Analysis of the Population Genetics and Polybrominated Diphenyl Ether (PBDE) Burdens of Otters in England and Wales: With Case Studies of Populations in South West England

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

…………………….

Abstract

Otter populations declined drastically across many areas of England and Wales during the 1960s to 1980s. The main cause of this decline is thought to have been high concentrations of organic pollutants, in particular PCBs and dieldrin. Here we look at the health of the present day otter population, focussing on the numbers of otters, the genetic diversity of populations and investigating a possible new organic pollutant threat, polybrominated diphenyl ethers (PBDEs).

A non-invasive spraint genotyping study of the otter population inhabiting the River Camel in Cornwall not only revealed that the river was capable of supporting a minimum number of 12 otters over a 9 month period, but gave insight into the ranges and genetic relationships of the individuals using the river system. A further population genetic study was carried out focussing on the River Itchen in Hampshire, a population which declined drastically to just a few isolated individuals before receiving otters through a captive breeding programme. Microsatellite genotyping of tissue samples showed the River Itchen population to be relatively diverse, indicating a successful population recovery, and haplotype analysis reveals that captive bred otters have successfully bred within the River Itchen population. However, haplotype analysis also indicates that the otters used to found the captive breeding programme were unlikely to have originated from a native British population.

Concentrations of PBDEs in otters rival the high concentrations observed in many marine mammal species and are approaching the concentrations of PCBs and DDTs already observed in otters. The profile of the PBDE congeners found shows that lower congeners show relative concentrations similar to those observed in many other species of biota, with high BDE-47 dominating the profile and BDE-99 and -100 also found at significant concentrations. Otters also contain relatively high concentrations of the congeners BDE-153 and BDE-209, a trend generally typical of terrestrial top predators.

In summary, the otter populations studied appear to be recovering well. However, increasing concentrations of PBDEs may cause problems for otter populations in the future.

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1. Introduction

1.1 Background

This is a study of otter population genetics and ecotoxicology, focusing in at different levels on otter populations in locations across England and Wales. The study addresses the various issues and problems faced by otters in the recent past, in the present and those they may face in the future.

1.1.1 The Eurasian Otter (*Lutra lutra*)

1.1.1i) Natural History and Distribution

The Eurasian otter (*Lutra lutra*) is one of Britain's most enigmatic, shy and elusive animals. As a semi-aquatic mammal, this beautiful and versatile animal is at home in terrestrial, freshwater and coastal semi-marine systems. The otters semi-nocturnal and crepuscular habits mean that it is rarely seen by humans, but signs of otter activity, such as spraint and pad marks can be easily spotted by anyone trained to know where and what to look for. The shy but playful nature of the otter has meant that over the last few decades these enigmatic creatures have moved from being seen as an animal of vermin to one whose place is properly recognised by the British public as an essential component of healthy freshwater ecosystems.

An otter's diet is generally rather varied and an otter will travel to different areas of a water system depending upon the food availability at the time. The mainstay of the otter's diet is fish, with otters feeding on a variety of species depending on availability (Simpson, 1998; Clavero *et al.*, 2003; Sales-Luís *et al.*, 2007). In areas where they are abundant, amphibians can make up a significant proportion of an otters diet (Taastrøm & Jacobsen, 1999; Jędrzejewska *et al.*, 2001) and otters will occasionally supplement their diet with small mammals, birds, crayfish and freshwater invertebrates (Taastrøm & Jacobsen, 1999; Jędrzejewska *et al.*, 2001). In coastal areas otters are also known to feed on marine life such as crab and sea urchins (Heggberget & Moseid, 1994). In short, it appears that fish are an easy favourite, but otters will eat anything that is available and can be opportunistic when required (Clavero *et al.*, 2003).

Being rather shy creatures, particularly across mainland England where encounters with humans are common and where in the past the persecution of otters was rife, otters require a certain amount of cover. Otters will spend much of the day sleeping and for this, being an air breathing mammal they must be out of the water. Often the otters will use a holt - a den-like structure, usually dug into the side of the river bank, with one or more tunnels leading in. An otter is likely to have several holts spread throughout its territory (Erlinge, 1967) and in particular mothers with young cubs choose to use them for their safety and security (Ruiz-Olmo *et al.*, 2005). In addition to holts, otters, particularly transient animals, commonly use couches (Erlinge, 1967). These shallow scrapes in the ground are often situated in areas of dense undergrowth, providing a degree of shelter and protection from predators.

As a species, otters do not have a set breeding season and in many areas otters will give birth at any time of the year (Hauer *et al.*, 2002b). Exceptions occur in locations where food availability becomes seasonal (Erlinge, 1967; Hyvärinen *et al.*, 2003). For example, in Shetland, where the fish that otters prey on are scarce in winter and early spring, the majority of females will give birth during the summer months to take advantage of the higher abundance of food (Kruuk & Conroy, 1991).

It is possible to calculate the age of a wild otter at death by counting the rings of dentine in the roots of their incisors and canines (Hauer *et al.*, 2002; Hyvärinen *et al.*, 2003). This method has been used in several studies to estimate the average life expectancy of wild otters to be somewhere approximately 4 years (Kruuk & Conroy, 1996). An accurate life expectancy is extremely difficult to calculate because of the difficulty in accurately estimating the number of otters that die in their first year and do not get reported. It is likely that a large number of otter cubs die whilst still in the holt, and it is suspected that many more drown whilst learning to swim and are swept out to sea before their bodies are discovered (Kruuk & Conroy, 1991).

Otters are just as at home on land as they are in water and will often travel cross-country to exploit neighbouring river systems and isolated ponds and lakes. This is particularly important for coastal otters having to travel inland on a regular basis in order to find a source of freshwater. As with many mammal species, otters exhibit male-biased dispersal (Clutton-Brock, 1989; Radespiel *et al.*, 2003). The young of both sex will leave their mother at about one year of age (Erlinge, 1967) and will become transient

animals, moving between territories until they find an area in which there is space and resource to allow for them to settle. Both males and females are territorial (Kruuk, 1992), although home ranges will often overlap, and in particular the home ranges of males, generally being larger in size, will often overlap with the home ranges of several females (Erlinge, 1967). Within an otters territory there is usually a core area containing the major resources of food and shelter and this area an otter will defend more vehemently (Erlinge, 1967; Ruiz-Olmo *et al.*, 2005).

When searching for a territory it is thought that males tend to move greater distances than females. In mammals in general greater dispersal in males is thought to have evolved as a mechanism for inbreeding avoidance (Perrin & Mazalov, 1999; Loew, 1999; Blundell *et al.*, 2002) with males generally moving further away from, and therefore being less likely to mate with female relatives (Clutton-Brock, 1989; Radespiel *et al.*, 2003). As resident males often occupy large territories, spanning those of several females (Erlinge, 1967), it is not uncommon for young males to remain transient for considerable amounts of time before finding an area to settle (Erlinge, 1967). Where space is at a premium fighting amongst otters is not uncommon and in extreme cases can lead to castration, in the case of the males, or death of the individuals involved (Simpson, 2007).

Worldwide there are twelve recognised species of otter, ranging from the 1.4m - 1.9m long (nose to tail) giant otters (*Pteronura brasiliensis*) of South America, to the 65cm - 100cm long Asian short-clawed otter (*Aonyx cinerea*) (Macdonald, 1984), the Eurasian otter (*Lutra lutra*) measuring 95cm – 120cm long , with females slightly smaller than the males (Figure 1.1). Otters also exhibit a wide range of life styles and behaviours from the sea otters (*Enhydra lutris*) that even go so far as to sleep floating in the sea, to the freshwater behaviour of the North American river otters (*Lontra canadensis*) (Macdonald, 1984). The range of the Eurasian otters expands from the western most tip of the Iberian peninsula, across mainland Europe and across the majority of the Asian continent south of the tundra line to Japan and the Far East (Hung *et al.*, 2004). The Eurasian otter is also found in some areas along the Mediterranean coast of North Africa (Macdonald, 1984).

