammals (Umetsu et al., 2006) but primarily invertebrates (Nunes et al., 2011).

Another traditional sampling method for monitoring mammals is camera trapping. It is a reliable, cost-effective and a standardized monitoring method which has been used in environmental monitoring since the 1920s (Kucera & Barrett, 2011). Although this method is deemed cost effective compared to other methods, the initial cost of purchasing the equipment can be expensive (Welbourne et al., 2020). This method is completely non-invasive which puts it in good stead to monitor endangered species. It can be applied to many different environments and climates which would otherwise be extremely difficult to monitor by person, e.g., tree canopies to monitor aboral species (Bowler et al., 2017; Kaizer et al., 2021), radioactive areas like the Chernobyl exclusion zone (Schlichting et al., 2020) and even subnivean habitats to monitor small mammal species (Soininen et al., 2021). When using camera traps in extreme weather climates (e.g., snow or humid tropical rainforest) the type of camera must be carefully selected to prevent breakages and loss of data. Deployment,

operational and data management issues are common in camera trapping studies. These include battery life, incorrect camera settings, synchronising time between multiple cameras, incorrectly positioned cameras and equipment failure (Newey et al, 2015). Camera trapping can also be incredibly informative of behaviours that are vital to understand for *in-situ* conservation (Caravaggio et al., 2017). The fact that the images and videos are saved and available to share means that the data can be communicated with other researchers and used for multiple scientific studies. Camera trapping has mainly been utilised for the study of mammals and can be biased when targeting multiple mammal species of different sizes as smaller species can go undetected (Burton et al., 2015). Smaller species can be targeted by attaching a box or tunnel to the camera and using bait, which enables the camera to catch close-up images of the animal for correct identification (Littlewood et al., 2021). When using camera trapping in urban environments, vandalism and theft can be a problem so choosing a discreet location with good camouflage is important but can still be a risk to lose expensive equipment and important data (Meek et al, 2019). Ultimately camera trapping has many more positive than negatives for monitoring species and when combined with other monitoring methods it is an invaluable method for research (Nuñez et al., 2019).

Visual surveys or direct counts are one of the most basic methods used for monitoring species and require minimal equipment. However, they can be time consuming and sometimes inaccurate as they can leave cryptic, elusive, and rare species undetected (Greenwood & Robinson, 2006). The surveyor needs prior knowledge to identify species correctly and often expert knowledge to identify down to species level. Transect sampling (Buckland et al, 2010), quadrat sampling (Dupuis & Goulard, 2011), animal counts (RSPB,

2021), and roadkill surveys (Ruiz-Capillas et al., 2014) are all types of visual surveys used for animal identification, presence/absence studies or population estimates. Visual surveys are also used for bat roost counts (Moosman et al., 2020), bird counts (RSPB, 2021) and marine mammal surveys (Lomac-MacNair et al., 2019) and have become a popular method to use for citizen scientists.

Field sign surveys are a useful, non-invasive method which can be used to monitor mammals. Here the observer will look for signs left behind by the animal in the form of tracks, faeces, hair, runways, disturbance in vegetation, burrows, or nests/dens (Krebs, 2006). Field sign surveys are versatile and have been used to monitor a variety of mammal species e.g., water voles (Aars et al., 2006; Oliver et al., 2008; Sales et al., 2020a), otters (Jeffress et al., 2010; Mowry et al., 2011), red fox (Cortázar-Chinarro et al., 2019; Earle, 2003), least weasel (Sidorovich et al., 2005), wolf (Marucco et al., 2008), badgers (Balestrieri et al., 2009) etc. These surveys require little equipment, but the observer will need prior knowledge around the target species to ensure correct identification of findings. Latrine surveys are commonly used to detect mammals, as scats will often be deposited around the species' habitat. In areas where scats can persist for a long-enough period, scat counts can be used for population and abundance estimations (Greenwood & Robinson, 2006). Field sign surveys can sometimes result in a species going undetected (false-negative) resulting in underestimation of a populations size or an over estimation where a sign is misidentified (false-positive) (Evans et al., 2010).

Unmanned aerial vehicles (UAVs) have become more common to use as remote sensing aerial platforms, to collect data on wildlife populations in real-time (Mangewa et al.,

2019). This monitoring technique uses high-definition video footage to identify species and estimate population size which can be used in both terrestrial (Lu & He, 2017) and marine (Žydelis et al., 2019) habitats and can cover large-scale landscapes. Flying heights should be considered to enable a species to be monitored successfully without being disturbed by the presence of a UAV. A study shows that certain species are more sensitive to the noise of UAVs therefore would require a higher-flying height (e.g., owls) to monitor them with little disturbance (Scobie & Hugenholtz, 2016). Thermal imaging is a valuable monitoring technique to use when studying nocturnal species or species that live in dense vegetation which would otherwise be nearly impossible to detect (Havens & Sharp, 2016). It has become more accessible as technologies improve and the cost is now more affordable. This method is completely non-invasive therefore is suitable to study endangered species in the field. Thermal cameras can be set up on the ground on fixed cameras or loaded on to UAVs for aerial surveys (Rahman & Rahman, 2021).

Molecular genetics is widely utilised in wildlife conservation and can unveil important information about a population's size, gene flow, genetic diversity, species hybridization, diet, or population bottle necks (Rowe et al., 2017). Collecting hair/fur or scat samples can be used for genetic non-invasive sampling (gNIS) to correctly identify a species (Ferreira et al., 2018) or to investigate the genetic diversity within a population (Chaves et al., 2006). Once this genetic information is obtained, it can be used by scientists to trace the place of origin and transport links of the invasive species (Browett et al., 2020). These methods initially used mitochondrial DNA (mtDNA) to gather genetic information about a species but more recently use microsatellites or Single Nucleotide Polymorphism (SNP) to get more in-depth results

about a populations demography, origin, and inter-specific relationships (Browett et al., 2020). This information can be used to prioritise populations or individuals of genetic importance and identify conservation hotspots where they inhabit.

3.2. Environmental DNA

Environmental DNA (eDNA) is described as the genetic material left over in the environment by living organisms which can then be collected in water, soil, sediment, or air (Bohman et al., 2014; Ficetola et al., 2008; Taberlet et al., 2012). When a species interacts with the environment genetic material gets left behind in the form of urine, faeces, saliva, skin, hair, or gametes (Teberlet et al, 2012). The DNA is then extracted, and species-specific primers are used for PCR, qPCR, or droplet digital PCR (ddPCR) to detect if a species is present or absent or its relative abundance in an environment (Bohman et al., 2014). eDNA can be used to detect a single species (Ficetola et al., 2008; Foote et al., 2012; Ma et al., 2016; Thomsen et al., 2012b; Williams et al., 2017) or multiple species which is known as eDNA metabarcoding (Harper, 2019; McDevitt et al., 2019; Sales et al., 2020a; Sales et al., 2020b; Ushio et al., 2019). eDNA metabarcoding is an advanced approach from DNA barcoding and uses next generation sequencing (NGS) to identify multiple species from a single environmental sample (Taberlet et al, 2012). The extraction and purification of DNA are carried out 'blind' without targeting a specific organism. Here a specific part of the genome is targeted to correctly identify a select group of organisms e.g., mammals, and instead of using species-specific primers, universal primers are used (Deiner et al., 2017). Once the sequences have been generated, they are matched using reference databases (GenBank) to correctly identify the species. This approach has widely been applied to aquatic systems to study both marine (Holman et al., 2019;

Thomsen et al., 2012a) and freshwater (Lawson et al., 2019; Thomsen et al., 2012b; Valentini et al., 2016) species, but only in the last few years has this become an effective tool for monitoring mammals (Harper et al., 2019; Sales et al., 2020a; Sales et al., 2020b; Ushio et al., 2017). eDNA has also been sampled from snow tracks to identify rare mammal species (Franklin et al., 2019; Kinoshita et al., 2019). Mammals encounter water frequently in their habitats as they drink, eat, defecate, or urinate close to or directly in water. Aquatic or semi-aquatic, mammals can swim or travel through water bodies which makes ponds, rivers, and lakes the perfect collection points for mammal eDNA sampling (Rodgers & Mock, 2015; Ushio et al., 2017; Williams et al., 2017). It is possible to detect not just terrestrial mammals but also arboreal and flying mammals by eDNA sampling (Sales et al., 2020b). Studies can aim to detect the presence or absence of a species or to try an indicate species abundance by analysing the concentration of DNA in a sample.

The first emergence of environmental DNA (eDNA) in biological studies dates back from 1987 where a protocol was used to extract and purify DNA from sediments (Ogram et al., 1987). Only three years later, metabarcoding was referred to by Giovannoni and colleagues (1990), investigating the genetic diversity in Sargasso Sea bacterioplankton. In the late 1990s the first metagenomic study was published identifying bacteria communities from soil (Handelsman et al., 1998). During the early 2000s there was a strong increase in eDNA studies looking into microorganisms based on cloning. A study used DNA metabarcoding to identify extinct species from permafrost showing how DNA can be preserved for long periods of time (Willerslev et al., 2003). In the late 2000s there was a breakthrough in next-generation sequencing (NGS) which meant metabarcoding was a lot faster and less costly. Following this

was the first study of microorganisms using eDNA metabarcoding for diet analysis (Valentini, 2009). eDNA analysis is now commonly used in environmental studies in both aquatic and terrestrial species. Ancient environmental DNA (aeDNA) has recently been acknowledged as a reliable tool for studying historical taxa (Epp, 2019). Successful studies have extracted DNA from frozen sediment in Siberia (Willerslev et al., 2003) and marine environments from ship ballast tanks (Shaw et al., 2019). The first aeDNA metabarcoding study was applied by Willerslev et al (2014) where they investigated the history of the Arctic circumpolar plant diversity over a 50,000-year period.

eDNA has now been established in the IUCN's guidelines for planning and monitoring corporate biodiversity performance (Stephenson & Carbone, 2021) as a cost-effective method of monitoring species (Smart et al., 2016). It has been found useful for monitoring elusive, invasive, rare, or endangered species which can easily go undetected in more traditional surveying methods (Deiner et al., 2017). The method for sample collection is quick, meaning it's less labour intensive than other methods and with the advancements in molecular genetics, NGS can be used to generate millions of reads in just one sequencing run (Ushio et al, 2017). eDNA is a completely non-invasive monitoring method that allows single or multiple species to be detected without encountering the animal (Taberlet et al, 2012). eDNA metabarcoding is more reliable than some of the traditional methods, such as camera traps and line transect sampling due to the genetic sequencing verifying species identification when compared to reference databases (Biggs et al., 2015; Harper et al., 2019; Leempoel et al., 2020; Smart et al., 2015; Ushio et al., 2017). Due to the simplicity of eDNA sample collection, the training aspect is straightforward and easy to follow which allows the method

to be used by non-experts (Biggs et al., 2015). Overall, eDNA metabarcoding has been found to outperform traditional surveying methods in terms of cost, detectability, and reliability (Fediajevaite et al., 2021).

Although eDNA metabarcoding has gained high significance for ecological monitoring, it does have challenges and limitations. eDNA can sometimes not work, meaning little or no DNA is found in the sample. This could be due to the rate in which DNA degrades and what abiotic factors where present when the samples were collected. It is found that the pH, salinity, temperature, and dissolved oxygen can all affect eDNA sampling (Saito & Doi, 2021; Stewart, 2019) as well as the distance eDNA can travel (Deiner & Altermatt, 2014). Due to eDNA metabarcoding being highly sensitive, contamination can become a problem during sample collection and laboratory processes (Sales et al., 2020a). For this reason, extreme caution needs to be taken when collecting eDNA samples as any slight contamination could invalidate the whole study. Other challenges come when extracting the DNA and when carrying out the PCR and sequencing. Some of these can be overcome by adjusting laboratory protocols and trial and error, but with this method being relatively new there isn't yet a standardized method to overcome all challenges (Beng & Corlett, 2020). It is generally found that carnivores are more difficult to detect using eDNA (Harper et al., 2019; Sales et al., 2020a) which could be due to their solidarity and large home ranges. To overcome this issue, the use of other monitoring methods could be used in conjunction with eDNA sampling e.g., camera trapping and visual surveys. Reference databases can have gaps in relation to understudied areas e.g., tropical rainforest (Sales et al., 2020b) meaning exact sequencing matches cannot be made.

3.3. Monitoring endangered and invasive species

Invasive non-native species are a great threat to biodiversity worldwide and human intervention is generally needed when this happens (Bellard et al., 2016). A species that has moved out of its area of natural occurrence is classed as an invasive species and are mainly introduced by anthropogenic transportation around the world (Lowry et al., 2013). A recent study shows how biological invasions are closely associated with threatened species and species extinction (Bellard et al., 2016). In the United Kingdom some invasive species e.g., grey squirrel is so widespread that it is now more abundant than the native the red squirrel (*Sciurus vulgaris*) which is currently in decline (Mathews et al., 2018). Invasive or alien species have often been deliberately brought to new areas to be used as pest control (Chen et al., 2021; Shine et al., 2018), or accidentally introduced because of escapees when kept as pets, in zoos, or used for farming (English & Caravaggio, 2020). The most effective way to manage biological invasions is to prevent an invasive species becoming established in an area. If an invasive species becomes established, it can be extremely difficult to eradicate and can spread to neighbouring areas. As well as using traditional methods to monitor and discover invasive species, a genetic approach is more appropriate to detect the invader earlier on (Browett et al., 2020). As described in section 3.1., gNIS is a favourable way to correctly identify a species but more recently can be done by eDNA metabarcoding (Miralles et al., 2016; Sales et al., 2020a), due to its high sensitivity it can pick up small traces of DNA from an invasive species. Native wildlife populations can become endangered and even extinct due to the impact of a non-native invasive species. Invasive species could affect the native wildlife directly by hybridization (Gómez et al., 2015), predation (Barbraud et al., 2021; Cooke et al., 2020; Lutz

et al., 2020), as toxic prey (Jolly et al., 2016) or indirectly by habitat modification (Fleri et al., 2021), habitat occupancy (Hegel et al., 2019), spreading disease (Browett et al., 2020; Nally et al., 2016) or extinguishing resources which other native species rely on (Montoya, 2015). Not all non-native species become invasive as they first must be introduced to an area, become established and then expand their population (Sakai et al., 2001).

Invasive species management plans need to be followed to prevent potential invasions and to manage current non-native species before they become invasive. Eradication campaigns are used when an invasive species is threatening local wildlife but is a lengthy and costly process (Croft et al., 2021). The most difficult part of an eradication programme is to know if the species is finally removed or not. This can be done by collecting data from visual sightings, scats, tracks, hair (Caley et al., 2015) and more recently eDNA sampling (Miralles et al., 2016).

3.4. Citizen Science

A tremendous amount of money is spent each year by the world's governments towards meeting global biodiversity conservation targets (McCarthy et al., 2012), therefore if more cost-effective monitoring techniques were used on a larger scale, it could increase the likelihood of reaching these targets. Scientists are limited by a lack of distribution and demographic data for many mammalian species due to the need for long-term, methodical monitoring that is mainly focusing on rare or endangered species (Massimino et al., 2018). To conserve wildlife, effective monitoring programmes need to be carefully thought out and followed with the priorities of both species and having their associated habitats taken into consideration (Pereira et al., 2013). Species with a higher risk of extinction will be of a higher

conservation importance, along with the areas they inhabit; but also, common species will be included as they play an important role in our ecosystems (Gaston & Fuller, 2008). With new changes to biodiversity arising, the collection of data has never been more paramount to help conserve and protect the world's fauna and flora and their habitats.