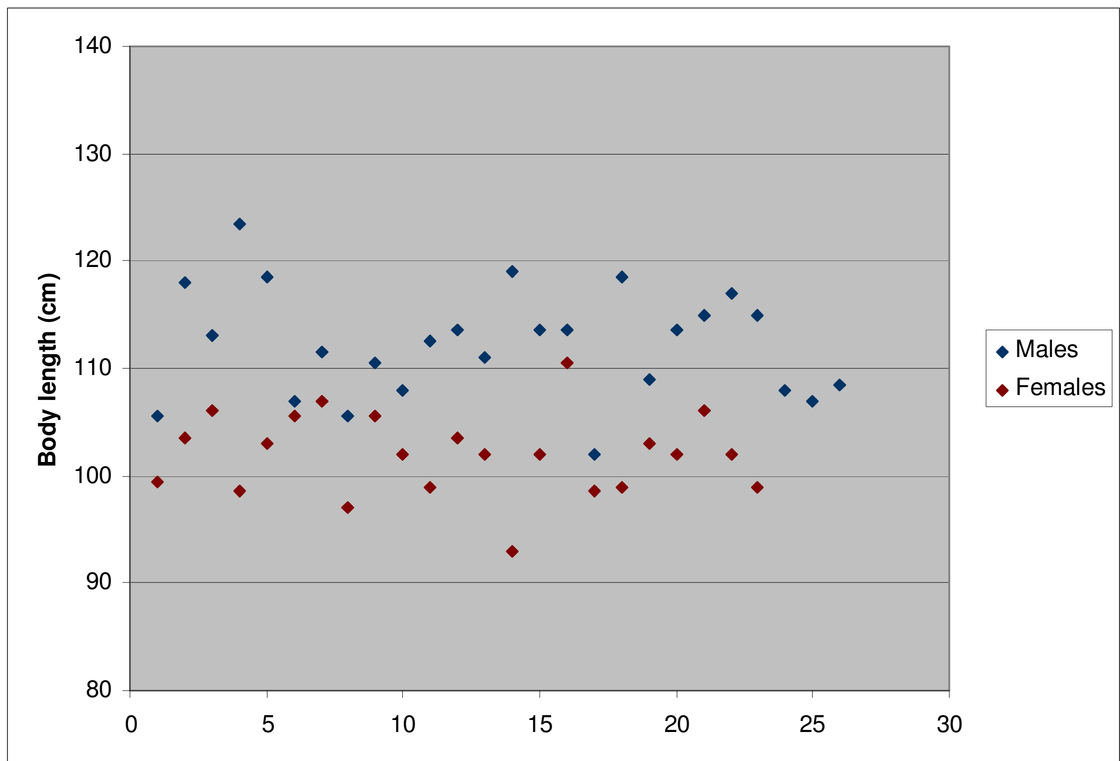

Figure 1.1 Size distribution of adult Eurasian otters. Data obtained from the UK Environment Agency Otter Project (Simpson, 2007; Chadwick, 2007).

1.1.2 The Declines of the 1950s and 60s

1.1.2i) Otter Declines

Steady declines have been occurring in otter populations across Europe since the end of the 19th century. In Britain this general decline was observed until the mid 1950s when an even more severe decline in otter numbers occurred (Chanin & Jefferies, 1978). Hunting records show that the decline was not uniform with hunting success rates in the south and east as well as midland locations dropping by approximately 50%, whilst declines in the north were less drastic (Chanin & Jefferies, 1978). In northern Wales there was no observed decline in hunting success, whilst in southern Wales drastic declines were observed in the number of otters found (Chanin & Jefferies, 1978).

Hunting records place the start of the declines at around 1957 to 1958 and indicate that the severe decline tails off around 1964 (Chanin & Jefferies, 1978), however, no significant recovery of the populations is observed for a couple of decades. In the late 1970s only an estimated 6% of sites across England deemed as suitable for otter habitation showed any sign of otter activity, with the South West and Welsh border Wye valley areas showing the highest estimates of activity at 23.5% and 17.1% of habitat occupied, respectively (Lenton *et al.*, 1980; Crawford, 2003). Scottish and Welsh otter populations on the whole fared better than English populations, however, populations in the more industrial regions of the Scottish Lowlands saw significant declines in numbers (Dallas *et al.*, 1999). In some areas of England, such as the Trent valley and the Thames valley areas, otter signs had become non-existent by the late 1970s, suggesting that otters had become extinct in these areas by this time (Lenton *et al.*, 1980; Crawford, 2003

In many areas across Europe otters did not fare much better with populations in many countries declining in the period between the 1950s and 1980s (Roos *et al.*, 2001). Otters became absent from many areas of central Europe, with otter populations disappearing from Central and Eastern France, Central and Western Germany (Effenberger & Suchentrunk, 1999) and across most of Denmark (Madsen *et al.*, 1999; Pertoldi *et al.*, 2001). In Italy otters became restricted to a small region in the south of the country (Ketmaier & Bernardini, 2005; Prigioni *et al.*, 2007). Otters remained in Western France and across much of the Iberian Peninsula, but their populations, even today, are often fragmented and otters are absent from many areas with high human

habitation (Barbosa *et al.*, 2001; Barbosa *et al.*, 2003; Pérez-Haro *et al.*, 2005). In Eastern Europe, from the eastern fringes of Germany down to Slovenia and eastwards towards Asia, otters remain fairly common. In contrast, in Scandinavia, in particular Sweden and Denmark, otter numbers declined drastically and populations have become sparse and fragmented (Mucci *et al.*, 1999; Arrendal *et al.*, 2004). In general, otter populations in Central Europe suffered the most, but in many areas across the continent otter populations became reduced and fragmented.

The cause of the otter declines across Britain and Europe is unclear, but it is generally accepted that the effect of several negative factors combined, causing the huge decline in numbers observed. Habitat destruction, persecution, pollution and a dip in prey availability are all likely to have played a role in the demise of the otters. The question is how much of a role did each of these factors play and are they likely to cause future problems for the now recovering populations?

1.1.2ii) Persecution

The first records of otters being hunted date back to the 13th century when otters were killed to protect stocks of fish kept in pools on the estates of the wealthy as a form of live food storage (Chanin, 1985). Hunting otters later became popular as a sport, generally carried out with a pair of specially bred otter hounds trained to find and track otters. Hunting certainly had an impact upon otter populations, particularly when firearms came into common use in the late 18th century. It is likely that local reductions or extinctions occurred in areas where hunting activity was greatest, whereas in other areas otter numbers would have remained relatively healthy. When hunting pressures reduced then otters from the less affected areas would move in to re-establish a population. Interestingly, it was the huntsmen themselves who first flagged up the problem of declining otter numbers as it became increasingly difficult for them to find their quarry. In the 1960s, when huntsmen began to recognise that otter populations were struggling, it became common practice for hunts to find their quarry but not to kill (Chanin & Jefferies, 1978), effectively allowing a continuation of the sport without exasperating the decline in otter numbers. As otters became scarcer and finding their quarry became more difficult, many hunts disbanded and by the time a ban on killing otters came into place in 1978 few hunts were still active.

Despite a move over the last century or two to viewing the otter as an enchanting marvel of the British countryside, and even after the decline in numbers and a ban on killing otters, to this day a substantial number of animals are still killed due to human activity. For example, in fish farming it is common practice to hold large numbers of fish in stock ponds. The huge concentrations of fish with relatively little cover obviously appear very easy prey for the otter and once an otter discovers such a pool it will return again and again. It is understandable that fish farmers get annoyed at the loss of stock (Adámek *et al.*, 2003; Lanszki *et al.*, 2007), which can total thousands of pounds particularly in the case of prize fish, such as coy carp. Recent efforts to mitigate the situation by introducing methods such as otter proof fencing, or by planting stocks of natural prey species to deter otters from the fish in stock pools, appear to be appeasing the situation (Lanszki *et al.*, 2007; Graham Roberts, pers. comm.); however, isolated incidents of otter persecution do still occur (Madsen *et al.*, 1999; Hauer *et al.*, 2002a; Simpson, 2007) and with otter populations on the rise again such incidents may become more common place in the future.

In the past, before the use of special otter guards became common place, it was common for otters to become stuck in flyke nets set to catch fish such as eel (Madsen *et al.*, 1999, Hauer *et al.*, 2002). Once in the nets the otters would not be capable of escaping and many otters drowned as a result.

The persecution and killing of otters by humans is unlikely to have been the main cause of the decline in otters. Otters have been hunted for centuries with little effect, and no distinct increase in the numbers of otters killed was seen before or during the decline. It is more likely that the persecution worked in combination with other factors to aid the decline (Prigioni *et al.*, 2007).

1.1.2iii) Habitat Destruction and Disturbance

In many river systems the availability of different prey types varies throughout the year (Taastrøm & Jacobsen, 1999; Jędrzejewska *et al.*, 2001). In response to this otters will use the different areas of the river system, or of their territory within the river system, at different times of the year (Erlinge, 1967; Jędrzejewska *et al.*, 2001; Ruiz-Olmo *et al.*, 2005). A healthy and varied river system is therefore vital if it is to sustain a healthy population of otters. The ideal habitat would contain healthy resident populations of salmon, trout, eel and other freshwater fish, as well as areas rich in waterfowl, with still pools and marshy areas suitable for amphibians to breed in and areas of dense cover suitable for small mammals. In reality it is rare to find the perfect river system, but for a river to maintain a healthy resident population of otters it must have a suitable food source of some kind or another available all year round.

With greater health care and better basic standards of living human population numbers have risen sharply over the last few decades, resulting in an increase in the number of people inhabiting the British Isles. This in turn has led to greater demands on land and resources, and many waterways have been drastically altered as a result. Rivers have often been physically altered, with dams to create water storage or instillations for flood defence. In some cases this has produced the benefit of creating further water systems, with the creation of reservoirs or the building of canals (Davies et al., 2004); in many cases though the alterations have been detrimental. The banks of many rivers have been

stripped of vegetation and altered to make way for livestock grazing, industry or residential areas, reducing the amount of cover available for otters to hole up in during the day and making them feel more vulnerable in and out of the water (Sales-Luís, 2007).

Until recently it was very rare to see an otter in a built-up area, and it is only within the last few years that increased sightings of otters in cities have occurred (Durbin, 1998). Increased sightings would suggest that otters are utilising the waterways of our cities, but the lower quality of these waterways in comparison to an out of city river site – in terms of vulnerability and lack of areas to rest – would suggest that these stretches of river could be considered to be sub-optimal habitat. Otters have relatively large home ranges (Erlinge, 1967; Ruiz-Olmo *et al.*, 2005) and it is likely that the city component may form just part of an otter's territory. It may also be that as sub-optimal habitat these stretches of river are relatively safe resting grounds for transient animals trying to avoid conflict with established otters or may naturally form part of the territories of more subordinate individuals. As the numbers of otters grows the pressure to use these sub-optimal stretches of river will also grow.

Humans have drastically altered and destroyed much of the otters habitat, putting extra pressure on populations and disrupting the natural behaviour of individuals. The use of city waterways by otters suggests that they are able to adapt, however, it is not known how disturbance and habitat availability may affect the breeding behaviour of females requiring greater levels of cover and lower instances of disturbance in order to successfully raise young (Ruiz-Olmo *et al.*, 2005). It is unlikely that habitat destruction and human disturbance caused otter declines, but otters are known to be less populous in areas of high human habitation. It is likely that these detrimental changes to habitat to some degree aided the decline in otters. Now that otters are recovering, an awareness of otters needs in terms of habitat quality and lack of human disturbance is becoming ever more important if we are not to hinder the effective recovery of British otter populations.

1.1.2iv) Road Deaths

Research suggests that traffic is one of the biggest killers of the modern day otter, with road traffic accidents being responsible for a high percentage of otter deaths in countries across Europe (36.1%, Norway, Heggberg (1991); 45%, Denmark, Madsen *et al.* (1999); 69.9%, Eastern Germany, Hauer *et al.* (2002)). The percentage of otter carcasses collected from the roads ahs been increasing over the years (Hauer *et al.*, 2002), however it is not clear whether this is due to an increase in road deaths or an increase in public awareness leading to an increase in the numbers of road deaths reported (Philcox *et al.*,1999). The UK, with some of the highest levels of road traffic in Europe, also has some of the highest levels of traffic related otter mortality. In South West England alone 83.6% of recorded otter mortalities between the years of 1996 and 2003 were as a direct result of road traffic accidents (Simpson, 2007). Even taking into account the fact that otters dying as a result of road traffic accidents are more likely to be found and reported, this is still a staggering and significant proportion.

Part of the natural behaviour of otters involves sprainting and marking areas that are clearly defined landmarks, such as large boulders or fallen trees in or on the banks of the river (Erlinge, 1967). The large concrete structure of a bridge makes an ideal substitute for natural landmarks and otter spraint are often found on large boulders near to a bridge, or under the bridge itself. As the territories of otters often overlap, and transient individuals commonly cross territorial boundaries, it is thought that these bridges become a valuable message board, advertising who is in the area, along with information about age, sex, sexual status and health (Erlinge, 1967). An otter may spend some time checking the spraint of others, as well as leaving their own spraint behind. Whether trying to avoid a direct confrontation with a neighbour or just out of curiosity, unfortunately this activity often also appears to include crossing over a bridge as well as under it. It appears that otters are also more likely to cross over bridges when rivers are in spate, with higher numbers of otters involved in road traffic accidents during the days following heavy rainfall (Simpson, 1998; Philcox *et al.*, 1999). The shear numbers of otters involved in road traffic accidents indicates a need for mitigation in areas where otters frequently get knocked down (Madsen *et al.*, 1999; Philcox *et al.*, 1999). In some areas work has already been carried out to incorporate ledges and walkways to aid the otters passage though the obstacle, although there is still little

evidence as to the effectiveness of mitigation in preventing road deaths (Clarke *et al.*, 1998).

Of note is the fact that the number of males being killed on the roads is significantly higher than the number of females (56%, Philcox *et al.* (1999); 58.2%, Madsen *et al.*, (1999)). This is more than likely an artefact of the male-biased dispersal, whereby males are moving between water systems more frequently and so are crossing roads and putting themselves in danger on a more frequent basis (Buskirk & Lindstedt, 1989; Blundell *et al.*, 2002). As far as looking at the effects of road traffic accidents on the population density, male deaths are of lower consequence than female deaths as it is the females that breed and give birth to the next generation. However, a reduction in the male population could still lead to a reduction in the effective population size (N_e) and genetic diversity, which may, if severe, lead to a reduction in population density. However, the death of a transient male will only lead to a direct reduction in the population density if the population the male was travelling to has no resident males of breeding ability. Conversely the death of a female would also lead to the death of any dependant young in her care, as well as the loss of any future offspring she is capable of producing.

Whether the incidents occur at a bridge or as an otter is crossing the countryside from one water-way to another, it seems to be an unavoidable fact that road traffic accounts for a large proportion of otter deaths, and certainly for the largest proportion of recorded deaths (Madsen *et al.*, 1999; Hauer *et al.*, 2002). The question is was it a major factor in the sharp decline in numbers in the 1950s and 60s? The evidence would suggest not. Roads nowadays are even busier than they were back then, and despite the continued incidence of road deaths otter numbers in Britain are now on the rise (Crawford, 2003).

1.1.2v) Otters and Mink

The first American mink (*Mustela vison*) farms were established in Britain in the early 1930s and the business reached its peak at around 700 farms active in England alone during the early 1960s (Bonesi *et al.*, 2006). Unsurprisingly accidental and deliberate releases of mink became common. Despite this it is thought that it was not until the 1950s that feral mink were first sighted in the wild, and not until several years after this that they began to breed and proliferate. It is not surprising that the timings of mink proliferation and otter decline would make it appear that the expanding mink populations were driving otter populations into decline, however, records indicate that the otters decline preceded the spread of mink (Chanin & Jefferies, 1978). Thus it seems that in reality mink took advantage of the available habitat recently vacated by otters, but were unlikely to have had a direct influence on otter numbers.

Evidence suggests that where healthy otter and mink populations compete for space and resource it is often the mink that loose out (Bonesi *et al.*, 2006; McDonald *et al.*, 2007), posing questions over what will happen to wild mink populations as the otter populations expand. Evidence from areas with high otter densities, such as the Scottish population or the re-establishing English South West populations, suggest that mink are ousted to the sub-optimal areas of habitat, such as the outer reaches of river systems or into the surrounding terrestrial environments (Bonesi *et al.*, 2006). In areas where otter and mink are competing for resources, mink diet often contains higher concentrations of prey such as small mammals (Bueno, 1996; Jędrzejewska *et al.*, 2001), putting added pressure on the populations of more vulnerable mammals such as the water vole (*Arvicola terrestris*).

In their native habitat mink naturally survive alongside American river otters (*Lontra canadensis*), suggesting that mink and otter can co-exist. However, there is evidence to suggest that a more direct form of competition may occur between mink and the smaller Eurasian otter (Chanin *et al.*, 1981; Jędrzejewska *et al.*, 2001), and over the last couple of decades reported incidence of fighting between mink and otters have become more common, particularly in areas where both species are thriving and competition for resources in greater (Simpson, 2007). With high levels of competition, both direct and indirect, and an apparent resulting alteration of mink diet to include greater numbers of small mammals, it is not know what effect an increase in otter numbers will have upon

populations of small mammals in areas where mink are still present. An apparent exodus of mink to less favourable habitat in the more peripheral freshwater habitats and even terrestrial habitats means that otter surveys, traditionally used as a sideline means of assessing numbers of mink, may no longer cover the range of this expansion in mink habitat use. Estimations of present day mink populations, and ultimately their effects on small mammal populations, are therefore potentially being underestimated in areas where otter numbers are high.

1.1.2vi) Nutrient Imbalances and Fish Stocks

Humans are capable of having large influence upon the health of water systems. As well as habitat destruction, which directly impacts upon the otters ability to inhabit a habitat, anthropomorphic effects can have less direct, but still as destructive influences over the suitability of a habitat for habitation by otters. One such influence is to alter the abundance of food resources available to the otter. Human influence in shaping freshwater ecosystems themselves can influence the abundance of prey species. Physical changes to the ecosystem, such as the construction of weirs and dams, alterations to the banks of watercourses and the removal of structures such as shade giving trees can all influence the composition and abundance of fish species present in the water system.

Human activity can alter prey availability in less direct ways. Anthropomorphically caused imbalances in nutrient compositions, in particular nitrogen and phosphorus can be a major threat to the ecological balance of freshwater ecosystems. Sources of excesses of nitrogen and phosphorus include the leaching and run off of excess fertilizer from agricultural land (Howarth *et al.*, 1996; Ayoub, 1999; Keeney, 2002), residential and industrial waste water disposal (Valiela *et al.*, 1997; Smith *et al.*, 1999), and organic waste from livestock (Garg & Bhatnagar, 1999; Smith *et al.*, 1999).