Citizen science (CS) is a useful and very much needed tool for conservation as it can drastically reduce the cost and workload of monitoring projects, surveys, and other scientific collection (Aceves-Buenoet al., 2015). With the collaboration of volunteers and scientists, huge data sets can be obtained from large spatial and temporal scales with less effort to gain a better understanding of the natural world (Bonney & Dickenson, 2012; Buxton et al., 2018). Volunteers can get involved with scientific research from data collection, to monitoring sites and sometimes can be involved with the whole research project. Members of the public have directly contributed to the collection and analysis of scientist research for many decades but has only recently been described as 'Citizen Science' (Cooper et al., 2021). CS is now widely used and accepted all over the world to gather data which contributes towards conserving wildlife. The acceleration in CS based research is shown by the increasing number of peer reviewed publications per annum as demonstrated by Huddart and colleagues (2016). Engaging the public to participate in CS projects is a great way to inform communities about the importance of conservation and for them to connect with their local wildlife, but also about bigger projects worldwide. CS based projects can involve visual surveys such as species identification (Sullivan et al, 2014), identifying species from camera trap data (Swanson et al., 2016), tracking population trends (Heres et al., 2021), invasive species removal/eradication (Carballo-Cárdenas & Tobi, 2016; Miralles et al., 2016) and eDNA sample collection (Biggs et

al, 2015). Although citizen science is now widely used in biological monitoring, using a citizen science approach for a long term eDNA project is relatively new (Biggs et al., 2015).

The question of credibility is constantly being asked when citizen volunteers are involved in scientific data collection, but with suitable training and education, reliability and accuracy can be maximised (Henderson, 2012). Citizen generated data can also result in biased findings due to variation between participants (Dickinson et al., 2010). Many volunteers already have a lot of prior knowledge in certain fields e.g., avid bird watchers, therefore they are keen to get involved and have high accuracy when identifying species. Whereas other volunteers have no previous knowledge in the field and will have a low accuracy when identifying species (Austen et al., 2016). Collaborative groups for citizen volunteers can be used to enable standardization and the exchange of knowledge and experience (Lawrence, 2009). When planning to standardise CS projects it is important to consider the nature of the project, number of volunteers, experience level and time commitments (Freitag et al., 2016). Many volunteers don't have the time to commit to extensive training programmes which in some studies is essential for data accuracy (e.g., identifying species) (Swanson et al., 2016), whereas simpler types of data collection do not require much training or prior knowledge. Just-in-time-training (JITT) is another type of short-term training which is becoming more popular in CS projects where on the day training is given (Crall et al., 2011; Katrak-Adefowora et al., 2020; Kelling et al., 2015).

For mammals, Mammal Web is a CS based platform that aims to get citizens involved with collection and identification of camera trap images to map the distribution of mammals across the UK (Mammal Web, 2021). Mammal web has been successful in getting participants

involved at the same time as giving online education. Another mammal focused, CS based platform is the Mammal Society's mammal mapper app. This enables citizens to track animals when seen in the wild giving them information on how to correctly identify them. Both platforms share their data with the National Biodiversity Network (NBN), which holds valuable data on UK species. The National Bat Monitoring Programme (NBMP) used volunteers to collect data for British bat population trends in the UK. Long term monitoring surveys include the Roost Count, Hibernation Survey, Field Survey and Waterway Survey. During the 2019 surveys, volunteers gave 17,313 hours which is equivalent to £254,943 (Barlow et al, 2015) which highlights the power of these types of projects. Nevertheless, biases have been found in the overall data as higher populated areas have a larger number of volunteers carrying out surveys and areas with lower populations are being surveyed the least. Volunteer participation may be irregular with a high volunteer turnover as well as different levels of experience (Barlow et al., 2015).

CS has now been applied to eDNA studies and was first successfully used in 2015 by Biggs et al. (2015) for the monitoring of the Great crested newt *(Triturus cristatus).* During this long-term project, volunteers applied online and were sent their own sample collection kits which included simple instructions on how to correctly collect an eDNA water sample from their selected sight. This was carried nationally covering 239 sites in the UK, where volunteers collected water samples from ponds. The study resulted in a high detection of 99.3% rate with little false negatives making the data highly reliable and demonstrating the effectiveness of volunteers collecting eDNA samples. The California environmental DNA programme (CALeDNA) was launched in 2017 by the University of California Conservation

Genomics Consortium which monitors the biodiversity of California through eDNA (Meyer et al, 2019). CALeDNA is a platform used for collaboration of the public, multi-institutional engagement for recruitment and training.

3.5. Mammal community of study site

This study focuses on two adjacent rivers in Essex, the Blackwater and Colne (Figure 1). The Blackwater runs off from the River Pant and continues through the South of Essex towards Maldon, whereas the Colne is a smaller river which passes through Colchester. There is a diverse community of mammals found in this area (Table 1) however many of them are currently in decline in the UK (water vole, hedgehog, rabbit, red squirrel, and hazel dormouse) (Mathews et al., 2018). Some species have seen an increase in population abundance/range over the recent years (beaver, otter, polecat, badger, red and roe deer). There are four invasive non-native mammal species found in the study area; American mink, grey squirrel (Sciurus carolinensis), Reeves's muntjac (Muntiacus reevesi) and brown rat (Rattus norvegicus) (highlighted in Table 1).

The European water vole *(Arvicola amphibius)* is a small, semi-aquatic, riparian, mammal whose populations are widely spread throughout Europe. Their preferred habitats are located along slow-flowing rivers, streams and marshes with steep banks and dense vegetation (Bonesi et al., 2002; Harrison & Bates, 1991). The species is classified as Least Concern (LC) by the ICUN Red List (Batsikhan et al. 2016) but is Endangered (EN) in England (Mathews et al., 2018). Populations of water vole are in decline in some European countries such as the United Kingdom, Italy, and the Netherlands (Battersby 2005). The Vincent Wildlife Trust conducted two national water vole surveys in 1989-90 (Astrakhan & Jefferies, 1993) and

1996-98 (The Vincent Wildlife Trust, 2003) which demonstrated a drastic decline in water voles across Britain. Findings from these surveys revealed that water vole populations had decreased by 88% over seven years. The people's trust for endangered species have enabled a long-term monitoring programme called the National Water Vole Surveying Project (NWVSP) where the public can complete surveys and enter their data online line to be part of the mass study (People's Trust for Endangered Species, 2021). Water vole populations in the UK are currently under threat by habitat loss, water pollution and predation, mainly from the American mink (Carter & Bright, 2003).

The American mink is a strictly a carnivorous species which prays on fish, amphibians, crustations and small mammals. It is an invasive species in the UK with their main food source consisting of water voles, birds, and other small mammals (Carter & Bright, 2003) and competes with native species such as the European mink (*Mustela lutreola*) for food and resources (Harrington et al., 2009). American mink, who also have the status of LC by the IUCN (Reid et al., 2016), are native to North America but are now widely spread across Europe. The species was first recorded in the UK in the 1920s when mink were regularly imported overseas for fur farming. Over time American mink were able to establish feral populations across the UK by escaping and deliberate releasing (Bonesi et al., 2007). The Essex Wildlife Trust Water Vole Recovery Project kicked off in 2007 to control the mink in and around Essex (EWT, 2021). Similar projects have been set up all around the country by trusts and councils.

The Eurasian Beaver *(Castor fiber)*, a large semi-aquatic rodent, is currently in a population increase as conservation efforts have helped boost its populations in the UK. The species has progressed from Near Threatened (NT) in 2002 to Least Concern (LC) in 2008

(Batbold et al., 2016) but is Endangered (EN) in England (Mathews et al., 2018). In the early 20th century, the global population on the Eurasian beaver had decreased to just eight populations with the total of 1200 individuals (Halley and Rosell, 2002). Hunting bans, re-introduction programmes and protected habitat have helped with the rapid recovery of this species. A pair of beavers were reintroduced to section of the river at Spains Hall Estate, Essex in 2019 as part of the 'Slow the Flow' Beaver and Natural Flood Management Project, Finchingfield (Essex Rives Hub, 2019). Since the reintroduction the pair have had a litter of two kits which proves how established the beavers are in their new habitat. The beavers have helped to modify the wetland habitat which could lead to a biodiversity boost and natural flood defences (BBC News, 2019).

The Eurasian otter *(Lutra lutra)* is another important species in the UK and was widespread in the 1950s but became locally extinct in Essex in the 1970s, along with many other counties, due to polluted waterways and lack of food (EWT, 2019). The otter has a status of Near Threatened (NT) globally (Roos et al., 2015), and Least Concern in England (Mathews et al., 2018). Legislation to protect this species has helped increase its populations and range in the UK. Captive breeding programs were set up in 1985 which led to reintroduction programs across Europe including the UK. The Otter Survey of England (2009-2010) revealed that populations have increased by 58.8% between 1977-2010 and their distribution is now widely spread across most of the UK (Crawford, 2011). This species continues to be monitored by the EWT and its volunteers (EWT, 2019).

Table 1: Species list and conservation status (England) of all wild mammal found in the study area, excluding bat species. Invasive non-native species are highlighted (Mathews et al., 2018).

Order	Scientific name	Species	Status
Artiodactyla	*Dama dama*	Fallow deer	LC
	Cervus elaphus	Red deer	LC
	Muntiacus reevesi	**Reeves's muntjac**	**NN**
	Capreolus capreolus	Roe deer	LC
Carnivora	***Neovison vison***	**American mink**	**NN**
	Lutra lutra	Eurasian otter	LC
	Meles meles	European badger	LC
	Mustela putorius	European polecat	LC
	Mustela nivalis	Least weasel	LC
	Vulpes vulpes	Red fox	LC
	Mustela erminea	Stoat	LC
Eulipotyphla	*Sorex araneus*	Common shrew	LC
	Sorex minutus	Eurasian pygmy shrew	LC
	Neomys fodiens	Eurasian water shrew	LC
	Erinaceus europeaus	European hedgehog	VU
	Talpa europaea	European mole	LC
Lagomorpha	*Lepus europaeus*	European brown hare	Nat
	Oryctolagus cuniculus	European rabbit	Nat
Rodentia	*Myodes glareolus*	Bank vole	LC
	Rattus norvegicus	**Brown rat**	**NN**
	Sciurus carolinensis	**Eastern grey squirrel**	**NN**
	Castor fiber	Eurasian beaver	EN
	Arvicola amphibius	European water vole	EN
	Microtus agrestis	Field vole	LC
	Micromys minutus	Harvest mouse	LC
	Muscardinus avellanarius	Hazel dormouse	VU
	Mus musculus	House mouse	Nat
	Sciurus vulgaris	Red squirrel	EN
	Apodemus sylvaticus	Wood mouse	LC
	Apodemus flavicollis	Yellow-necked mouse	LC

LC – Least concern, VU - vulnerable, NT – Near threatened, EN – Endangered, CE – Critically endangered, NN - Non-native, Nat – Naturalised (IUCN, 2021)

3.6. Essex Wildlife Trust

The Essex Wildlife Trust (EWT) have been working on increasing numbers of water voles along the river Colne. The River Colne Water Vole Translocation Project was set up (Essex Rivers Hub, 2021) in 2007 aiming to bring back the water vole colonies to this area. Here, they released around 600 water voles on the river Colne between 2010-2012. They also closely monitored the presence of American mink with the use of camera trapping, mink rafts and visual sightings. The mink rafts are small floating boxes with clay or sand as a base. This allows footprints to be captured of any mink that enter the raft (Essex Wildlife Trust, 2021)

3.7. Aims and objectives

The study had two main aims. Firstly, it aimed to investigate if citizen science sampling could be a reliable source of sampling for eDNA based investigations. This was assessed by comparing eDNA results obtained from CS sampling (winter) with those from Broadhurst et al. (2021) (summer). Consistency patterns between sampling regimes in the same area would support the reliability of CS sampling. Secondly, the study aimed to investigate the ecology of mammal communities on the two rivers in Essex (Southeast England) over two seasons (summer and winter). It was predicted that (i) less species would be detected during the winter due to a colder climate and harsher weather conditions as well as species seasonal ecological behaviours and (ii) Mammals who live in groups and utilise the water bodies more e.g., semi-aquatic species, will be expected to be detected at a higher density than solitary and terrestrial mammals.

4. Methods

4.1. Sample collection

This study is a follow on from Broadhurst et al. (2021) where sampling was carried out in the summer (July 2019) at the same sampling points using the same sampling methods. Sites were labelled slightly differently from Broadhurst et al. (2021) due to less sites being sampled on the river Blackwater during the winter (Table S1). Sampling took place in December 2019 - January 2020 along the river Blackwater and the river Clone located in Essex, Southeast of England. 15 locations were sampled along the river Colne and 12 along the river Blackwater (Figure 1). The sites Kelvedon (mink raft) and Marks Hall, Robin Brooke (B14 & B15) were not sampled due to weather conditions /sediment and the site Brooklands, Little Bardfield (B04) was not sampled due to not having landowner permission. From here on the July 2019 sampling will be referred to as the summer sampling and the December 2019 – January 2020 will be referred to as the winter sampling. A citizen scientist approach was used to carry out the winter sampling with volunteers from the Essex Wildlife Trust (EWT) doing the sample collection. The volunteers were given a sampling kit and a demonstration for eDNA sample collection by the EWT, highlighting the importance of eDNA sensitivity and contamination. Before sampling took place the collection and storage equipment were labelled accordingly with the date and sample site. Five replicates were taken at each sample point labelled A-E with A and B being sampled upstream, C at the exact GPS point and D and E further downstream. Sterile gloves were used for sample collection and were changed between each sample site. For sample collection, sterile 500ml water bottles were emptied out just before use. Samples were taken by standing at the edge of the riverbank and completely submerging

the collection bottles, only letting the glove and bottle touch the water. Field controls were not taken due to different volunteers sampling each different sampling site. The samples were then filtered using sterile single-use syringes (60ml) into Sterivex pressure filters with a 0.45 µm pore size (Merck). This was done by pushing as much of the 500ml sample as possible through the filters which was then stored in the labelled bags and kept frozen at -20 °C.

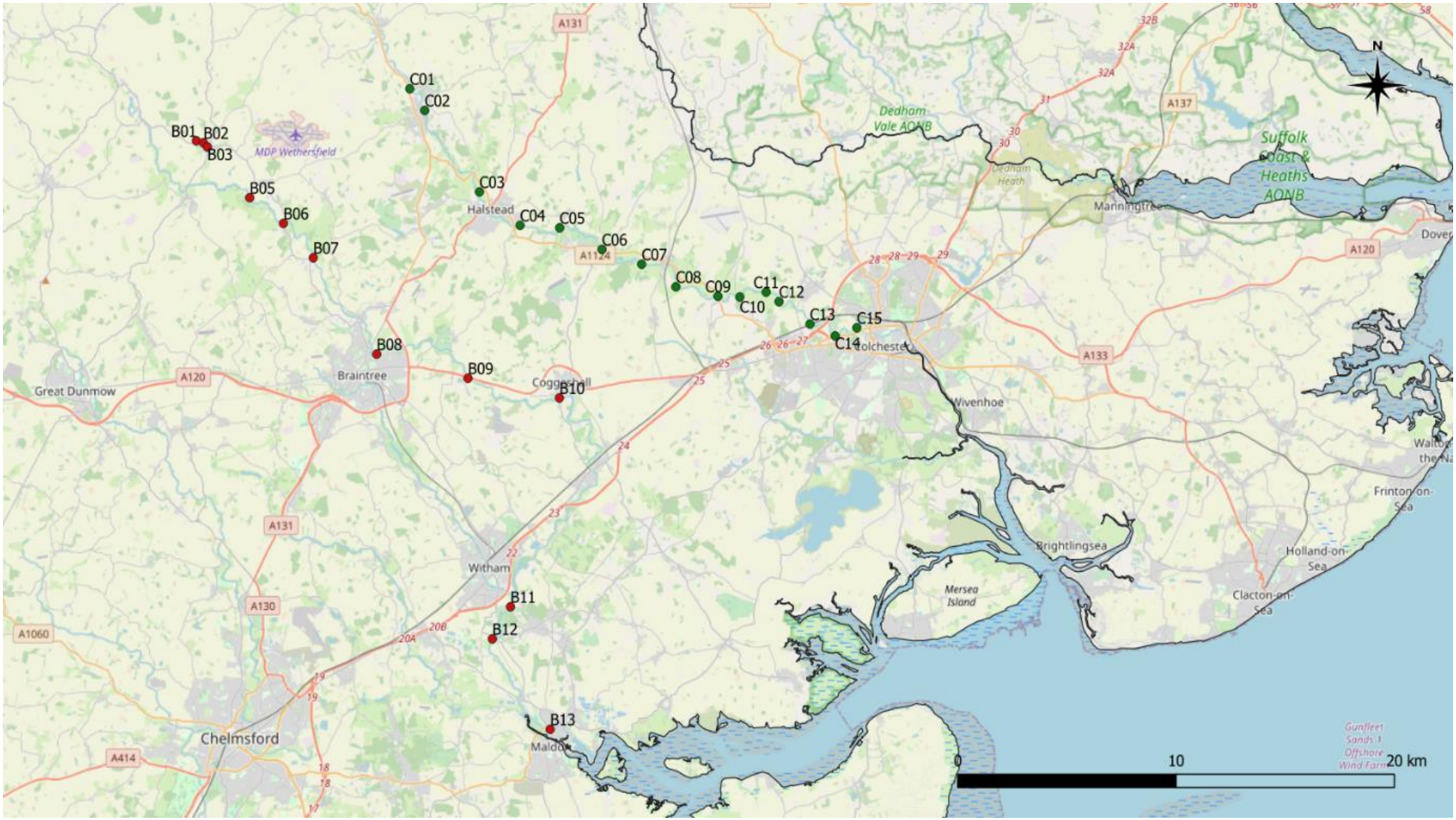


Figure 1: Map of Essex showing sampling points along the river Colne (green) and the river Blackwater (red) (QSIS).

4.2. Laboratory Methods

eDNA was extracted in a dedicated eDNA laboratory ('DNA-free room') which is set up in the University of Salford. Here the mu-DNA water protocol v.0219 (Sellers et al., 2018) was used. For each eDNA extraction samples were thawed to room temperature before removing the filter. Laboratory extraction blanks were taken each day DNA extractions took place. One modification was made in the wash stage of the protocol on step 1. Here, 600 µl of wash solution was added instead of 500 µl. A decontamination process was used to clean surfaces and equipment between each sample site and replicate. 10% bleach and 80% ethanol were used to clean down surfaces and DNA-away used on equipment. A UV Stratalinker® was also used to clean small equipment before and after use to prevent cross-contamination between sample sites. A UV light was put on at night to clean the whole room. After eDNA extraction 100 µl of eluted DNA was stored at -20°C. All extractions are shown on table (S2).

DNA samples were tested on a Qubit fluorometer (Thermo Fisher Scientific) for quantification. Polymerase chain reaction (PCR) was then carried out to amplify all DNA samples. The PCR plates were prepared in a specialised cabinet within the eDNA laboratory. All samples were organised into two libraries where controls were spread out evenly across both libraries. We included 2 positive controls in each library to monitor contamination by using a non-target species. A concentration of 5 ng/µL of DNA was used, extracted from faecal samples of Northern muriqui (*Brachyteles hypoxanthus)*, a South American primate. Negative controls were also used containing distilled water (ddH20) for the 8 PCR blanks and 8 extraction blanks (extraction buffer) were used from each extraction day. Samples from each river and the beaver enclosure were also spread out evenly between both libraries to prevent

sequencing bias. We used a universal primer set, MiMammal (MiMammal-U-F, 5'-GGGTTGGTAAATTTCGTGCCAGC-3' and MiMammal-U-R, 5'-CATAGTGGGGTATCTAATCCCAGTTTG-3'; Ushio et al., 2017) to target a ~171bp amplicon of the variable mitochondrial 12S rRNA gene. Sample-specific multiplex identifier tags (MIDs) were used with the primers to carry out the multiplex (Macrogen).

The PCRs were carried out in a total reaction volume of 20 µl, containing 2 µl of DNA, 1 µl of each primer (MiMammal-U-F and MiMammal-U-R), 0.16 µl of BSA, 5.84 µl of distilled H_2O and 10 µl of Amplitaq. The PCRmax Thermal Cycler was used for amplifications with a specific program designed for this study. The PCR conditions consisted of an initial, denaturing phase of 95°C which lasted 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 45 seconds and 72°C for 30 seconds then a final extension of 72°C for five minutes, with a cover temperature set to 110°C. If the PCR ran over night, it would be set to a continuous cycle of 10°C for cooling. PCRs were done in triplicates and then pooled at a later stage.

Gel electrophoresis was used to check the fragment size of the amplified DNA. Gels were prepared using 1.2% agarose gel and buffer TBE, diluted 1 in 10 to make 100 ml. The gel was heated until the mix cleared, and the agarose was completely diluted. Once cooled 5µl of red gel dilute buffer was added, mixed then poured into a gel rack and left to set. After 30 minutes the gel was ready to use. 2 µl of each sample was pipetted into separate wells followed by 2 µl of hyperladder 50 bp. We used DNA Qubit® dsDNA HS Assay Kit to quantify

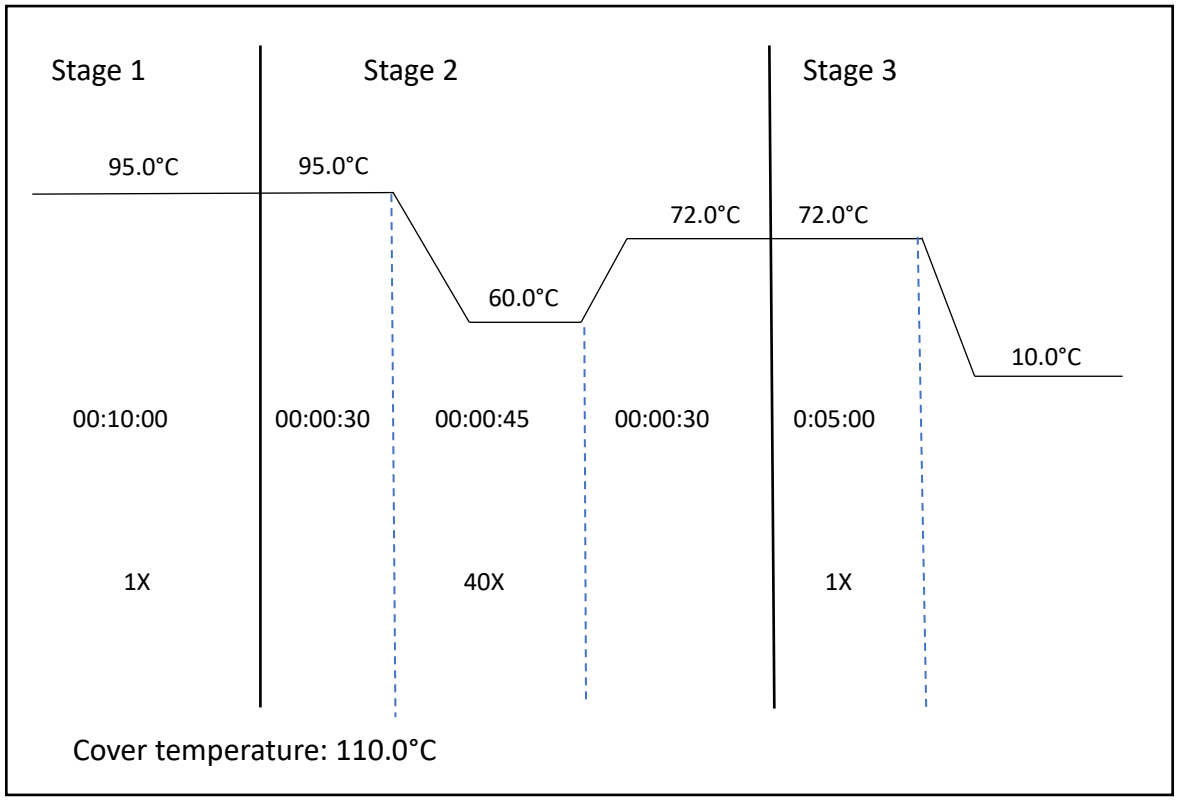


Figure 2: Breakdown of each stage of thermocycler program used for PCR in this study.

the DNA and calculate the DNA concentrations. The DNA was cleaned using a left side size selection of 1.1x Agencourt AMPure XP (Beckman Coulter). This is done to remove all irrelevant DNA and non-target fragments. Dule-Index adaptors (Illumina) were added to each library using KAPA Hyper Prep kit (Roche) including a fragmentation step, end repair and A-tailing, adapter ligation and a bead clean-up. Before pooling the libraries in equimolar concentrations quantification was carried out using NEBNext Library Quant Kit (New England Biolabs). Both libraries were sequenced on one MiSeq sequencing run using an v2 300 reagent kit. The final output resulted in two csv files.

4.2. Bioinformatics

Bioinformatics was carried out using the *OBITools* metabarcoding package (Boyer et al., 2016), following the same pipeline as Sales et al., (2020a). The files containing the raw data were downloaded from BaseSpace (basespace.illumina.com) in csv format. Fast QC (Andrews, 2014) was used to check the quality of sequences before proceeding. Forward and reverse sequences were aligned with illumina paired-end and then sorted into 'good' or 'bad' sequences based on alignment score (>40) with '*obiannotate'.* Good sequences were then assigned their sample ID using '*ngsfilter*'. Both libraries were then filtered to a sequence length between 166-176bp using '*obigrep*'. '*Obiuniq'* clustered identical sequences and chimeras were removed using '*vsearch'* (Rognes et al., 2016). Lastly taxonomic assignment was preformed using '*ecotag'* using the same reference database as Sales et al. (2020a).

A further filtering procedure was then carried out to remove any sequencing errors or false positive MOTU reads. A tag-jumping proportion was calculated for each library by dividing the total amount of positive control (PC) reads in the eDNA samples and negative controls (extraction blank & PCR blank) by total number of PC reads in PC samples (Table 2). This proportion was the taken off each sample to account for possible tag-switching during PCR. Next, the highest number of reads recorded from a MOTU found in any of the negative (extraction blanks & PCR blanks) controls was subtracted from all samples. Non-target MOTUs, human reads and domestic species were also removed. Domestic species removed included Pig *(Sus)*, cow *(Bos)*, Horse *(Equus)*, Sheep *(Ovis),* Dog (Canis) and Cat *(Felis).* MOTUs with >5 reads were removed and MOTUs only with a best identity <0.98% were included.

4.3. Analysis

Statistical analysis was preformed using R version 3.6.3 (R Core Team, 2020) and Microsoft Excel (Microsoft Corporation, 2018). Venn diagrams were made using Microsoft word displaying relationships between species detected (Figures 4 & 5). Clustered column charts were created on Excel showing number of sites in which each species was detected on both the river Colne and the River blackwater comparing the winter and summer sampling. For this all sites were used from both sampling seasons including the beaver pond samples (Figure 6). QGIS version 3.6.1-Noosa (QGIS Development Team, 2021) was used to create a map of Essex with site locations from the winter sampling on the rivers Colne and Blackwater (Figure 1). For all other analysis B06-B13 and C1-C15 form the winter sampling. B05-B13 and C01-C15 from the summer sampling were used as the beaver pond samples were excluded and B05 from the winter analysis was excluded as the samples didn't detect any species. The iNEXT (Hsieh, Ma & Chao, 2020) and ggplot2 packages (Wickham & Chang, 2016) were used to create both species accumulation curves (Figure 7) to determine if the sampling effort was sufficient to detect all mammalian species along each river and then combined. The Vegan package (Oksanen, 2019) was used to create five non-metric multidimensional scaling plots (NMDS) using a Jaccard index to calculate similarity between samples (Figure 8). Both rivers and sampling seasons are compared to explore similarities and differences between the variables. To calculate the proportion of human reads in each sample, the total amount of human reads was divided by the total amount of reads in each sample. This was done after removing the positive control tag switching proportion, positive control samples and all blanks (extraction blanks and PCR blanks). R (R Core Team, 2020) base functions were used

to calculate the mean, median and standard deviation using R's basic functions and then a two-sample t-test to compare the proportion of human reads in December and July. Finally, box jitter plots were created to show the difference between species richness on each river and sampling season using the *tidyverse* package in R (Wickham et al., 2019) (Figure 9).

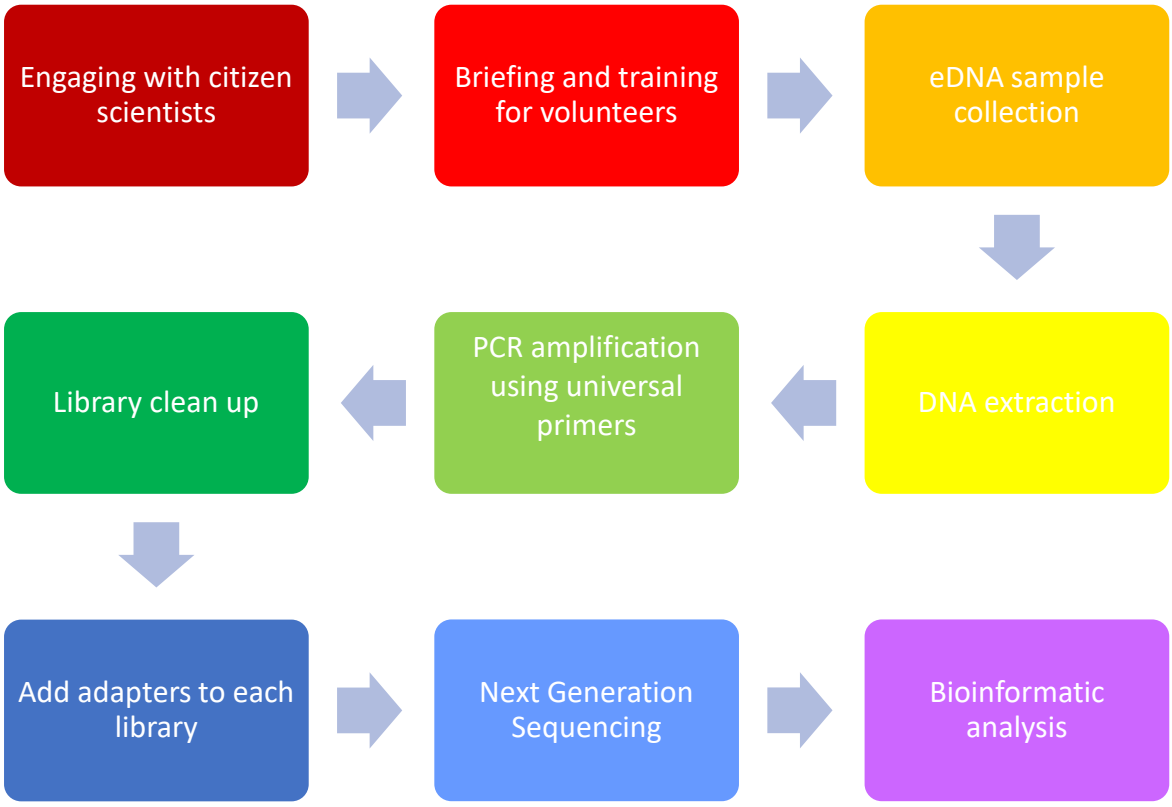


Figure 3: The workflow of eDNA metabarcoding using a citizen science approach.