This increase in nutrient availability in the system is known as eutrophication (Ayoub, 1999). The increase in nutrient availability frequently results in an initial increase in numbers of primary and secondary producers, such as phytoplankton and zooplankton (Breitburg, 2002; Camargo & Alonso, 2006), which in turn produces favourable feeding conditions for species further up the food chain, such as fish species (Garg &

Bhatnagar, 1999). However, a less favourable result is the proliferation of one or two dominant species, as happens during algal bloom events (Vitousek *et al.*, 1997; Ayoub, 1999; Anderson *et al.*, 2002). Nitrates and nitrites can, at sufficient concentrations, be toxic to several species of freshwater invertebrates, fishes and amphibians (Camargo & Alonso, 2006). In closed water systems, such as ponds and lakes, areas of low water flow in rivers and streams and in coastal habitats, resulting biochemical processes can also lead to a decrease in levels of dissolved oxygen within the water system (Garg & Bhatnagar, 1999), leading to further disruptions and alterations in the biodiversity and ecology of the system (Breitburg, 2002; Rabalais *et al.*, 2002). Ultimately the imbalance in nutrient structure usually leads to an imbalance in the natural ecology of the ecosystem (Jefferies & Maron, 1997).

The affects of nitrogen and phosphorus excesses varies widely and depends on the overall concentrations added to the system (Garg & Bhatnagar, 1999) as well as the biochemical, biological and physical nature of the receiving system (Jefferies & Maron, 1997; Keeney, 2002). This variability means that, although it is possible that fluctuations in prey availability (both increases and decreases) may have occurred in isolated areas of otter habitat, it is unlikely that the large, nation wide decline in otter numbers was purely as a result of nutrient release into the freshwater environment (Crawford, 2003). However, alterations in prey abundance resulting nutrient enrichment, as well as physical alterations of water systems may have added further stress to otter survival.

1.1.2vii) Heavy Metals

Otters in Shetland have a naturally high dietary intake of mercury as many of the fish in the area contain high concentrations. As would be expected, concentrations of mercury in the livers of otters in Shetland increased with age (Kruuk and Conroy 1991). Despite the high intake, relatively few otters reached an age at which mercury levels would be considered toxic. Exceptions to this include two cases in which otters with high mercury concentrations were observed shortly before death exhibiting symptoms, such as lack a of coordination of movement, that would be consistent with mercury poisoning (Wren 1986, cited by Kruuk and Conroy 1991; Mierle *et al.*, 2000).

The high mercury intake of otters in Shetland is an extreme case and otters in most areas of the British Isles and Europe are not exposed to such high levels of metal intake (Mierle *et al.*, 2000; Fortin *et al.*, 2001; Hyvärinen *et al.*, 2003). Otters are often found to contain a range of different heavy metals at detectable concentrations (Mason & Stephenson 2001; Hyvärinen *et al.*, 2003), however there is evidence to suggest that these are obtained from natural sources rather than anthropomorphically (Mason & Stephenson 2001).

1.1.3 Otter Declines and Chlorinated Hydrocarbons

1.1.3i) DDTs

DDT came into common use around the time of the Second World War and has been used world wide, both as a method of controlling insect vectors of disease and as an agricultural insecticide (Walker, 2001). Despite bans on use coming in to force in the 1960s and 70s, due to the compounds persistent nature DDT, and in particular one of its major metabolites *p,p′*-DDE are still present in many environments today and are often found in biota at relatively high concentrations (Walker, 2001; Chadwick, 2007).

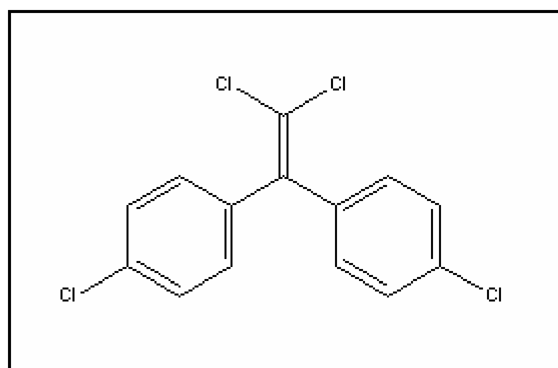

Figure 1.2 Chemical structure of pp'-DDE.

DDT and its metabolites can be acutely toxic to birds (Jefferies & Walker, 1966; Mayne *et al.*, 2005), but there is little evidence to suggest that DDT is toxic to mammals (Jensen *et al.*, 1977).

In the Swedish otter population concentrations of DDT and its metabolites decreased significantly over time between the beginning of the decline in otter numbers in Sweden in 1970 and 1994 (Roos, 2001). More recently concentrations of *p,p′*-DDE have been shown to be decreasing significantly over time (1992 – 2003) in otters from England and Wales ($F_{1,243} = 20.85$, $p < 0.001$; Simpson, 2007). Despite this decline, concentrations of *p,p′*-DDE are still found at relatively high concentrations in otter liver tissue (mean; males 317.6 $ng.g^{-1}$ wet wt (n = 189), females 290.0 $ng.g^{-1}$ wet wt (n = 121); calculated from data provided in Chadwick, 2007).

1.1.3ii) Dieldrin

In 1956 a new group of insecticides, known as cyclodiene chlorinated hydrocarbons, were brought into use, the two most prolifically used being dieldrin and aldrin (Walker, 2001). The active ingredient of dieldrin, also the primary metabolite of aldrin, is HEOD.

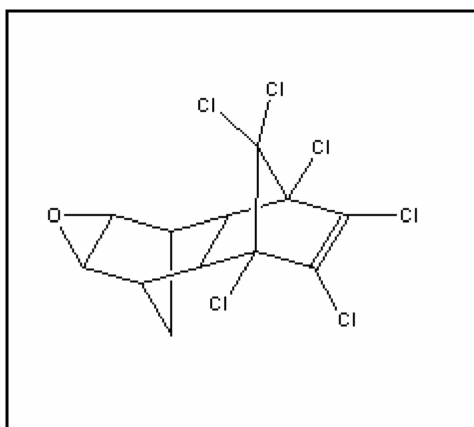

Figure 1.3 Chemical structure of dieldrin ($C_{12}H_8Cl_6O$).

Almost immediately after dieldrin and aldrin were first used a profound effect was seen in local wildlife. The insecticides were not only toxic to invertebrates, but were found to also be highly toxic to mammals and birds (Walker, 2001).

Hunting records indicate that severe declines in English and Welsh otter populations occurred between the periods of 1950-55 and 1966-71 (Chanin & Jefferies, 1978). The records suggest that the most severely affected areas were in the south, east and midlands and that in the majority of areas the decline began around 1957 to 1958, with variations ranging from 1956 to 1960 (Chanin & Jefferies, 1978). This would suggest that the declines occurred in the years immediately following the beginning of widespread dieldrin application in 1956, implicating the use of dieldrin as the primary cause of the declines. In the south and east of England dieldrin was widely used as seed dressing (Walker, 2001), where as in the north west of England and north Wales, where otters declined to a lesser extent, dieldrin was more commonly used in sheep dips, suggesting that the slow leaching of dieldrin from crops into water supplies had a greater effect than the more isolated but more concentrated pollution events caused by the releases after sheep dipping (Chanin & Jefferies, 1978).

In a study of Scottish otters, carried out by Kruuk & Conroy (1996) otters collected between 1987 and 1992 contained relatively low concentrations of dieldrin (mean 0.08ppm, maximum 0.28ppm) when compared to those found previously in British otters (mean 0.5ppm, maximum 13.9ppm) (original data from Jefferies et al., 1974). Whether this reflects the difference over time or a difference in geographical location is unclear.

Studies of otters collected from the south west of England show a significant decline in dieldrin concentrations between 1996 and 2003 ($F_{1,243} = 17.3$, $p < 0.001$; Simpson, 2007) and in the previous period of 1988 to 1996 this decline with time was shown to be highly significant ($r = -0.170 \pm 0.036$, $p \leq 0.001$, $n = 56$; Simpson, 1998). In a sister study of otters covering the rest of England and Wales, no significant decline in levels of dieldrin were observed during the study period 1992 to 2003 (Chadwick, 2007). However, otters in this study exhibited significantly lower levels of dieldrin (mean; males 171.1 $ng.g^{-1}$ wet wt ($n = 189$), females 130.7 $ng.g^{-1}$ wet wt ($n = 121$); calculated from data provided in Chadwick, 2007) than those presented in previous studies carried out in earlier decades (original data from Jefferis & Hanson, 2000).

Although the circumstantial evidence suggests that declines in otters occurred as a result of dieldrin and aldrin there is little direct evidence to substantiate this. Dieldrin is known to be capable of reducing vitamin A levels in the body. Simpson (2000) showed that in otters in South West England there was a significant negative correlation between levels of vitamin A and dieldrin concentrations in the liver. Dieldrin concentrations showed a significant decrease over the study period of 1988-1996 (see above), and vitamin A levels showed a significant increase over time ($r = 0.28 \pm 0.064$, $p < 0.001$, $n = 44$; Simpson, 1998). However, when time was added into the equation the relationship between vitamin A and dieldrin was no longer significant, suggesting that there are likely to be other factors involved in the increasing vitamin A levels observed (Simpson, 2000).