5. Results

5.1. Filtering steps

A total of 134 eDNA samples and 20 laboratory control samples were sequenced. The MiSeq run generated 10,732,216 reads (5,283,888 from library 1 and 5,448,328) which filtered down to 3,183,175 during the bioinformatics clean up steps (Table 2). The number of reads deducted during each filtering step are shown in below Table 2. During the filtering process 4,190,060 reads were deducted from Library 1, leaving 1,093,828 true reads with a tag

Table 2: Number of reads retained after each filtering step during the bioinformatics, per library and total.

Filtering step	**Library 1**	**Library 2**	**Total**
Total reads before filtering	5,283,888	5,448,328	10,732,216
After PC corrected	5,124,225	5,352,427	10,476,652
After PC removed	5,124,223	5,352,404	10,476,627
After Blanks removed	4,863,933	5,025,459	9,889,392
After removing all non-mammals	4,487,988	4,373,394	8,861,382
After removing human reads	2,396,497	2,293,282	4,689,779
After removing domestic species (Sus, Bos, Equus, Ovis, Canis, Felis)	2,318,903	2,135,367	4,454,270
MOTU's with a best identity 0.98 (min), reads >5	1,093,828	2,089,347	3,183,175
Tag-switching proportion	0.0198	0.00035	

switching proportion of 0.0198%. 3,358,981 reads were deducted from Library 2 leaving a total of 2,089,347 with a tag switching proportion of 0.00035%.

5.2. Species detection

A total of 21 species were detected over both rivers with 20 on the river Colne, 17 on the river Blackwater and 12 species in and around the beaver pond (Figure 5, Table 3). Overall, the winter sampling detected five less species than the summer sampling as Polecat *(Mustela putorius)*, Least weasel *(Mustela nivalis)*, American mink, Common shrew *(Sorex araneus)* and Red squirrel were not detected (Figure 4). Red deer *(Cervus elaphus)* was detected on the Blackwater during the winter sampling which was not found in the summer (Figure 4). The clustered column charts in (Figure 6) show species presence and absence at each site, comparing both sampling seasons and rivers. Brown rat was the most abundant species

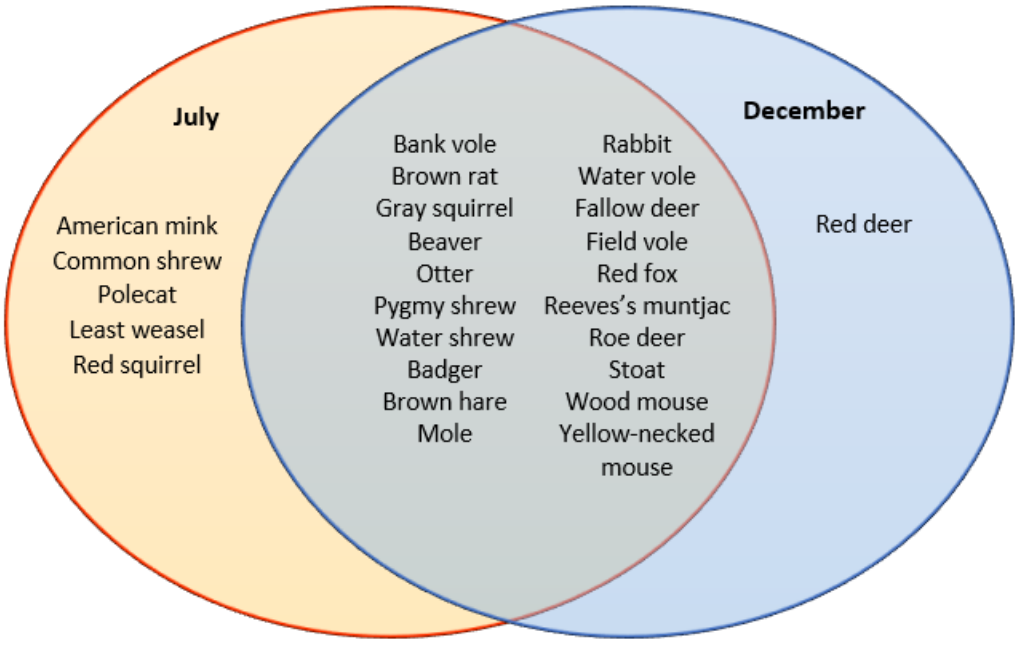


Figure 4: Relationship between species detected in the summer and species detected in the winter displayed on a Venn diagram.

detected in the winter sampling over both rivers found at 14 sites on the river Colne and at nine on the Blackwater (Figure 6 & Table 3). Species that were only detected at a single site in the winter sampling on the river Colne included Red deer, Eurasian pygmy shrew and European mole *(Talpa europaea)* and on the river Blackwater (including the beaver sampling) included Red deer, otter, European hare *(Lepus europaeus)* and stoat *(Mustela erminea)*.

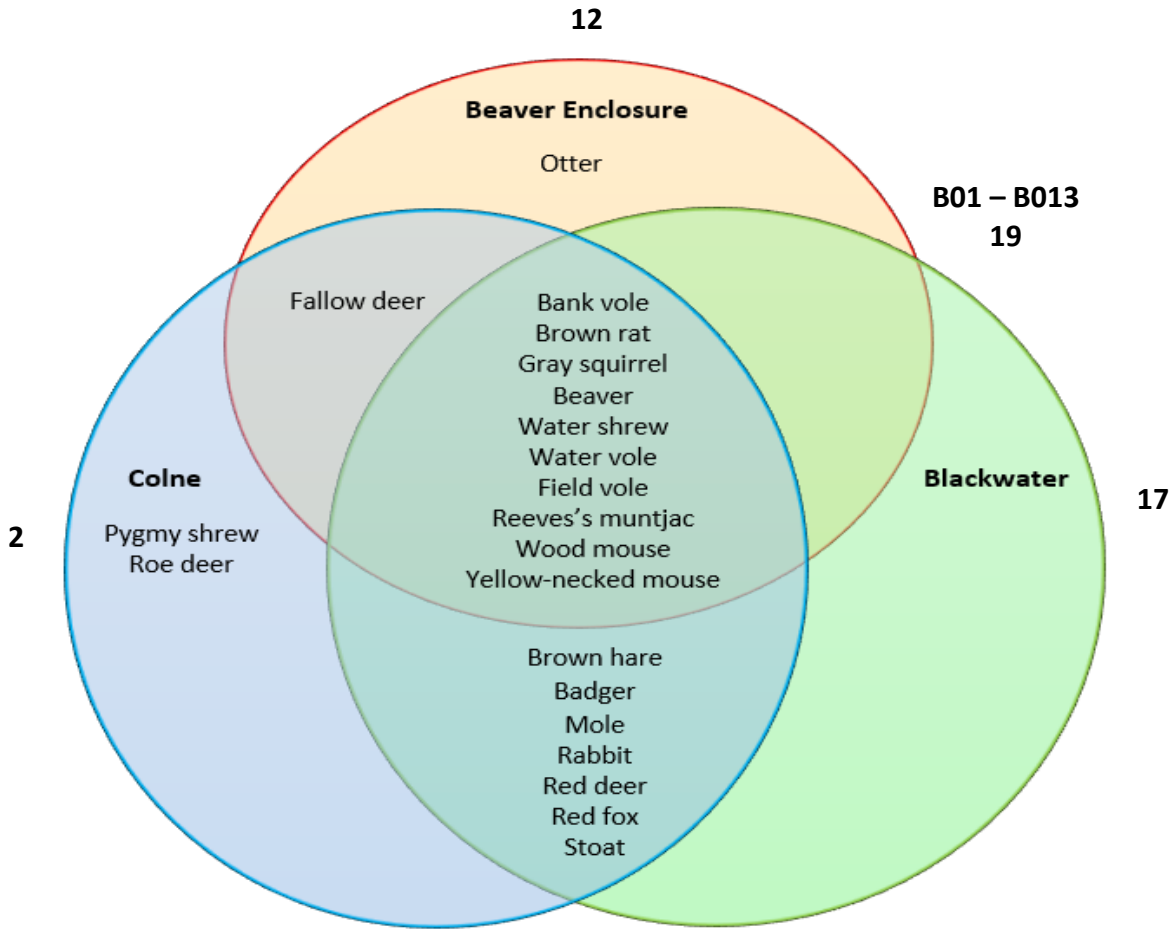
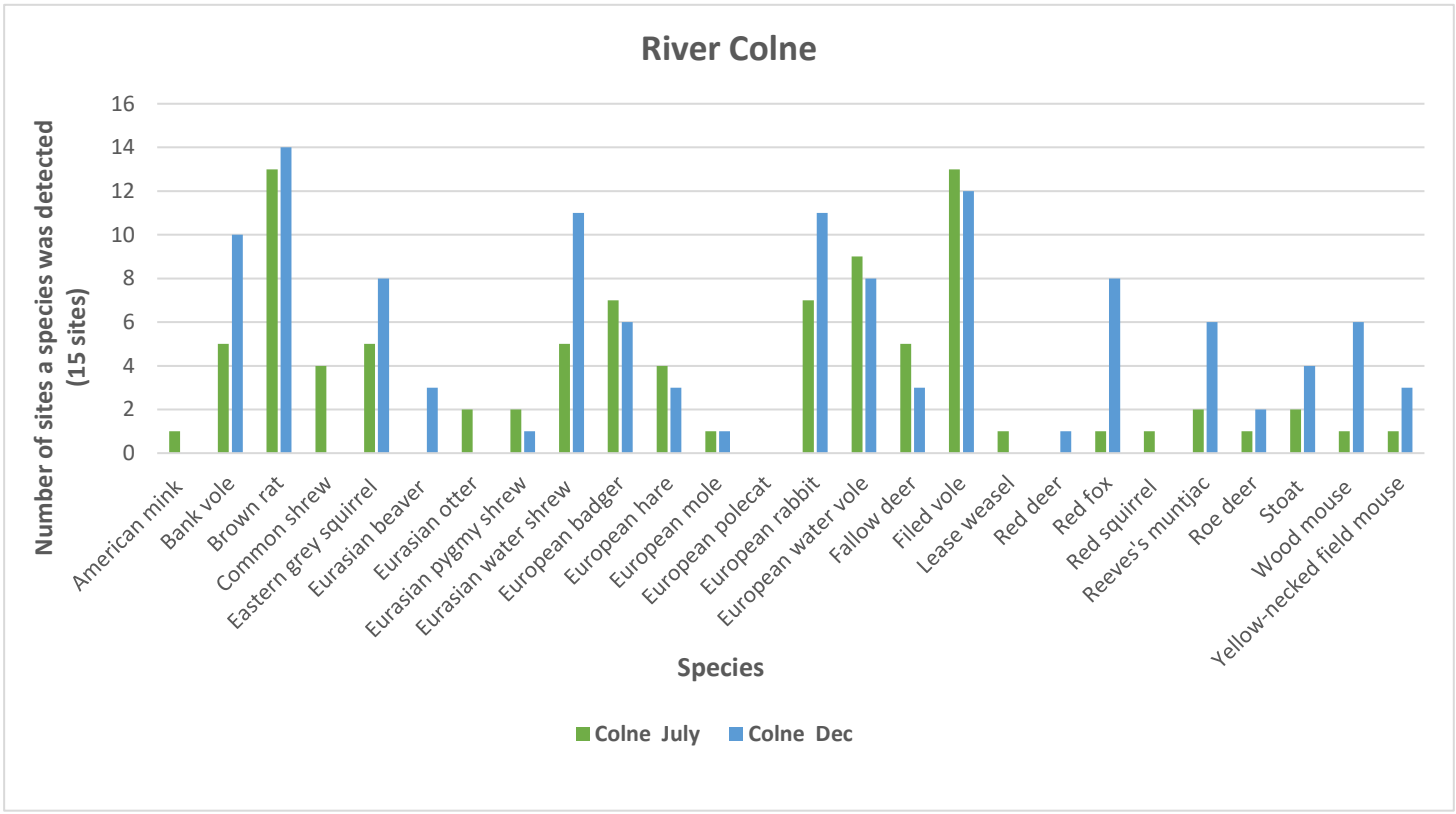


Figure 5: Relationship between species detected on the river Colne, Blackwater and in the beaver enclosure displayed on a Venn diagram.



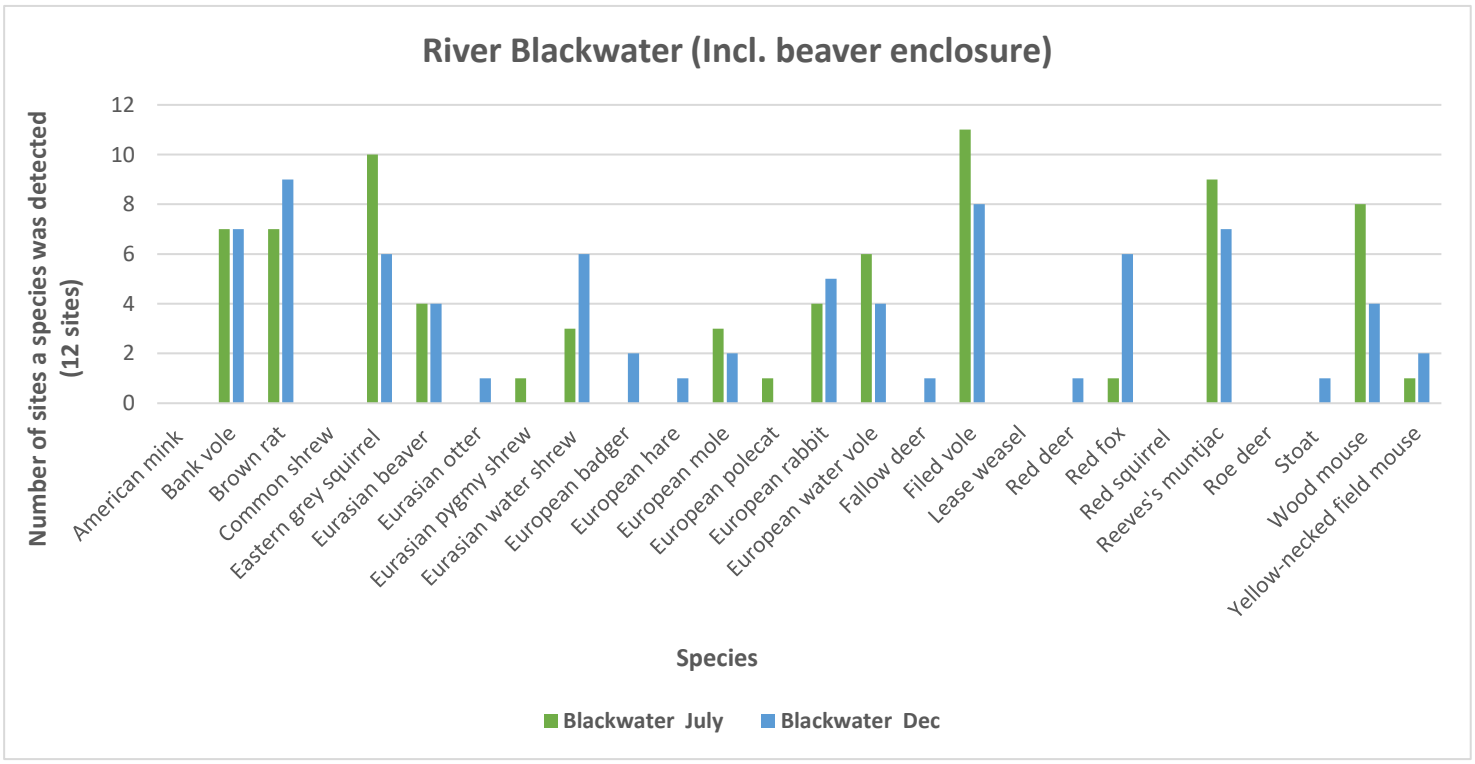


Figure 6: Number of sights each species was detected on the river Colne (top) and the river Blackwater (bottom), showing sampling periods July and December. The sampling points for the beaver were included.

Table 3: Species list with taxonomic order, conservation status (England) and number of sites where each species was detected on rivers Blackwater, Colne and the Beaver enclosure (B01 – B03, see Table S1 for ID and site names and Table S3 for individual site detections).

				Colne		Blackwater		Beaver	
Order	**Scientific name**	**Species**	**Status**	**July**	**Dec**	**July**	**Dec**	**July**	**Dec**
Carnivora	***Neovison vison***	**American mink**	NN	1	0	0	0	0	0
Rodentia	*Myodes glareolus*	Bank vole	LC	9	10	4	4	2	3
Rodentia	*Rattus norvegicus*	Brown rat	NN	13	14	5	6	2	3
Eulipotyphla	***Sorex araneus***	**Common shrew**	LC	4	0	0	0	0	0
Rodentia	*Sciurus carolinensis*	Eastern grey squirrel	NN	5	8	7	5	3	1
Rodentia	*Castor fiber*	Eurasian beaver	CE	0	3	0	2	4	2
Carnivora	*Lutra lutra*	Eurasian otter	LC	2	0	0	0	0	1
Eulipotyphla	*Sorex minutus*	Eurasian pygmy shrew	LC	2	1	0	0	1	0
Eulipotyphla	*Neomys fodiens*	Eurasian water shrew	LC	5	11	2	3	1	3
Carnivora	*Meles meles*	European badger	LC	7	6	0	2	0	0
Lagomorpha	*Lepus europaeus*	European brown hare	Nat	4	3	0	1	0	0
Eulipotyphla	***Erinaceus europeaus***	**European hedgehog**	VU	0	0	0	0	0	0
Eulipotyphla	*Talpa europaea*	European mole	LC	1	1	3	2	0	0
Carnivora	***Mustela putorius***	**European polecat**	LC	0	0	1	0	0	0
Lagomorpha	*Oryctolagus cuniculus*	European rabbit	Nat	7	11	3	5	1	0
Rodentia	*Arvicola amphibius*	European water vole	EN	9	8	4	2	2	2
Artiodactyla	*Dama dama*	Fallow deer	LC	5	3	0	0	0	1
Rodentia	*Microtus agrestis*	Field vole	LC	13	12	8	5	3	3
Rodentia	***Micromys minutus***	**Harvest mouse**	LC	0	0	0	0	0	0
Rodentia	***Muscardinus avellanarius***	**Hazel dormouse**	VU	0	0	0	0	0	0
Rodentia	***Mus musculus***	**House mouse**	Nat	0	0	0	0	0	0
Carnivora	***Mustela nivalis***	**Least weasel**	LC	1	0	0	0	0	0
Artiodactyla	*Cervus elaphus*	Red deer	LC	0	1	0	1	0	0
Carnivora	*Vulpes vulpes*	Red fox	LC	1	8	0	6	1	0
Rodentia	***Sciurus vulgaris***	**Red squirrel**	EN	1	0	0	0	0	0
Artiodactyla	*Muntiacus reevesi*	Reeves's muntjac	NN	2	6	5	5	4	2
Artiodactyla	*Capreolus capreolus*	Roe deer	LC	1	2	0	0	0	0
Carnivora	*Mustela erminea*	Stoat	LC	2	4	0	1	0	0
Rodentia	*Apodemus sylvaticus*	Wood mouse	LC	1	6	5	3	3	1
Rodentia	*Apodemus flavicollis*	Yellow-necked mouse	LC	1	3	1	1	0	1
			Totals	**23**	**20**	**12**	**17**	**12**	**12**

LC – Least concern, VU - vulnerable, NT – Near threatened, EN – Endangered, CE – Critically endangered, NN - Non-native, Nat – Naturalised (ICUN Red List, 2021).

Figure 5 shows the relationship between species detected on both rivers and the beaver enclosure in the winter sampling. It shows that a total of 12 species were detected in or around the beaver enclosure including otter *(Lutra lutra).* Otter was only detected in or around the beaver enclosure. The beaver enclosure consisted of only three sample sites (Table S1). Pygmy shrew and Roe deer *(Capreolus capreolus)* were only detected on the river Colne and there were no species that were detected only on the river Blackwater (Figure 5).

Tables 4 and 5 show the percentage of sites each species was detected based on the number of sampling sites per season. In the summer sampling both rivers Colne and Blackwater had 15 sites each whereas in the winter sampling the river Colne had 15 sites and the Blackwater had 12 sites. This shows the species detected at the most sites overall in the summer sampling was the field vole *(Microtus agrestis)* found at 80% of sites followed by the brown rat which was detected at 67% of sites which is highlighted as an invasive species. In the winter sampling brown rat was detected the highest, found at 82% of the sites. Other invasive species which are highlighted include the grey squirrel which is detected at 50% of sites in both sampling seasons and Reeves's muntjac which was found at 37% of sites in the summer sampling and 43% of sites in the winter sampling.

Table 4: Species found in the December sampling study area with percentage of sites detected. Species are ranked in descending order of sites detected. Invasive non-native species are highlighted. There were 27 sites in total including the beaver sampling.

Order	Scientific Name	Species	Sites detected
Rodentia	*Rattus norvegicus*	Brown rat	82%
Rodentia	*Microtus agrestis*	Field vole	71%
Rodentia	*Myodes glareolus*	Bank vole	61%
Eulipotyphla	*Neomys fodiens*	Eurasian water shrew	61%
Lagomorpha	*Oryctolagus cuniculus*	European rabbit	57%
Rodentia	*Sciurus carolinensis*	Eastern grey squirrel	50%
Carnivora	*Vulpes vulpes*	Red fox	50%
Rodentia	*Arvicola amphibius*	European water vole	43%
Artiodactyla	*Muntiacus reevesi*	Reeves's muntjac	43%
Rodentia	*Apodemus sylvaticus*	Wood mouse	36%
Carnivora	*Meles meles*	European badger	29%
Rodentia	*Castor fiber*	Eurasian beaver	25%
Carnivora	*Mustela erminea*	Stoat	21%
Rodentia	*Apodemus flavicollis*	Yellow-necked mouse	18%
Artiodactyla	*Dama dama*	Fallow deer	14%
Lagomorpha	*Lepus europaeus*	European brown hare	14%
Eulipotyphla	*Talpa europaea*	European mole	11%
Artiodactyla	*Capreolus capreolus*	Roe deer	7%
Artiodactyla	*Cervus elaphus*	Red deer	7%
Carnivora	*Lutra lutra*	Eurasian otter	4%
Eulipotyphla	*Sorex minutus*	Eurasian pygmy shrew	4%
Carnivora	*Mustela nivalis*	Least weasel	0%
Carnivora	*Mustela putorius*	European polecat	0%
Carnivora	*Neovison vison*	American mink	0%
Rodentia	*Sciurus vulgaris*	Red squirrel	0%
Eulipotyphla	*Sorex araneus*	Common shrew	0%
Eulipotyphla	*Erinaceus europeaus*	European hedgehog	0%
Rodentia	*Mus musculus*	House mouse	0%
Rodentia	*Micromys minutus*	Harvest mouse	0%
Rodentia	*Muscardinus avellanarius*	Hazel dormouse	0%

Table 5: Species found in the July sampling study area with percentage of sites detected. Species are ranked in descending order of sites detected. Invasive non-native species are highlighted. There were 30 sites in total including the beaver sampling.

Order	Scientific name	Species name	Sites detected
Rodentia	*Microtus agrestis*	Field vole	80%
Rodentia	*Rattus norvegicus*	Brown rat	67%
Rodentia	*Myodes glareolus*	Bank vole	50%
Rodentia	*Sciurus carolinensis*	Eastern grey squirrel	50%
Rodentia	*Arvicola amphibius*	European water vole	50%
Lagomorpha	*Oryctolagus cuniculus*	European rabbit	37%
Artiodactyla	*Muntiacus reevesi*	Reeves's muntjac	37%
Rodentia	*Apodemus sylvaticus*	Wood mouse	30%
Eulipotyphla	*Neomys fodiens*	Eurasian water shrew	27%
Carnivora	*Meles meles*	European badger	23%
Artiodactyla	*Dama dama*	Fallow deer	17%
Eulipotyphla	*Sorex araneus*	Common shrew	13%
Rodentia	*Castor fiber*	Eurasian beaver	13%
Lagomorpha	*Lepus europaeus*	European brown hare	13%
Eulipotyphla	*Talpa europaea*	European mole	13%
Eulipotyphla	*Sorex minutus*	Eurasian pygmy shrew	10%
Carnivora	*Lutra lutra*	Eurasian otter	7%
Carnivora	*Vulpes vulpes*	Red fox	7%
Carnivora	*Mustela erminea*	Stoat	7%
Rodentia	*Apodemus flavicollis*	Yellow-necked mouse	7%
Carnivora	*Neovison vison*	American mink	3%
Carnivora	*Mustela putorius*	European polecat	3%
Carnivora	*Mustela nivalis*	Least weasel	3%
Rodentia	*Sciurus vulgaris*	Red squirrel	3%
Artiodactyla	*Capreolus capreolus*	Roe deer	3%
Eulipotyphla	*Erinaceus europeaus*	European hedgehog	0%
Rodentia	*Micromys minutus*	Harvest mouse	0%
Rodentia	*Muscardinus avellanarius*	Hazel dormouse	0%
Rodentia	*Mus musculus*	House mouse	0%
Artiodactyla	*Cervus elaphus*	Red deer	0%

5.3. Sampling effort/ Species accumulation curves

The number of sampling sites needed to successfully detect all species on the river Blackwater in both summer and winter sampling is shown to be adequate (Figures 7A & 7B). However, the sampling effort for the river Colne in both sampling seasons is shown to not be sufficient as if more samples were taken, more species could be detected. It can also be seen that more species have been detected in the winter sampling on the river Blackwater than the summer (Figure 7B).

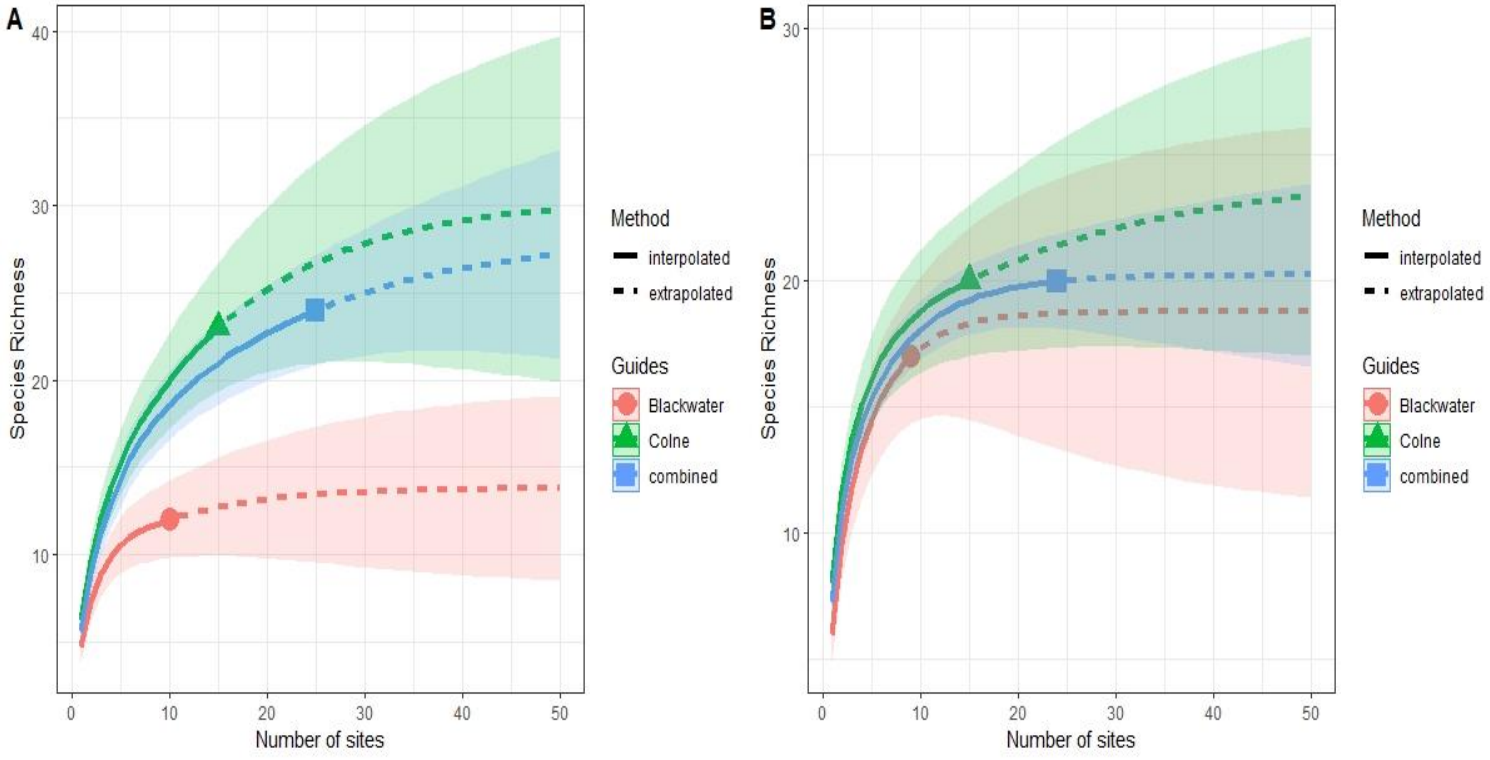


Figure 7: Species accumulation curves showing species richness compared to number of sights sampled, (A) July sampling and (B) December sapling.

5.4. Species richness and β-diversity

β-diversity is represented by the ordination plots (Figure 8), showing community data and how closely related each sample is from another. The Jaccard distance considers presence/absence and uses a distance matrix to measure how different samples are from one another. The sites in the Rivers Blackwater and Colne in the winter sampling period were more different in composition than in July (Figures 8A and 8B). There was more similarity in community composition in the River Colne between sampling periods (Figure 8C) than the Blackwater (Figure 8D). When comparing all variables together the plot displays tight ellipsis with a high quantity overlap meaning there is less variability between the sampling seasons and rivers (Figure 8E). The River Colne had higher overall mean species richness than the Blackwater in both sampling periods (Figure 9).