1.1.3iii) PCBs

Polychlorinated biphenyls (PCBs) are among the suite of compounds implicated as factors in the decline of the otters. The basic structure of PCBs is made up of two hydrocarbon rings joined by a carbon bond (Figure 1.4). The number and location of chlorine atoms around the carbon rings dictates the congener. In total there are 209 different combinations, or 209 different congeners, however some are much easier to manufacture than others due to the nature of varying strengths of electron repulsion based on the positions of various atoms around the molecule.

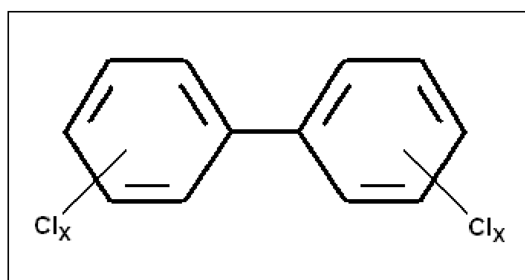

Figure 1.4 The basic structure of PCBs.

PCBs came into common use in the 1930s and were used in many materials including cooling and insulating fluids for transformers and capacitors, hydrolic fluids, coatings for copy paper, sealants and synthetic resins, rubbers, paints, asphalt and lubricating oils (Walker, 2001). It was not until the 1960s that the extent of PCBs effect on wildlife became apparent as more and more materials containing PCBs were incinerated, releasing their PCB content into the environment. In the 1970s and 80s the use of PCBs was phased out as successive bans on using PCBs in specific materials were introduced.

Being a top freshwater predator otters have been shown to build up high concentrations of PCBs. Congeners CB-153, -138 and -180 are often found at high concentrations in otters, with congeners CB-170, -118, -128, -156, and -105 also found at significant concentrations (Kruuk and Conroy, 1996; Simpson 2007; and see Table 1.1, Chadwick, 2007). PCB concentrations have been measured in otter populations from a range of countries around Europe. In Scotland average ∑PCB concentrations range from 0.797 $\mu g.g^{-1}$ (SE 0.712-0.892) wet weight, 27.4 $\mu g.g^{-1}$ (SE 24.29-30.92) lipid weight in a sample of 116 otters collected between 1987 and 1992 (Kruuk and Conroy, 1996). In a studies of otters from France concentrations range from 1.24 to 145.31 $\mu g.g^{-1}$ dw (means of 33.94 $\mu g.g^{-1}$ dw (n = 22) and 26.19 $\mu g.g^{-1}$ dw (n = 32))(Ruiz-Olmo *et*

al., 2002), and a range of 0.6 to 970 µg.g-1 lipid wt was detected in otters sampled from Sweden, 1970–1994 (Roos et al, 2001). In Spain some of the highest and the lowest concentrations were detected, as concentrations ranged from below the detection limit to 1005.59 $\mu g.g^{-1}$ dw (means of 2.44 $\mu g.g^{-1}$ dw (n = 5), 6.15 $\mu g.g^{-1}$ dw (n = 10) and 78.30 $\mu g.g^{-1}$ dw (n = 41))(Ruiz-Olmo *et al.*, 2002).

Table 1.1 Median PCB congener concentrations in the liver tissue of 178 otters sampled from England and Wales (Chadwick, 2007).

PCB congener	Median concentration ($ng.g^{-1}$ wet weight)
PCB-153	72
PCB-138	52
PCB-187	52
PCB-180	45
PCB-170	22
PCB-118	18
PCB-128	9
PCB-156	7
PCB-105	6

PCBs have been cited as a possible cause of decline in several otter populations (Ruiz Olmo *et al.*, 2002; Barbosa *et al.*, 2003). In Sweden for example, the spatial variations in PCB concentrations in otter tissue mirror the spatial variations in otter declines (Roos *et al.*, 2001). In Scotland Kruuk and Conroy (1996) observed some of the highest PCB concentrations in samples collected from close to the English border, an area of Scotland known to have suffered a significant drop in otter numbers during the 1960s-80s (Dallas *et al.*, 1999). However, the amount of PCB contamination is likely to be a measure of human activity in an area (Barbosa *et al.*, 2001; Roos *et al.*, 2001) and may in turn represent areas of heavy use of other environmental contaminants as well as areas of heavy habitat disruption and human disturbance (Barbosa *et al.*, 2003). This situation highlights the fact that care must be taken when drawing conclusions about the effects that one particular factor may have had upon the otters decline; however, studies such as this add weight to the argument that the toxicity of PCBs in the environment is likely to have been a contributing factor in the decline seen in otter numbers.

In several studies, otters dying from disease or starvation have been found to contain higher concentrations of PCBs than those dying a violent or traumatic death in incidents such as road traffic accidents or accidents with fishing nets (Kruuk and Conroy, 1991;

Roos *et al.*, 2001). Body condition has also been found to correlate with PCB concentrations, with animals in poorer condition containing higher concentrations of PCBs (Kruuk and Conroy, 1996; Ruiz Olmo *et al.*, 2002). The question this raises is: are otters losing condition because of the high concentrations of PCBs? In which case, are some otters more susceptible to taking on the contaminants, or are otters building up higher concentrations of contaminants because they are in a poor condition?

Otters, being a protected species, are not ethically suitable for experimentation to look at effects of contamination on the health of individuals or populations. However, several studies have used mink (*Mustela vison*) as a substitute, considering them to be similar enough in evolutionary and physiological terms to be used as a suitable comparison to otters. Studies of female mink exposed to commercial mixes and combinations of fractions thereof show no significant decreases in implantation success but do show significant increases in foetal deaths in the womb, resulting in a significant decrease in the numbers of live young born to females exposed to mixtures of PCB fractions (Jensen et al., 1977; Bäcklin & Bergman, 1992; Kihlström *et al.*, 1992). No significant alterations to reproductive output are observed in females exposed to single fractions of PCBs (Kihlström *et al.*, 1992). Overall, the results suggest that PCBs have little or no effect on female reproduction in mink in terms of ovulation or implantation, but have significant effect on the survival rates of foetuses and that these effects are due to mixture effects when mink are exposed to two or more PCB fractions at once.

In one study, the commercial mixtures Clophen A50 and Aroclor 1254 appeared to have differing levels of toxicity (Kihlström *et al.*, 1992; Håkansson *et al.*, 1992; Bergman et al, 1992). Both commercial mixtures increased foetal death significantly, but Clophen A50 had the greatest effect with the group of nine females producing just one stillborn pup between them, the rest of the reproductive output being foetal deaths (Kihlström *et al.*, 1992). Females exposed to Clophen A50 expressed significantly lower concentrations of vitamin A in both their pulmonary and hepatic tissue (Håkansson *et al.*, 1992) as well as showing significant differences in their liver histology (Bergman et al, 1992), suggesting that Clophen A50 has a greater ability to significantly disrupt normal bodily functions.

The increased toxicity of Clophen A50 is most likely due to the higher content of fractions of non-*ortho*-CBs and mono-*ortho*-CBs in this commercial mix (see Figure 1.5

for the locations of *ortho*, *meta* and *para* positions). Non-*ortho*-CBs and mono-*ortho*-CBs are dioxin like in their toxicity and are known to lower concentrations of retinol (vitamin A) (Murvoll *et al.*, 2006) and levels of the thyroid hormone thyroxine (T^4) (Thomas, 2000; Baldridge *et al.*, 2003), all of which are processes involved with important bodily functions such as growth and development, reproduction and resistance against infectious disease (Murk *et al.*, 1998; Baldridge *et al.*, 2003).

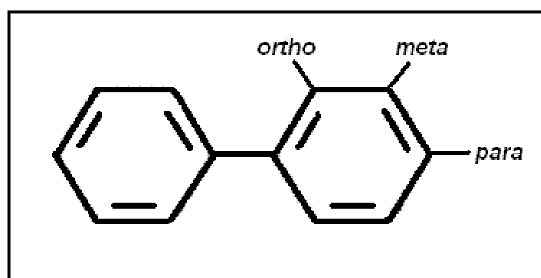

Figure 1.5 Structural positions for *othro*-, *meta*- and *para*-PCBs.

PCBs have also been shown to significantly increase hepatic cytochrome P450 concentrations, to increase liver weights and even to significantly increase levels of cadmium within the kidneys of mink exposed to very high doses (Jensen 1977; total dose of 218mg over 66 days). PCBs clearly have many and varied effects upon the physiology of mink, affecting many bodily functions from reproduction to development. Many of these effects suggest serious disruption of vital physiological bodily processes and still further effects and mechanisms of PCB action remain to be understood in mink and other species. In addition, there may still be unknown and untested effects of PCBs on both mink and otter.

Despite mink being a useful study species, there are questions over their comparability to otters. Species vary in their susceptibility to different compounds (Yamaguchi *et al.*, 2003) and despite otters and mink being close in evolutionary terms, and despite their similarities in terms of habits, diet and basic physiology it appears that there may be large differences in their susceptibility to various environmental pollutants; Kruuk and Conroy (1996) found a mean PCB concentration in otters higher than that stated as causing reproductive failure in mink. Otters with PCB concentrations close to or significantly higher than those shown to cause reproductive failure in mink have also been found expressing clear signs of breeding successfully (Kruuk and Conroy, 1991) and from this it can be concluded that otters have a higher tolerance to PCBs than mink,

at least with respect to reproductive parameters. However, there is evidence that high PCB concentrations may affect the health and reproductive potential of otter populations. For example, Roos *et al.* (2001) found that otters from Southern Sweden contained significantly higher PCB concentrations than those from Northern Sweden ($p < 0.001$), and in parallel they noted low numbers of juveniles and sub-adults collected from the south compared to the north. Although not conclusive evidence of a link, this trend does indicate that there may be a correlation between PCB concentrations and reproductive success in wild otters from Sweden.