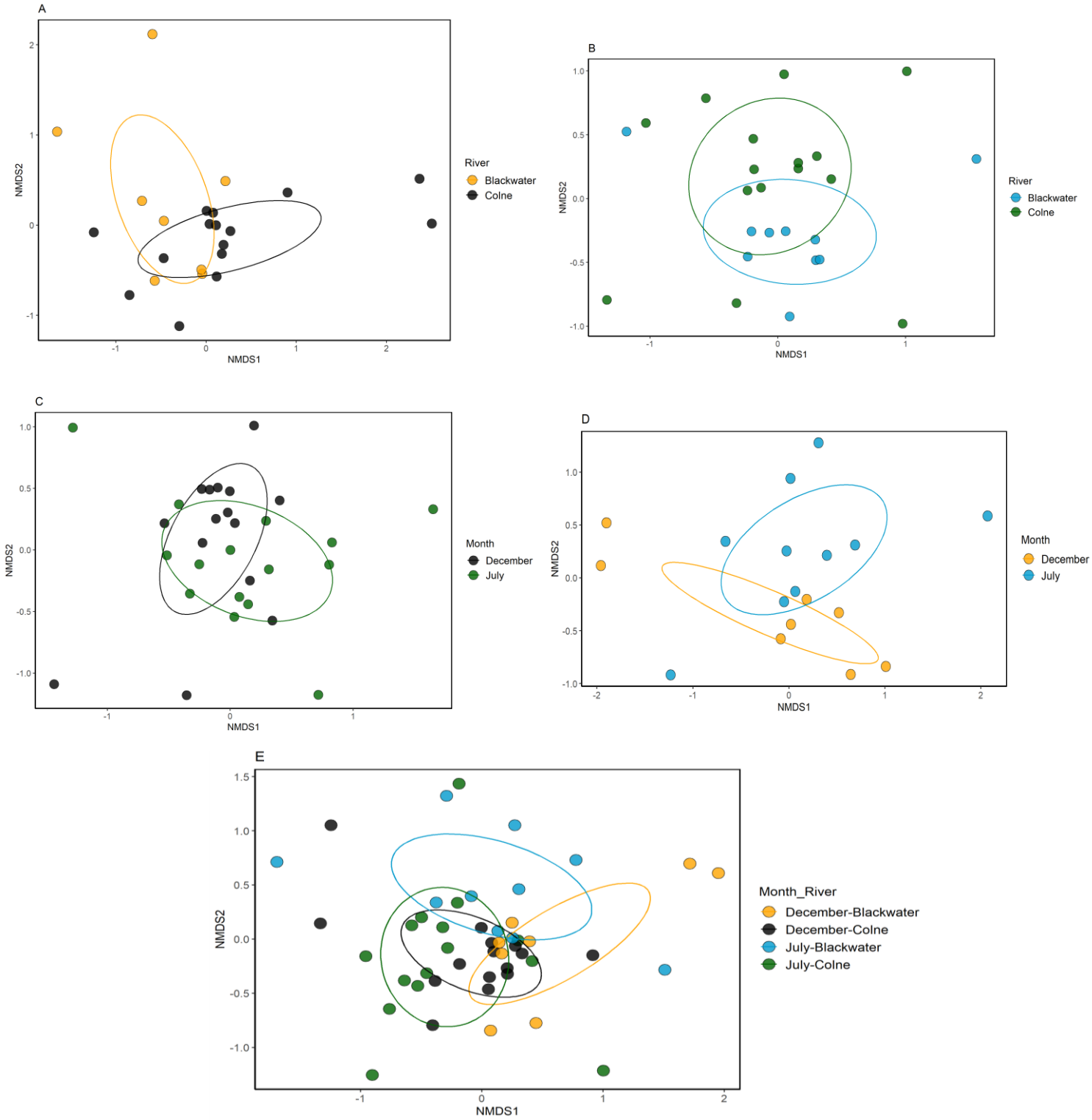


Figure 8: NMDS ordination plots with ellipses showing β-diversity using Jaccard distancing, A) December, B) July, C) Colne, D) Blackwater, E) comparing all variables

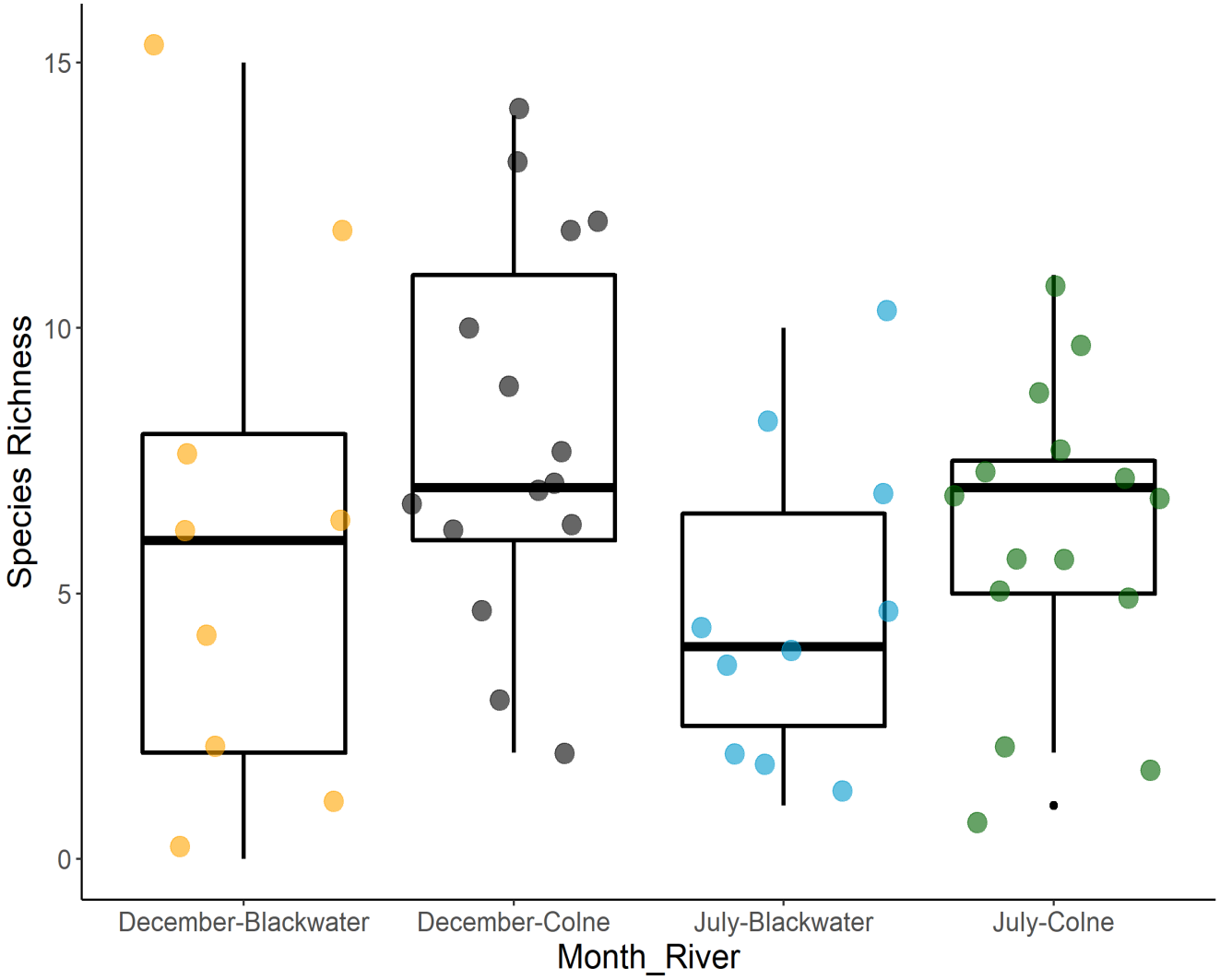


Figure 9: Box jitter plot showing overall species richness for each river and sampling season.

5.5. Human read proportions per sample

In July there were a total of 149 samples (excluding PC and blanks) with a total of 10,571,299 reads in which 3,113,787 were *Homo sapiens* reads with a best identity of 1 (Table 6). This gave an average of 29% of reads in each sample that were human with a median value of 29% and a standard deviation of 18%. In December, a total of 130 samples (excluding PC and blanks), with a total of 9,889,392 reeds had 4,165935 being *Homo sapiens* reads with a best identity of 0.994152047. This gave an average percentage of 27% human reads found in each sample and a median value of 30% with a standard deviation of 15%. Proportions were calculated using the csv files after positive controls and blanks removed shown in Table 6 for December and Broadhurst et al., (2021) supplementary material for July.

Table 6: Proportion of human reads in all samples in both July and December sampling.

	July	**December**
No of samples	149	130
Total number of human reads	3113787	4165935
Total number of reads	10571299	9889392
Mean	0.2874534 (29%)	0.2709303 (27%)
Median	0.294947 (29%)	0.3044886 (30%)
Standard deviation	0.1777924 (18%)	0.1546323 (15%)

We found that 14 out of 130 of the December samples had 0 reads compared to just 3 out of 149 of the July samples had 0 reads meaning there was no DNA found in these samples. The two-sample t-test gave an output of: t-value = 0.45979, degrees of freedom = 277 and p-value = 0.646. The null hypothesis was that there is no significant difference between human read proportions in July and December. The t-test resulted in the null hypothesis being accepted since the p-value was larger than 0.05.

6. Discussion

Here, eDNA metabarcoding was used to show that citizen science sampling is just as effective as professional eDNA sampling to understand species distributions and mammalian communities across two river systems. With the need to expand data on the geographical distributions of UK mammal populations (Croft et al., 2017; Mathews & Harrower, 2020), citizen science can be used to collect samples for accurate eDNA analysis. Despite citizen science being widely used for monitoring surveys, it has only recently been used for the collection of eDNA sampling (Biggs et al., 2015; Larson et al., 2020b; Lin et al., 2021; Wilken et al., 2018). As suggested by Broadhurst et al. (2021), the use of the local conservation groups, in this case the Essex Wildlife Trust, has been used to engage with local volunteers which has enabled this sampling season to be even more time and cost-effective. Due to the impact of Covid-19, a parallel sampling in December (eDNA specialists vs citizen scientist) could not be carried out as desired. However, the winter citizen science sampling alone has generated valuable data on the mammal community present.

6.1. Species detection using eDNA

The species detected in the winter sampling provide an accurate representation of the mammal community that you would expect to find in this area with a few exceptions. Non-riparian mammalian species: European hedgehog *(Erinaceus europesus)*, harvest mouse *(Micromys minutus)*, hazel dormouse *(Muscardinus avellanarius)* and house mouse *(Mus musculus)* were absent from both sampling seasons, although other non-riparian species, such as grey squirrel were frequently detected, most likely due to its sheer abundance and invasive nature. Brown rat was the most abundant species detected in December (82% of

sites) whereas in July field vole was detected at the most sites (80%; Tables 4 & 5). We expected to detect less mammals during the winter sampling than the summer due to species ecology and environmental factors which reflect in the data. From the 28 known mammal species in the area (excluding bats; Mathews et al., 2018) the summer sampling detected 25 of these species, whereas the winter detected 21. It was expected that terrestrial and arboreal species will have a lower detection rate than aquatic or semi-aquatic species due to them having less contact with the water (Harper et al., 2019; Ushio et al., 2016). Some terrestrial or arboreal species will naturally have more contact with water than others due to their lifestyle and behaviours (Rodgers & Mock, 2015; Ushio et al., 2016).

When considering the species accumulation curves (Figure 7B), it suggests that most mammals have been detected by the eDNA sampling on the river Colne and nearly all mammals on the river Blackwater as this river has a lower species richness (Broadhurst et al., 2021). Some species we found had particularly low detection rates in this study e.g., pygmy shrew and otter were only detected at one sampling site and red deer only detected at two sites overall in the winter sampling period. There were also species detected with extremely low read counts of <10 e.g., badger *(Meles meles)*, hare, red deer, stoat, yellow-necked mouse *(Capreolus capreolus),* and roe deer. Some of these species are rarer e.g., yellow-necked mouse, or elusive species e.g., stoat, which were picked up by the eDNA sampling and perhaps wouldn't have been detected by more traditional methods.

Red deer was detected in the winter sampling on both rivers but with extremely weak detection rates of only two and five reads (shown in Table S3*)*. As the read counts were so low for this species and it wasn't detected in the summer sampling speculation can be asked

whether this is a true detection or not. False positives have found to be common when using Illumina sequencing platforms (Sales et al.,2020a), hence the intensive filtering process after bioinformatic analysis. As red deer are present in the Essex area (Lovari et al., 2018; Mathews et al., 2018) the low read count could be due to the DNA being highly diluted in the water when collected. A larger volume of water could be used in future studies to increase the load of DNA in the sample, although this could become problematic to filter when using a citizen science approach due to the specialist equipment needed (Broadhurst et al., 2021; Cantera et al., 2019; Mächler et al., 2016; Lyet et al., 2021).

Following on from the beaver experiment in Broadhurst et al. (2021) which investigates the ecology of eDNA, beaver was detected in the winter sampling on both rivers (Figure 6 & Table 3). As the beaver enclosure is located on sites B01-B03 on the river Blackwater the positive detections on the river Colne are probably due to contamination (all 3 detections on the Colne had read counts <10; Table S3). Out of the four positive detections on the Blackwater only one site had a substantial proportional read count (B01; Table S3), which indicates a strong detection. However, a total of 12 species were detected in and around the beaver enclosure (B01-B03; Figure 5) including both otter and water vole. This could be an indication of the positive effect the recently introduced beavers are having on the local environment as seen in other reintroduction programmes (Stringer & Gaywood, 2016).

eDNA detection is known to be affected by the seasonal activity of species (de Souza et al., 2016). Sampling in winter only can limit the ability to detect certain species, therefore this kind of eDNA sampling should be carried out in each season to allow all species to be detected throughout the year. Other species are less active in the colder months e.g., water

voles, who spend more time in their burrows consuming stored food (Batsaikhan et al., 2021). This is shown in our findings as water voles were detected in 15 sites (50%, Table 5) in the summer sampling but only 12 sites (43%, Table 4) in the winter sampling. Species which were detected in the summer sampling but not the winter sampling are American mink, common shrew, polecat, least weasel and red squirrel (Figure 4), three of which are carnivores.

Carnivore species have frequently been found at lower detection rates than other groups in various eDNA studies (Harper et al., 2019; Lyet et al., 2021; Sales et al., 2020a; Thomsen et al., 2012b). The findings in both sampling seasons support this as stoat, polecat, least weasel, American mink and otter were only detected at one or two sampling sites or were completely absent in a season. Other studies have used species-specific primers with a qPCR approach to generate high detectability in carnivores (Franklin et al., 2019). Species with low abundances or solitary species with large home ranges e.g., red fox, can also be problematic to detect using eDNA but can have higher detection rates using other methods e.g., camera trapping or field signs (Broadhurst et al., 2021; Harper et al., 2019; Sales et al., 2020a). This suggested that a combination of field methods should be used alongside eDNA metabarcoding to generate higher detections in this group.

Otter had very low detection rates in both summer (two sites) and winter (one site) sampling seasons when compared to other semi-aquatic mammals e.g., water vole and beaver. Proportional read counts were also low in otter detections from the winter sampling (Table S3). This is supported in other studies which also find low detection rates and read counts in otters when using eDNA metabarcoding (Broadhurst et al., 2021; Harper et al., 2019; Sales et al., 2020a). Species found in high abundance may lead species of low abundance to

go undetected during sequencing, which could be the result in low species detection or absent species (Harper et al., 2018). Triplicates of all samples have been used in this study to try keep this bias to a minimum. As otters utilise water bodies frequently, it is thought that detection rates would be higher than terrestrial or arboreal mammals, which is not the case in either of these studies. A lot of genetic material originates from faeces and otter latrines are generally found on land and not in water. Together, with low detection rate in carnivores, this could indicate why otters are generally underrepresented in metabarcoding datasets. Again, species-specific assays have been developed to target otters which will lead to higher detection rates and would be more beneficial to survey river otters (Padgett-Stewart et al., 2016).

In this study it was found that three of the four non-native invasive species: Brown rat, Grey squirrel and Reeves's muntjac had high detection rates from eDNA sampling in both the summer and winter (excluding the invasive American mink which showed low and no detections; Tables 4 & 5). Not only were they detected at multiple sample sites, but many of the samples had very high read counts which could indicate there are many individuals. American mink is thought to now be eradicated in this area due to the success of mink control carried out by the EWT (Essex Wildlife Trust, 2021). As non-native invasive species can be monitored and detected early by the application of eDNA it can be presumed that by adding CS to this collaboration, it would magnify the benefits. If eDNA sampling was to be continued alongside other mink surveys such as mink rafts and camera traps, it could contribute to the mink control efforts. eDNA has been used for invasive species surveillance in other studies

(Dejean, et al, 2017; Williams et al., 2017) but with a targeted approach to only detect the specific species.