Murk et al. (1998) used a measure of PCBs and their toxic equivalents (GC-TEQs) to show that PCBs caused disruption of the Ah receptor and altered levels of T^4 in the plasma of living captive otters. The comparison of GC-TEQs to ratios of retinol (vitamin A) over retinylpalmitate in wild otters suggests that the storage capacity or metabolism of vitamin A is disrupted in a PCB dose related manner (Murk *et al.*, 1998). Simpson (1998) identified significant increases in vitamin A concentrations over time whilst concentrations of PCB congeners decreased significantly over the same time period. It would appear therefore, that there may be a link (Simpson *et al.*, 2000), despite the fact that no significant correlations between PCB congener concentrations and levels of vitamin A in otters were observed (Simpson, 1998).

1.1.3iv) Conclusion

It is likely that several organochlorine compounds played a part in the demise of the otters by combining to disrupt the biological functions of individuals, and consequently altering otter populations as a whole. The gradual declines seen in the early 20th century are likely to have been partially caused by the effects of organochlorines on populations and PCBs in particular have been shown to have major physiological effects upon many species, not just otters. These effects are likely to have worked in conjunction with other threats to otter survival such as habitat destruction and pressures from persecution (Prigioni *et al.*, 2007), slowly reducing otter populations as human populations and needs grew. The severe decline in otter numbers across England and Wales in the 1950s is most likely to have been caused by the heavy use of the insecticide dieldrin during this period (Chanin & Jefferies, 1978), a chemical known to be toxic to many birds and mammals and known to have been the cause of many other wildlife mortalities during its widespread use.

Continued monitoring of contaminant levels in the otter population is essential if a repeat decline in otter numbers is not to become a reality again in the future. Concentrations of PCBs and organochlorines such as dieldrin and DDT appear to be decreasing in the environment and in top predators such as the otter since bans on their use have been put in place. With an ever increasing suite of chemicals and compound mixtures being used by humans, as one suite of compounds are deemed an environmental and human health risk it seems that yet another set are brought in to replace them.

1.1.4 Reintroductions

As discussed, from the mid 1950s, the numbers of wild otters in England declined drastically and after several decades of decline there was no apparent recovery. In many areas across central, southern and eastern England numbers of otters were extremely low and in some areas they were thought to be extinct (Crawford, 2003). A captive breeding program was set up Norfolk by the Otter Trust and releases of captive otters began in 1983. The majority of otters released originated from the Norfolk captive breeding programme. However, several releases of rehabilitated wild otters were carried out by the Vincent Wildlife Trust and RSPCA (Crawford, 2003).

Captive breed otters were released by the Otter Trust between 1983 and 1998, and the Vincent Wildlife Trust between 1990 and 1996, into those areas with consistently low numbers of otters. Areas of focus for these releases were the Anglian Region, with at least 99 otters released between 1983 and 1998, the Yorkshire Region with 29 and the Thames with 23 otters released (Crawford, 2003). Smaller numbers were released into the South West, Dorset, Southern and Northumbrian Regions and the Trent catchment.

The areas of greatest focus for the release programme were areas where otter numbers were low or non-existent. In the 1977-79 survey of the Anglian Region only 3.2% of sites were positive for the presence of otters, and in the 1984-86 survey this had dropped to just 1.1% positive. In Yorkshire only 1.8% of sites showed to be positive for otter activity in the 1977-79 survey, whilst the Thames catchment was void of all otter activity until the 1991-94 survey (Crawford, 2003).

In areas such as the Anglian Region and the Thames valley the otter population was unlikely to recover alone. With the surrounding catchments also depleted of otters it would be unlikely that the populations would have recovered due to an influx of otters from surrounding source populations (Barbosa *et al.*, 2001), hence recovery in these areas is likely to be due to the releases of captive bred otters. The Dorset Region, on the other hand, would have been likely to receive an influx of otters being displaced from the more highly populated South West Region neighbouring along its western border, in cases such as this it is unclear to what extent the population recovery is due to natural influx or re-introduction events (Copp & Roche, 2003). This, combined with the releases in this area, is likely to have caused the greater increase in numbers of otter

signs seen in later surveys of the Dorset Region. Figure 1.6 amalgamates the results of the four English otter surveys to provide a picture of otter rehabilitation in the different regions across England. From the graph it can be seen that otter numbers in the Anglian and Yorkshire Region have risen substantially in the last decade or two and that otters have returned to the Thames since the releases on this catchment. In contrast, numbers of otters in the Southern Region, which received relatively few captive released otters, have remained low with numbers of positive sites peaking at around 5%, the majority of those occurring on the Itchen catchment, close to the western border with Dorset and the release site of at least four otters.

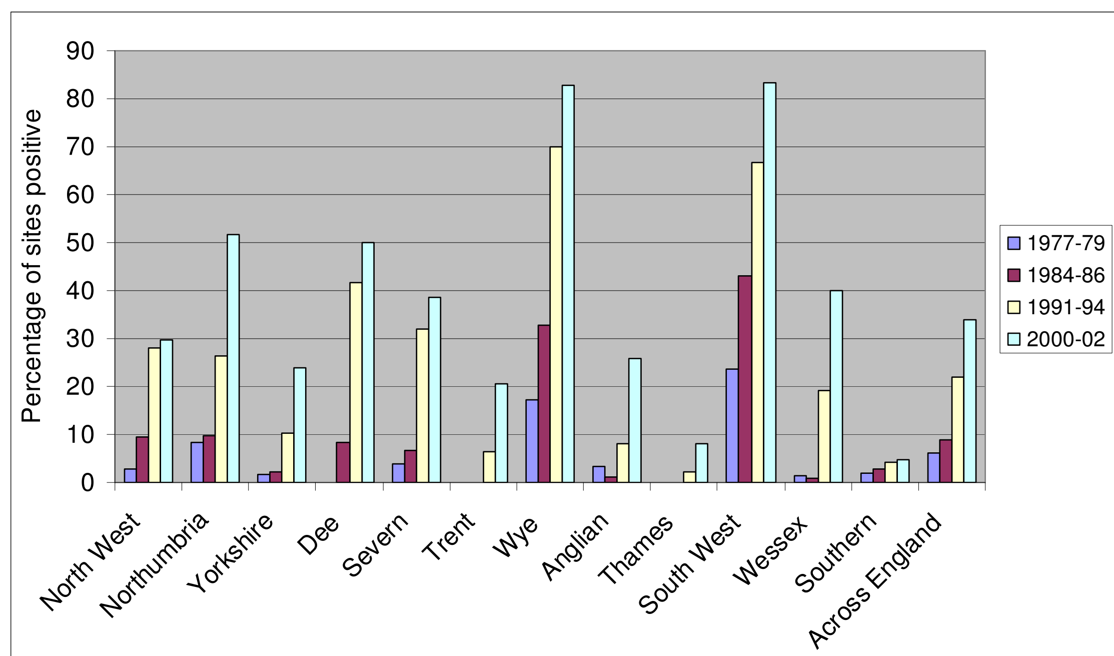

Figure 1.6 Shows the change in percentage of positive sites for otters by region in England. Graphic has been adapted using data extracted from the Fourth Otter Survey of England (Crawford, 2003).

The data indicate that the otter populations have recovered steadily over the last couple of decades. In areas where numbers remained fairly high the population of otters has risen substantially to reach a level nearing habitat saturation. In the South West alone, numbers have risen from 23.5% of sites showing signs of otter activity in the 1977-79 survey to 83% in 2000-2002 (Crawford, 2003). In the Wye Valley occupancy has also risen to 83% in 2000-2002, and in both these areas the population has risen with little or no intervention or additions of captive releases. In the majority of areas otter numbers have risen more slowly, and even after a high number of captive releases the total percentage of suitable habitat showing signs of otter occupancy totals less that 35% when England is considered as a whole (Crawford, 2003).

1.1.5 Monitoring Population Size

1.1.5i) Why Monitor Otter Populations

Discrepancies over what caused the decline in otter numbers in the past highlights the need for thorough monitoring of contemporary otter populations if a similar decline in numbers is to be prevented in the future. If a trend is noticed early enough then perhaps this will give time to act to prevent further declines in the already depleted populations. A better understanding of the processes associated with otter population decline in the past will hopefully help us to prevent future population declines for both the otter and other vulnerable species.

Monitoring numbers of individuals in otter populations is vital to ensure that any significant dip in numbers is detected early enough for remedial action to be taken. Simple observations of population fluctuations can be unreliable. In order to obtain accurate knowledge about the health of populations in terms of the number of animals present and the fluctuations in population size the use of proper, well structured census techniques is essential.

1.1.5ii) Methods of Population Monitoring

Direct census of the population is a much more accurate method for detecting population change, where 'counts' are made at strategic periods in time. There are many different ways to census a wild population of mammals including: direct counts of the number of individuals present in a certain representative area of their habitat; using traps along with a capture-recapture approach (van Apeldoorn , 1992; Efford, 1998); using camera traps; counting signs such as tracks and faecal samples (Erlinge, 1967; Prigioni *et al.*, 2007); or using genetic techniques combined with the finding of faecal samples or collection of hair samples with the capture-recapture approach. Obviously not all of the above methods are suitable for every species. For example, direct count is often impractical if not impossible for the census of otters.

A capture-recapture method would be impractical for use with otters as trapping would be considered inhumane, and if some individuals, for example, females with cubs, are naturally more wary of the traps it may also provide biased and therefore inaccurate

information. Radio-tracking has been used successfully to plot home range size and/or habitat use in species with similar territorial behaviour, such as the red squirrel, *Sciurus vulgaris* (Andrén and Delin, 1994) and white-footed mice, *Peromyscus leucopus* (Ormiston, 1985). As before this process involves trapping animals to fit a radio collar. In some studies it has been suggested that this stresses the animal and although sensibly designed radio collars do not appear to affect the animal in terms of lack of weight gain or reproductive fecundity (Ormiston, 1985), there is evidence to suggest that more subtle affects on dominance relationships (Berteaux et al., 1994) or vulnerability to disease (Morell, 1995) may occur if the animal is stressed during the fitting process. The need to subject the animal to an anaesthetic during the fitting of a radio collar subjects the animal to further risks, particularly in the event of an adverse reaction to the anaesthetic drug (Ormiston, 1985).

Radio tracking of individual otters has been carried out, and has produced some interesting findings in terms of otter movement and special behaviour. In the case of otters the method is perhaps better suited to situations when trapping is not necessary, for example, when an animals is already in human care and being released back into the wild after a period, thus minimising any unnecessary handling. Due to the need to trap and fit the radio-tracking device this method on its own is not a suitable censusing technique, but is a technique better suited to obtaining data pertaining to the behaviour of the individual animal being tracked.

In the past, methods using field signs, such as tracks and faecal samples to score the percentage of a suitable habitat occupied by otters have been used with relative success. The method may not give a direct count of the numbers of otters, and there will be a degree of discrepancy based on variations in levels of activity of otters in different areas. Despite this, when used over a large enough area, as in the English surveys of otters carried out in 1977-79 (Lenton et al, 1980), 1984-86 (Strachan et al, 1990), 1991-94 (Strachan and Jefferies, 1996) and 2000-2002 (Crawford, 2003), large scale counting of signs of otter presence provides an overall picture of what is going on in a population in a particular area as opposed to a direct count of every individual present.

On a smaller scale the method of counting field signs can be a little ambiguous. Variations in otter sprainting behaviour caused by variations in prey availability, breeding status and territorial disputes will influence the frequency with which spraint

are found and the weather too will influence the count, with flood waters often washing otter signs away. On a more fine scale, such as on a river basis, the counting of field signs can prove to be inaccurate in terms of counting the numbers of otters present. The method is more suitable for providing positives for sites used by otters and in providing a rough count for river system populations.

One way to ensure an accurate count of the number of otters depositing the spraint found in an area is to genetically type DNA extracted from the sprain to identify the individual otter that produced it. A distinct advantage of this method is that it is non-invasive, causing very little disturbance to the individuals being counted (Morin *et al.*, 1993). In recent years this has become a method commonly used for many species in order, not only to census population numbers, but also to obtain an insight into animal behaviour.

1.1.6 Non-Invasive Genetic Census

1.1.6i) Non-Invasive Genetic Census Methodology

The genotyping of DNA extracted from faecal material is a censusing method which has been developing fast over the last few years, both in terms of technical development and in terms of the number of species for which it is used. The method relies on the use of species specific microsatellite primers to amplify the genotype of the individual animal that laid down the faecal deposit (Taberlet *et al.*, 1997). Individual animals can be distinguished from one another via differences in their genotypes and so ultimately a census of the population can be taken by counting the number of different genotypes obtained (Kohn *et al.*, 1997).

This type of analysis will only provide a minimum count for the population as there are likely to be individuals resident in the target area that have not been picked up in the survey (Frantz *et al.*, 2003). The count may also be inaccurate if two individuals contain the same allelic composition for the suite of microsatellite loci analysed (Dallas *et al.*, 2003). However, the technique still provides a reasonably accurate estimate of the population size inhabiting a particular area, with little resulting disturbance to the population. One drawback of the method is cost, with non-invasive genetic censusing

generally costing far more than other methods and so requiring a greater justification for its use over other less robust but more cost-effective methods (Arrendal *et al.*, 2007).

1.1.6ii) Faecal Genotyping in Other Species

Non-invasive studies, using DNA extracted from faecal or shed hair samples, have been used to census populations of several different species (Taberlet *et al.*, 1997; Frantz *et al.*, 2003), providing a count of the number of individuals inhabiting an area from the number of different microsatellite genotypes obtained (Kohn & Wayne, 1997). If such surveys are carried out over several points in time they can also provide insight into levels of local population fluctuation (Prugh *et al.*, 2005; Piggott *et al.*, 2006).

At a more fine scale, non-invasive genetic data can be combined with information regarding the locations of sample collections to provide information about behavioural aspects such as home range use (Kohn *et al.*, 1999). If the level of genetic variability at the loci analysed is great enough, then calculations of genetic relatedness and paternity can be used to provide insight into the kinship structure of the population (Kohn *et al.*, 1999; Lucchini *et al.*, 2002).

At a wider scale, it is possible to use non-invasive sampling to study levels of genetic variation across and between populations by assessing variation in mitochondrial haplotypes or by using microsatellite genotype data to carry out population genetic analysis (Kohn *et al.*, 1995; Lucchini *et al.*, 2002; Idaghdour *et al.*, 2003). Mitochondrial DNA sequencing and microsatellite allele ranges can be used for species identification in situations where it is not possible to ascertain in the field which species produced a particular faecal sample (Ernest *et al.*, 2000; Palomares *et al.*, 2002). This method of species identification is not only useful for studies of the population dynamics of species, but can also be integral in providing proof of the presence of a species in a particular geographic region (Wan *et al.*, 2003).

1.1.6iii) Sexing Faecal Samples

Gender specific genetic markers are useful for gaining information about the sex of the animal that produced a faecal sample (Kohn & Wayne, 1997). One of the most commonly used markers for genetic sexing is the SRY gene (Kohn *et al.*, 1999), which sits on the Y-chromosome and so is only amplified in male individuals. However, there are potential problems with the use of the SRY region, particularly when used with the low quantities of DNA typically obtained from faecal material.

A negative result from the PCR of the SRY gene will indicate that the sample originated from a female. However, with samples such as faeces, which produce low quantities of DNA it may simply be that the SRY region was not successfully amplified in a particular PCR reaction. Similarly a positive result would indicate that the sample originated from a male individual, but it may also have arisen from a contamination event. The SRY region is mammal specific and so even redesigning the primers to make them more species specific does not completely eliminate the possibility of amplifying DNA products from other prey species (Whittier *et al.*, 1999; Murphy *et al.*, 2003) or via contamination in the laboratory from other species, including humans.

1.1.7 Spraint Genotyping in Otters

The microsatellite genotyping of otter spraint material is a technique which has been used in an increasing number of studies to aid our understanding of population sizes, otter behaviour and relationships. Below are case studies of some of the most integral studies to date, highlighting the various uses of the non-invasive genotyping technique for the study of otters.

1.1.7i) Spraint Genotyping of Otters in South West England (Coxon *et al.*, 1999; Dallas *et al.*, 2003)

One study to use the microsatellite genotyping of spraint material did so as a tool for censusing the otter populations on four of the rivers in South West England (Coxon *et al.*, 1999; Dallas *et al.*, 2003). The study utilized nine microsatellite loci and the SRY gene to identify 57 otters from the spraint samples collected and genotyped. The use of

the SRY region meant that males and females could be easily identified (Dallas *et al.*, 2000), with the samples split into 31 males and 26 females (Coxon *et al.*, 1999), and as a result it was possible to observe differences in the ranges of otters of different sexes.

The results of the spraint genotyping study showed that 13 different otters used the River Itchen in Hampshire at some point between the months of January 1997 and July 1998. The timings of spraint collections suggest that at least four of these otters, one male and three females were resident on the river during this period, with each of these individuals genotyped on several different occasions during the sampling period. For the Rivers Brue in Somerset and the Torridge in Devon 11 and 10 otters respectively were genotyped from samples collected between the months of October 1997 and July 1998 (a further otter was genotyped from a sample collected from the River Brue in May 1997). The final river to be sampled was the River Tone in Somerset where 22 otters were observed between the months of June 1997 and July 1998.