As the sample collection was carried out by different individuals, the sampling period was spread across December 2019 to January 2020. The collection dates range from the 10/12/19 – 05/01/20 (Table S2). This winter sampling was conducted over a four-week period compared to the summer sampling that was executed over just two days (Broadhurst et al., 2021). This can be a common problem in citizen science project as volunteers have to fit their sampling around their personal lives and jobs. Also, weather conditions were a problem in the winter sampling with rain and high river flow/flooding preventing sample collection on some days. Cogshell Hall Bridge (Site B10) had high river flow and continuous rain at the time of sample collection (shown in the notes column in Table S2) which reflects in the results as there were very low detection rates at this site (Table S3).

6.2. Species richness and β-diversity

It is important to understand species richness within a habitat to be able to optimize management plans for species and community conservation. Knowledge on species communities over spatial and temporal scales is necessary to gauge how species interact and to understand why species which are expected in the area but are absent from a particular site (Fontana et al., 2020). Species richness and β-diversity (variation of species composition) relates to the population size and the diversity of species in a site. By the absence of five species in the winter sampling compared to the summer and the gain of one extra species detection in the winter, it shows the dissimilarity in mammalian composition between the two seasons (Figure 4, 8a & 8b). There are also dissimilarities in mammalian communities

between rivers in both seasons as overall, the Colne shows greater species richness than the Blackwater and with a difference in species detected. Interestingly, some species that were absent from the Blackwater in the summer, were present there in the winter. Otter was detected at two sites on the Colne in the summer and absent from the Blackwater but in the winter sampling it was absent from the Colne but detected at one site on the Blackwater. This again highlights the importance of sampling multiple rivers within a landscape to capture the overall community diversity (Broadhurst et al., 2021).

6.3. Citizen Science

Citizen science should be used to increase conservation efforts of UK mammals as there is a lack of ongoing, large-scale monitoring projects (Dickenson et al., 2010). As eDNA sampling is a relatively simple method to follow it should be used more by citizen science-based programmes to monitor mammals. The use of active volunteers from the EWT has enabled this project to cut out the recruitment step which can be very time consuming and costly (Merenlender et al., 2016).

There are many positive aspects when it comes to volunteering or getting involved with CS projects to both the project and the citizen scientist. Firstly, the underlying benefit is the contribution towards helping conserve local and global habitats and species. As educating the public is vital for conservation, getting the public involved not just educates but immerses them into the scientific world of research and data collection (Larson et al., 2020a). Another major benefit of CS is time. The time put in by volunteers is invaluable as many of these projects would never have the funding or resources to man these large-scale, long-term projects. CS can also speed up the progress of research projects by adding more

observers/participants (Liebenberg et al., 2016). Benefits to the volunteer go way beyond learning about science as there are also many physical and mental benefits. They can improve people's mental health by giving them hobbies and opportunity to socialise with other people. Being outside and around the natural environment can be soothing and therapeutic for the volunteers who wouldn't usually be in these environments.

Some species can be easier than others to gain involvement from volunteers. Birds for instance, have a large set of volunteers who regularly enjoy going out and collecting data around the species and have many different monitoring programs (Audubon's Christmas Bird Watch, Big Garden Bird Watch, Britain's Common Bird Census, Cornell Lab of Ornithology's eBird, NestWatch & Project Feeder Watch, etc.), whereas other groups may not be as favourable to volunteers e.g., plants or rodents (Larson et al., 2020a). Citizen science research has mainly focused on species distributions with a strong leaning towards sampling vertebrates. Theobald et al. (2015) found that 97% of citizen science research monitored taxonomic diversity whereas genetic studies have less citizen science involvement. As citizen science data collection can become taxonomically biased (Theobald et al., 2015), the use of eDNA metabarcoding sets out to overcome this limitation as it has the power to target multiple taxonomic groups in the same study. By using eDNA metabarcoding, it can be used to target different groups, for instance mammals, but then different metabarcodes can be used with the same samples e.g., Lin et al., (2021) used five metabarcodes to target different groups in the same study. This enables one sampling period to be used for targeting and surveying multiple taxonomic groups/species.

Because eDNA is completely non-invasive and human-animal encounters or sightings are never made, it could make this method less appealing to volunteers who are interested in wildlife. They are more likely to get involved with a survey where they can count or identify an animal (bat counts, bird watching, small mammal trapping, camera trapping) or volunteer at an animal sanctuary rather than indirectly sample animals (eDNA sampling). Other motivations include social needs where volunteers can interact with other volunteers which can increase longevity in involvement (West & Pateman, 2016). If group meetings were held after the eDNA samples had been processed a presentation could be given showing what species were found at each sampling site. This will then show the volunteers what they have achieved by carrying out the eDNA sampling. More communication is needed with the volunteers who collected the samples to keep them in touch with the project and make them want to continue this type of work in the future. The publication of citizen science data is incredibly low compared to that of professionals (Theobald et al., 2015) which could mean results on this important data are not being recognized.

Although there are many successfully reliable citizen science-based studies there are still some that result in inaccurate and less accurate data when compared to scientific data (Hoyer et al., 2012; Oldekop et al., 2011). As the human proportion read count in samples was quantified and compared to those sampled by eDNA specialists, no difference was found (Table 6). This suggests that unlike other methods, eDNA has relatively low error rate when sampled by citizen scientists. Due to the eDNA sample collection method being relatively simple and does not need masses of prior knowledge, given the correct training there is little room for error. Most issues, other than contamination or incorrect storage of samples, comes

in the laboratory and bioinformatic processes which are then carried out by eDNA specialists (Thomsen & Willerslev, 2015). Issues found within The National Amphibian and Reptile Recording Scheme (NARRS) was caused by an uneven representation of volunteers, as some sampling areas are richer in participants than others. The 1st six-year survey cycle found that after the first year the number of surveys carried out by volunteers started to decrease which may contribute to biased results in the overall data (Wilkinson & Arnell, 2013) similarly to the basis found in the NBMP (Barlow et al., 2015). Other trending issues that are occurring are keeping participants involved and motivated to commit to long-term projects and repeat sampling (West & Pateman, 2016). Building relationships with participants is a key component to keep them active and engaged (Penner, 2002). In the present day more and more online platforms are available which is good for engagement and makes things more accessible. Although online platforms are great it is still vital to get people interacting in person and in the natural environment.

6.4. Future suggestions

As using citizen science for eDNA metabarcoding is relatively new, there is a lot to be learnt and improved for future research. Many mammalian eDNA studies have found using multiple surveying methods (camera traps, latrine or field sign surveys) alongside eDNA metabarcoding, can be beneficial to maximise detections for problematic species (Harper et al., 2019; Lyet et al., 2021; Sales et al., 2020a). This could help to rule out any false negatives or false positives found in eDNA sampling. Although we were able to look for contamination within the eDNA samples (positive and negative controls, extraction, and PCR blanks), field blanks were not taken in this study. Due to the nature of this volunteer programme, it would

have been difficult to take field blanks as different volunteers collected individual sampling sites over a longer sampling period. It is vital that field blanks are collected on the day of each sampling session to test for any potential contamination in the field which can lead to false positive detections (Thomsen & Willerslev, 2015). For example, beaver was detected on the River Colne in December, and this is extremely unlikely. This could have come from contamination in the field which could have been controlled for if the appropriate field blanks had been taken. If management programs are implicated according to datasets containing false detections, it can have a negative impact on the conservation of species and waste valuable money and resources. Citizen science projects should and need to have engagement between the organization and the volunteers (Penner, 2002). In this study there was a lack of communication to inform volunteers on the data they collected which needs to be improved in the future. For the eDNA surveying to be continued with the EWT more training and engagement must be developed to both improve the survey technique and keep the volunteers engaged and connected. A meet-up could be arranged where the volunteers, the EWT staff and the eDNA specialists can interact and maximise relationships which will help move the project forward. This survey could also be expanded to a national scale involving different wildlife trusts or NGOs throughout the country following its successful data collection here in Essex, or at least expanded to areas of importance to monitor endangered or reintroduced mammals.

7. Conclusion

This study demonstrates that citizen science is a readily successful tool to use with eDNA sampling given the correct training. This study should be followed up with a parallel sampling in the same season to look deeper into specific site locations when comparing species detection. Overall, the mammalian community that was revealed in the winter sampling was backed up by similar findings in the summer sampling and the known mammals that we could expect to find in the area. As the volunteers from the EWT are keen participants, this new approach to monitor the mammal communities should be continued to track the presence/absence of the invasive American mink, the recovering water vole populations, reintroduced beavers, and to monitor any changes in the distribution of local species.

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10. Supplementary Material

Table S1. Sites co-ordinates for both winter (A) and summer (B) sampling in Essex of both rivers Blackwater and Colne showing how site IDs have been labelled. The sampling sites for the beaver experiment are highlighted in bold which were not used for the analysis.

A.	ID	Site	Long	Lat
	C01	Crouch Green, Castle Hedingham	0.585352	51.99125
	C02	Maiden Ley, Sible, Hedingham	0.595289	51.98272
	C03	Box Mill, Halstead	0.631664	51.95057
	C04	Bluebridge Farm Halstead	0.658667	51.93738
	C05	Ford Mill Colne Engaine	0.684973	51.93641
	C06	River House White Colne	0.713134	51.92786
	C07	Old Hall Farm White Colne	0.739529	51.92202
	C08	Bacons Farm Chappel	0.762268	51.91311
	C09	Woodland Trust Ford Street	0.790220	51.90938
	C10	Woodland Trust Fordham Hall	0.804775	51.90909
	C11	Gt Porters Farm Fordham	0.822283	51.91102
	C12	Cooks Hall West Bergholt	0.830873	51.90725
	C13	Seven Arches Farm	0.851409	51.89839
	C14	Spring Lane Colchester	0.868179	51.89372
	C15	Cymbeline Meadows	0.882572	51.89693
	B01	**Spains Hall beaver enclosure**	**0.443449**	**51.97074**
	B02	**Spains Hall footpath**	**0.448137**	**51.96994**
	B03	**Finchingfield Village**	**0.450784**	**51.96828**
	B04	----------	----------	----------
	B05	Cooks Farm Cottage	0.478914	51.94827
	B06	Wethersfield Mill	0.501315	51.93815
	B07	Road Bridge, Codham Hall	0.521184	51.92456
	B08	Straits Mill Fishery	0.563379	51.88651
	B09	Bridge Cottage, Bradwell	0.624036	51.87698
	B10	Coggeshall Road Bridge	0.684858	51.86917
	B11	Blue Mills, Witham	0.652309	51.78655
	B12	Wickham Hall, Road Bridge	0.640241	51.77385
	B13	Maldon A414, Road Bridge	0.678681	51.73801

B.	ID	Site	Long	Lat
	C01	Crouch Green, Castle Halstead	0.585352	51.99125
	C02	Maiden Ley Sible Halstead	0.595144	51.98299
	C03	Box Mill Halstead	0.631664	51.95057
	C04	Bluebridge Farm Halstead	0.658667	51.93738
	C05	Ford Mill Colne Engaine	0.684973	51.93641
	C06	River House White Colne	0.713134	51.92786
	C07	Old Hall Farm White Colne	0.739529	51.92202
	C08	Bacons Farm Chappel	0.762268	51.91311
	C09	Woodland Trust Ford Street	0.790220	51.90938
	C10	Woodland Trust Fordham Hall	0.804775	51.90985
	C11	GT Poters Farm	0.822283	51.91102
	C12	Cooks Hall	0.831107	51.90659
	C13	Seven Arches Farm	0.851240	51.8987
	C14	Spring Lane Colchester	0.868179	51.89372
	C15	Cymbeline Meadows	0.882602	51.89672
	Beaver1	**Spains Hall upper**	**0.443479**	**51.97076**
	Beaver2	**Spains Hall lower**	**0.443474**	**51.97066**
	Beaver3	**Spains Hall brook**	**0.443416**	**51.97067**
	B02	**Spains Hall footpath**	**0.448080**	**51.96989**
	B03	**Finchingfield village**	**0.450840**	**51.96824**
	B04	Brooklands Little Bardfield	0.432440	51.95223
	B05	Cooks Farm Cottage	0.479190	51.94822
	B06	Wethersfield Mill	0.501420	51.93818
	B07	Road Bridge	0.521330	51.92452
	B08	Straits Mill Fishery	0.563410	51.88646
	B09	Bridge Cottage Bradwell	0.624080	51.87695
	B10	Coggeshall road bridge	0.684800	51.86927
	B11	Blue Mills, Witham	0.653020	51.78711
	B12	Wickham Hall road bridge	0.640270	51.77378
	B13	Maldon A141 road bridge	0.678730	51.73787

Table S2. Sample extraction sheet showing sample ID, extraction date and notes about sites and samples which were taken by volunteers when collecting.

Extraction No.	Site ID	Site name	Replicate	River	Extraction Date	Notes	Library
1	**EB1**	**-**	**-**	-	22/09/2020		1
2	C07.1	Old Hall Farm	1	Colne	22/09/2020		1
3	C07.2	Old Hall Farm	2	Colne	22/09/2020		1
4	C07.3	Old Hall Farm	3	Colne	22/09/2020	Date collected: 11/12/19	1
5	C07.4	Old Hall Farm	4	Colne	22/09/2020		1
6	C07.5	Old Hall Farm	5	Colne	22/09/2020		1
7	C05.1	Ford Mill	1	Colne	22/09/2020		1
8	C05.2	Ford Mill	2	Colne	22/09/2020		1
9	C05.3	Ford Mill	3	Colne	22/09/2020	Date collected: 11/12/19	1
10	C05.4	Ford Mill	4	Colne	22/09/2020		1
11	C05.5	Ford Mill	5	Colne	22/09/2020		1
12	C02.1	Maiden Lea	1	Colne	22/09/2020	Date collected: 07/01/20 Partially defrosted before being re-frozen	1
13	C02.2	Maiden Lea	2	Colne	22/09/2020		1
14	C02.3	Maiden Lea	3	Colne	22/09/2020		1
15	C02.4	Maiden Lea	4	Colne	22/09/2020		1
16	C02.5	Maiden Lea	5	Colne	22/09/2020		1
17	C06.1	River House	1	Colne	22/09/2020		1
18	C06.2	River House	2	Colne	22/09/2020		1
19	C06.3	River House	3	Colne	22/09/2020		1
20	C06.4	River House	4	Colne	22/09/2020		1
21	C06.5	River House	5	Colne	22/09/2020		1
22	**EB2**	**-**	**-**	-	23/09/2020		1
23	C03.1	Box Mill Halstead	1	Colne	23/09/2020		1
24	C03.2	Box Mill Halstead	2	Colne	23/09/2020		1
25	C03.3	Box Mill Halstead	3	Colne	23/09/2020		1
26	C03.4	Box Mill Halstead	4	Colne	23/09/2020	Date collected: 31/12/19	1
27	C03.5	Box Mill Halstead	5	Colne	23/09/2020		1

28	C09.1	Woodland Trust, Ford Street	1	Colne	23/09/2020		1
29	C09.2	Woodland Trust, Ford Street	2	Colne	23/09/2020		1
30	C09.3	Woodland Trust, Ford Street	3	Colne	23/09/2020		1
31	C09.4	Woodland Trust, Ford Street	4	Colne	23/09/2020		1
32	C09.5	Woodland Trust, Ford Street	5	Colne	23/09/2020		1
33	C014.1	Spring Lane	1	Colne	23/09/2020		1
34	C014.2	Spring Lane	2	Colne	23/09/2020		1
35	C014.3	Spring Lane	3	Colne	23/09/2020		1
36	C014.4	Spring Lane	4	Colne	23/09/2020		1
37	C014.5	Spring Lane	5	Colne	23/09/2020		1
38	C01.1	Crouch Green	1	Colne	23/09/2020		1
39	C01.2	Crouch Green	2	Colne	23/09/2020		1
40	C01.3	Crouch Green	3	Colne	23/09/2020	Partially defrosted before being re-frozen	1
41	C01.4	Crouch Green	4	Colne	23/09/2020		1
42	C01.5	Crouch Green	5	Colne	23/09/2020		1
43	**EB3**	**-**	**-**	-	28/09/2020		2
44	C10.1	Fordham Hall	1	Colne	28/09/2020		2
45	C10.2	Fordham Hall	2	Colne	28/09/2020	TL9302027197	2
46	C10.3	Fordham Hall	3	Colne	28/09/2020		2
47	C10.4	Fordham Hall	4	Colne	28/09/2020	Date collected: 05/01/20	2
48	C10.5	Fordham Hall	5	Colne	28/09/2020	14:40	2
49	C08.1	Bacons Farm Chappel	1	Colne	28/09/2020		2
50	C08.2	Bacons Farm Chappel	2	Colne	28/09/2020		2
51	C08.3	Bacons Farm Chappel	3	Colne	28/09/2020	TL9008327447	2
52	C08.4	Bacons Farm Chappel	4	Colne	28/09/2020	Date collected: 10/12/19	2
53	C08.5	Bacons Farm Chappel	5	Colne	28/09/2020	Replicates 3,4,5 re-frozen	2
54	C11.1	GT Poters Farm, Fordham	1	Colne	28/09/2020		2
55	C11.2	GT Poters Farm, Fordham	2	Colne	28/09/2020		2
56	C11.3	GT Poters Farm, Fordham	3	Colne	28/09/2020	TL9421927373	2
57	C11.4	GT Poters Farm, Fordham	4	Colne	28/09/2020		2
58	C11.5	GT Poters Farm, Fordham	5	Colne	28/09/2020	Date collected: 10/12/19	2
59	C12.1	Cooks Hall West Bergholt	1	Colne	28/09/2020	TL9482626977	2
60	C12.2	Cooks Hall West Bergholt	2	Colne	28/09/2020		2

61	C12.3	Cooks Hall West Bergholt	3	Colne	28/09/2020	Date collected: 10/12/19	2
62	C12.4	Cooks Hall West Bergholt	4	Colne	28/09/2020		2
63	C12.5	Cooks Hall West Bergholt	5	Colne	28/09/2020		2
64	**EB4**	**-**	**-**	-	29/09/2020		2
65	C15.1	Cymbeline Meadow	1	Colne	29/09/2020		2
66	C15.2	Cymbeline Meadow	2	Colne	29/09/2020	TL9842725969	2
67	C15.3	Cymbeline Meadow	3	Colne	29/09/2020		2
68	C15.4	Cymbeline Meadow	4	Colne	29/09/2020	Date collected: 11/12/19 10:00	2
69	C15.5	Cymbeline Meadow	5	Colne	29/09/2020		2
70	C13.1	Seven Arches Farm	1	Colne	29/09/2020		2
71	C13.2	Seven Arches Farm	2	Colne	29/09/2020	TL9627726047	2
72	C13.3	Seven Arches Farm	3	Colne	29/09/2020		2
73	C13.4	Seven Arches Farm	4	Colne	29/09/2020	Date collected: 05/01/20 14:20	2
74	C13.5	Seven Arches Farm	5	Colne	29/09/2020		2
75	C04.1	Bluebridge Farm, Halstead	1	Colne	29/09/2020		2
76	C04.2	Bluebridge Farm, Halstead	2	Colne	29/09/2020		2
77	C04.3	Bluebridge Farm, Halstead	3	Colne	29/09/2020	TL82862988	2
78	C04.4	Bluebridge Farm, Halstead	4	Colne	29/09/2020		2
79	C04.5	Bluebridge Farm, Halstead	5	Colne	29/09/2020	Date collected: 30/12/19	2
80		**EB5**			30/09/2020		1
81	B09.1	Bridge Cottage, Bradwell	1	Blackwater	30/09/2020		1
82	B09.2	Bridge Cottage, Bradwell	2	Blackwater	30/09/2020		1
83	B09.3	Bridge Cottage, Bradwell	3	Blackwater	30/09/2020	TL8072223078	1
84	B09.4	Bridge Cottage, Bradwell	4	Blackwater	30/09/2020		1
85	B09.5	Bridge Cottage, Bradwell	5	Blackwater	30/09/2020		1
86	B05.1	Cook's Farm Cottage	1	Blackwater	30/09/2020		1
87	B05.2	Cook's Farm Cottage	2	Blackwater	30/09/2020		1
88	B05.3	Cook's Farm Cottage	3	Blackwater	30/09/2020	TL7046430655	1
-		**No replicate 4**	-	-	-	Date collected: 31/12/19	1
89	B05.5	Cook's Farm Cottage	5	Blackwater	30/09/2020		1
90	B06.1	Wethersfield Mill, Wethersfield	1	Blackwater	30/09/2020		1
91	B06.2	Wethersfield Mill, Wethersfield	2	Blackwater	30/09/2020	TL7204229582	1
92		Wethersfield Mill, Wethersfield	3	Blackwater	30/09/2020	Date collected: 31/12/19	1

	B06.3						
93	B06.4	Wethersfield Mill, Wethersfield	4	Blackwater	30/09/2020		1
94	B06.5	Wethersfield Mill, Wethersfield	5	Blackwater	30/09/2020		1
95	B13.1	Maldon A414	1	Blackwater	30/09/2020		1
96	B13.2	Maldon A414	2	Blackwater	30/09/2020		1
97	B13.3	Maldon A414	3	Blackwater	30/09/2020	TL8505107762	1
98	B13.4	Maldon A414	4	Blackwater	30/09/2020		1
99	B13.5	Maldon A414	5	Blackwater	30/09/2020	Date collected: 11/12/19	1
100	**EB6**	**-**	**-**	-	01/10/2020		2
101	B12.1	Wickham Mill Bridge	1	Blackwater	01/10/2020		2
102	B12.2	Wickham Mill Bridge	2	Blackwater	01/10/2020	TL8225311650	2
103	B12.3	Wickham Mill Bridge	3	Blackwater	01/10/2020		2
104	B12.4	Wickham Mill Bridge	4	Blackwater	01/10/2020		2
105	B12.5	Wickham Mill Bridge	5	Blackwater	01/10/2020	Date collected: 11/12/19	2
106	B08.1	Straits Mill Fishery	1	Blackwater	01/10/2020		2
107	B08.2	Straits Mill Fishery	2	Blackwater	01/10/2020		2
108	B08.3	Straits Mill Fishery	3	Blackwater	01/10/2020	TL7651023989	2
109	B08.4	Straits Mill Fishery	4	Blackwater	01/10/2020		2
110	B08.5	Straits Mill Fishery	5	Blackwater	01/10/2020	Date collected: 11/12/19	2
111	**EB7**	**-**	**-**	-	05/10/2020		2
112	B10.1	Cogshell Hall Bridge	1	Blackwater	05/10/2020		2
113	B10.2	Cogshell Hall Bridge	2	Blackwater	05/10/2020	TL8494022362	2
114	B10.3	Cogshell Hall Bridge	3	Blackwater	05/10/2020		2
115	B10.4	Cogshell Hall Bridge	4	Blackwater	05/10/2020	River level high, following	2
116	B10.5	Cogshell Hall Bridge	5	Blackwater	05/10/2020	continual rain	2
117	B07.1	Road Bridge, Codham Hall	1	Blackwater	05/10/2020		2
118	B07.2	Road Bridge, Codham Hall	2	Blackwater	05/10/2020		2
119	B07.3	Road Bridge, Codham Hall	3	Blackwater	05/10/2020	TL7346028118	2
120	B07.4	Road Bridge, Codham Hall	4	Blackwater	05/10/2020		2
121	B07.5	Road Bridge, Codham Hall	5	Blackwater	05/10/2020	Date collected: 31/12/19	2
122	B11.1	Blue Mills Road Bridge	1	Blackwater	05/10/2020		2
123	B11.2	Blue Mills Road Bridge	2	Blackwater	05/10/2020		2
124	B11.3	Blue Mills Road Bridge	3	Blackwater	05/10/2020		2

125	B11.4	Blue Mills Road Bridge	4	Blackwater	05/10/2020		2
126	B11.5	Blue Mills Road Bridge	5	Blackwater	05/10/2020		2
127	B03.1	Finchingfield Village	1	Blackwater	05/10/2020		2
128	B03.2	Finchingfield Village	2	Blackwater	05/10/2020		2
129	B03.3	Finchingfield Village	3	Blackwater	05/10/2020	TL6845632815	2
130	B03.4	Finchingfield Village	4	Blackwater	05/10/2020	Date collected:	2
131	B03.5	Finchingfield Village	5	Blackwater	05/10/2020	10/12/19	2
132	**EB8**	**-**	**-**	-	06/10/2020		1
133	B02.1	Spains Hall Footpath	1	Blackwater	06/10/2020		1
134	B02.2	Spains Hall Footpath	2	Blackwater	06/10/2020		1
135	B02.3	Spains Hall Footpath	3	Blackwater	06/10/2020		1
136	B02.4	Spains Hall Footpath	4	Blackwater	06/10/2020		1
137	B02.5	Spains Hall Footpath	5	Blackwater	06/10/2020		1
138	B01.1	Spains Hall Beaver Enclosure	1	Blackwater	06/10/2020		1
139	B01.2	Spains Hall Beaver Enclosure	2	Blackwater	06/10/2020		1
140	B01.3	Spains Hall Beaver Enclosure	3	Blackwater	06/10/2020		1
141	B01.4	Spains Hall Beaver Enclosure	4	Blackwater	06/10/2020		2
142	B01.5	Spains Hall Beaver Enclosure	5	Blackwater	06/10/2020		2

Table S3. Species list displaying which site on the river Blackwater and Colne they were detected in with total number of reads per site and total number of species per site. B01-B03 were separated due to the beaver experiment. B05-B13 and C01-C15 are used for the overall analysis.

Common name	B01	B02	B03	Total	B05	B06	B07	B08	B09	B10	B11	B12	B13	Total	B01-B13
American mink	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bank vole	1	12569	97196	3	0	514	8299	0	0	0	5362	49	0	4	7
Brown rat	8129	973	17163	3	0	63	142	0	12966	0	68812	97	9385	6	9
Common shrew	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Eastern grey squirrel	1974	0	0	1	0	115	53	0	6416	0	2	12	0	5	6
Eurasian beaver	91536	0	7	2	0	0	0	0	0	0	0	3	2	2	4
Eurasian otter	0	0	231	1	0	0	0	0	0	0	0	0	0	0	1
Eurasian pygmy shrew	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Eurasian water shrew	7342	19479	29143	3	0	197	0	0	0	0	5763	7	0	3	6
European badger	0	0	0	0	0	0	0	0	0	0	5	9	0	2	2
European hare	0	0	0	0	0	0	0	0	0	7	0	0	0	1	1
European mole	0	0	0	0	0	0	0	0	0	0	3	7	0	2	2
European polecat	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
European rabbit	0	0	0	0	0	0	24	0	15479	0	25223	16	9	5	5
European water vole	4	0	17388	2	0	0	0	0	0	0	5469	27	0	2	4
Fallow deer	0	0	9114	1	0	0	0	0	0	0	0	0	0	0	1
Field vole	616	38	5317	3	0	24	5	0	0	0	18367	42	2	5	8
Least weasel	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Red deer	0	0	0	0	0	0	0	0	0	0	0	0	2	1	1
Red fox	0	0	0	0	0	8268	41	0	23975	0	3729	854	45	6	6
Red squirrel	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Reeves's muntjac	574	0	13344	2	0	0	161	13	0	6	4448	12	0	5	7
Roe deer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stoat	0	0	0	0	0	0	0	0	0	0	0	4	0	1	1
Wood mouse	5	0	0	1	0	0	56	0	0	0	11935	12	0	3	4
Yellow-necked mouse	0	0	5386	1	0	0	0	0	0	0	0	12	0	1	2
No. species per site	**9**	**4**	**10**	**12**	**0**	**6**	**8**	**1**	**4**	**2**	**12**	**15**	**6**	**17**	**19**

Common name	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10
American mink	0	0	0	0	0	0	0	0	0	0
Bank vole	6182	0	0	7468	0	3443	386	0	0	2412
Brown rat	7281	1511	2763	72497	14155	13367	36798	0	18236	17571
Common shrew	0	0	0	0	0	0	0	0	0	0
Eastern grey squirrel	0	527	0	0	0	1935	595	0	0	111
Eurasian beaver	0	2	0	0	0	0	0	0	7	0
Eurasian otter	0	0	0	0	0	0	0	0	0	0
Eurasian pygmy shrew	0	0	0	0	0	5139	0	0	0	0
Eurasian water shrew	3	7957	7329	6	0	6	3	0	0	6251
European badger	0	0	3	844	0	0	0	0	3	3
European hare	0	0	0	7	0	0	0	0	0	0
European mole	0	0	0	4285	0	0	0	0	0	0
European polecat	0	0	0	0	0	0	0	0	0	0
European rabbit	12475	6464	773	0	0	65817	126	0	294	7
European water vole	2	0	1258	47255	2278	0	0	5	0	67
Fallow deer	0	0	0	2595	0	0	0	0	0	22
Field vole	4	2	39	96164	0	7231	16481	0	7	142
Least weasel	0	0	0	0	0	0	0	0	0	0
Red deer	0	0	0	0	0	0	0	0	0	0
Red fox	0	0	0	6	0	596	12292	0	0	12
Red squirrel	0	0	0	0	0	0	0	0	0	0
Reeves's muntjac	0	0	0	254953	0	0	0	0	0	42944
Roe deer	0	0	0	0	23	0	0	0	2	0
Stoat	0	0	6	1917	0	0	0	0	4	14
Wood mouse	0	0	0	2667	0	0	0	4119	0	27
Yellow-necked mouse	0	0	0	0	0	0	0	0	0	7
No. species per site	**6**	**6**	**7**	**13**	**3**	**8**	**7**	**2**	**7**	**14**

Common name	C11	C12	C13	C14	C15	Total
American mink	0	0	0	0	0	**0**
Bank vole	15583	6677	7229	1129	5399	**10**
Brown rat	51157	79891	3323	1117	5292	**14**
Common shrew	0	0	0	0	0	**0**
Eastern grey squirrel	4	3238	632	64789	0	**8**
Eurasian beaver	0	0	0	9	0	**3**
Eurasian otter	0	0	0	0	0	**0**
Eurasian pygmy shrew	0	0	0	0	0	**1**
Eurasian water shrew	3	11896	2	0	4	**11**
European badger	0	6	19481	0	0	**6**
European hare	0	429	6	0	0	**3**
European mole	0	0	0	0	0	**1**
European polecat	0	0	0	0	0	**0**
European rabbit	13	46	6532	21155	0	**11**
European water vole	0	0	588	3416	0	**8**
Fallow deer	7	0	0	0	0	**3**
Field vole	11737	1379	9	5565	0	**12**
Least weasle	0	0	0	0	0	**0**
Red deer	0	0	0	5	0	**1**
Red fox	2	34	33431	56694	0	**8**
Red squirrel	0	0	0	0	0	**0**
Reeves's muntjac	27254	16925	57277	0	26928	**6**
Roe deer	0	0	0	0	0	**2**
Stoat	0	0	0	0	0	**4**
Wood mouse	12415	157	14662	0	0	**6**
Yellow-necked mouse	0	4	0	0	16584	**3**
No. of species per site	**10**	**12**	**12**	**9**	**5**	**20**