The accuracy of the spraint genotyping method was tested using comparisons to genotypes obtained from tissue samples collected from otters found dead in the region. Among the methods used for comparisons were assignment tests (Prichard *et al.*, 2000), which showed spraint and tissue genotypes from the same region as assigning to the same population This result indicated that there was little or no error in the genotyping of spraint samples analysed in this study (Dallas *et al.*, 2003). The genotypes obtained from the tissue samples were also used to calculate probability of identity, a measure of how likely it is, given the allele frequencies observed in the population, that two individuals will share the same genotype at all the microsatellite loci analysed (Waits *et al.*, 2001; Valière *et al.*, 2002). The results suggested that the nine microsatellite loci and SRY region used to genotype the tissue and spraint samples were effective at distinguishing between individuals in the population, with a relatively low probability of two individuals expressing the same allelic composition at each of the loci analysed (PI = $1.5\text{-}2.3 \times 10^{-5}$). Calculations based on the chances of siblings sharing an exact genotype suggest that, even given this close genetic relationship, the method was powerful enough to distinguish between siblings ($PI_{sib} = 5.6\text{-}6.8 \times 10^{-3}$; Dallas *et al.*, 2003). However, the study did note one incident where an identical genotype was obtained from a carcass as well as from spraint samples collected before and after the death of the otter (Coxon *et al.*, 1999; Dallas *et al.*, 2003).

1.1.7ii) Comparisons with Other Census Methodologies (Arrendal *et al.*, 2007)

The accuracy of the spraint genotyping method of censusing otter populations was analysed in more depth by Arrendal *et al.* (2007). In this study the method of snow tracking, one of the most commonly used and most effective methods of censusing otters (Erlinge, 1967), was compared to that of genotyping spraint samples collected across the same area of central Sweden (approximately 2500km^2). Arrendal *et al.* (2007) found that, depending on the stringency of the methods used, 10 to 15 otters were identified using the snow tracking methods, while 20 to 23 otters were identified from the same geographical region using the genotyping of spraint material. This would suggest that the size of otter populations may be underestimated using the snow tracking methodology. The snow tracking method works by measuring the size of footprints and the length between them to distinguish different individuals. However, with a limited size range, and the fact that adult females often have similar footprint sizes to sub-adult males, it is often difficult to tell individuals apart and as a result there is a certain amount of subjectivity to the method.

Arrendal *et al.* (2007) found that both methods provided maps of dates and locations for individual otter observations. The findings from the spraint study were more accurate in their identification of individual otters, with a specific genotype providing date and location data for a specific otter. Conversely, the data obtained from the snow tracking study was more dubious, with several instances of confusion as to which otter left a particular track. The conclusions of the study carried out by Arrendal *et al.* (2007) was that spraint genotyping was the more accurate method of census, however the monetary cost of the spraint genotyping study was far greater and so this method would only be useful in instances where the necessary funds were available.

1.1.7iii) Inferring Levels of Genetic Relatedness (Hung *et al.*, 2004)

As with other studies, Hung *et al.* (2004) were able to use genotypes obtained from spraint samples to pinpoint dates and locations for individual otters. Samples were collected during specific months of the year (February, May, August and November) along two stretches of river on Kinmen Island, off the coast of mainland China. The result was the mapping along transects of the two rivers of the ranges of the different

otters. Information about the months in which each otter was observed gave timeframes for otter occupancies. The data provided information about the patterns of otter movement, with clearly defined home ranges often used by several otters at once and with little or no overlap between the home ranges.

Hung *et al.* (2004) took the study of otter movement and behaviour one step further, using the genotypic data to calculate levels of genetic relatedness between the otters. The analysis showed that levels of genetic relatedness were higher between individuals using the same river (R = 0.160, SE = 0.059) than between otters from the two different river catchments (R = -0.041, SE = 0.035, p <0.001). To help define this point the levels of relatedness were investigated further, using measures of pairwise genetic relatedness to focus more specifically on the relationships between pairs of individuals. A tree was constructed to better illustrate the structuring resulting from the inferred levels of genetic relatedness. The analysis indicated that individuals occupying the same geographical range - both in terms of the river of origin and in terms of home range occupancy within the river system - tended to be more closely related to each other, although there were still many instances of unrelated individuals sharing the same home range.

1.1.7iv) Using the Genetic Data for Population Genetic Analysis (Hájková *et al.*, 2007)

The genotypes obtained from spraint material do not only provide information about individual otters; the technique can also be used to gain the sample numbers necessary to carry out population comparisons. Hájková *et al.* (2007) found that the accuracy of the genotypes obtained from spraint were such that they could be used alongside genotypes obtained from tissue in order to increase the overall sample numbers available for analysis. The genotypes from both sample sets were then used to compare otter populations from the Czech and Slovak Republics.

Spraint samples collected over a 100km^2 study site in the Czech Republic were used to gain an additional 50 genotypes, which were added to the 82 genotypes obtained from tissue samples collected over a much larger area of the country. Similarly, 13 otters were genotyped from spraint samples collected over a 100 km^2 region of the Slovak

Republic and these were added to the 52 genotypes obtained from tissue samples collected over a much wider area. The genotypes obtained from spraint and tissue samples were combined in order to calculate allelic richness, heterozygosity, Hardy-Weinberg equilibrium and F-statistics for the populations of the two countries.

Genetic assignment tests were carried out using the data from spraint and tissue combined. As with the study carried out by Dallas *et al.* (2003), Hájková *et al.* (2007) found that the populations clustered according to location, with the only exceptions being three individuals which appeared to represent the signature of captive released individuals. The fact that populations did not cluster according to the type of sample used indicates that the use of spraint samples is a legitimate and effective way of obtaining an accurate genotype for use in population studies.

1.1.8 Population Genetics of Otters

Haplotypes based on sequences of mitochondrial DNA are useful for looking at large scale population structuring used in phylogenetic and phylogeographic studies. For more fine scale and informative population information more variable nuclear markers such as microsatellites are more suitable. Dallas and Piertney (1998) designed a suit of microsatellite primers for the Eurasian otter (*Lutra lutra*) and since then these markers have been used in several studies of otter population genetics.

1.1.8i) Populations in Scotland

Perhaps some of the best studied otter populations are those in Britain. Demographic data suggests that during the otter population declines, seen between the 1950s and 1980s, populations in the south of Scotland became isolated and fragmented, with geographic separation from the Northern Scottish population and populations in the North of England dropping to just 1-10% habitat occupancy (Crawford, 2003). Despite this when comparing genetic diversity of Scottish southern and northern populations it appears that populations in the south have sustained only minor losses of genetic diversity (Dallas et al., 1999). In addition, rather than consisting of two populations the Scottish mainland appears to consist of a metapopulation structure, with distinct

populations of otters and greater levels of isolation by distance between than would be expected in a continuous population (Dallas et al., 2002). Despite this, for the Scottish mainland otter population as a whole the genetic data shows high levels of microsatellite polymorphism in all sub-populations, with healthy levels of gene flow between them (Dallas et al., 2002). The majority of Scottish island populations also showed high levels of genetic diversity comparable to those seen in mainland populations, with gene flow appearing to be the main mechanism for maintaining this diversity (Dallas et al., 1999).

The only island otter populations not to show levels of genetic diversity comparable to those on the mainland are populations on the Shetland Isles, and to a lesser degree Orkney. The most likely reason for the lower genetic diversity found on these islands is distance and isolation. Shetland and Orkney, at 110km and 24km respectively, are the islands furthest from the mainland and for Shetland in particular this is likely to prevent migration to and from the islands that make up this archipelago.

1.1.8ii) Populations in England and Wales

When compared to Scottish mainland populations, otter populations from Wales and the south west of England showed significantly lower levels of genetic diversity, although they showed significantly higher levels than the Shetland otter population (Dallas et a., 2002). Interestingly the otter population in the south west of England is split into two genetically distinct populations with no apparent barrier to otter movement separating them (Dallas et al., 2002). The population split seen in south west England ties with the population separation seen in Scotland, with populations split into genetically distinct groups with no obvious barriers to dispersal present between them. There are several possible explanations for this phenomenon, such as philopatry in males as well as females or unconsidered habitat barriers, but the main consideration for future studies of otter populations should be how this population separation impacts upon the populations themselves in light of conservation measures, re-introductions and further population declines.

1.1.8iii) Mitochondrial Haplotype Assessment of Otters in Europe

Several studies have used mitochondrial DNA to study the genetic diversity of otter populations across Europe. In the majority of these studies 249 to 365bp from the 5´ end of the control region have been sequenced to obtain haplotypes. Results from these haplotypes suggest that there is relatively little genetic diversity across Europe (Pérez-Haro et al., 2005). The findings from four such studies (Mucci et al., 1999; Cassens et al., 2000; Ferrando et al., 2004; Pérez-Haro et al., 2005), surveying populations all the way from the Iberian Peninsula in the west to Belarus and Russia in the east, are presented in Table1.2. In addition, Arrendal et al. (2004) and Ketmaier and Bernardini (2005) studied populations in Scandinavia and Italy respectively and, although haplotype information is not easily comparable to those in the above studies, both populations show comparably low levels of genetic variation based on the 5´ end of the control region.

Across Europe Lut 1 is by far the most common haplotype and this combined with low overall levels of genetic diversity has led many to predict that during the Pleistocene period otter populations, as happened with many other species (Taberlet *et al.*, 1998; Hewitt, 2000; Sommer and Benecke, 2004; Sommer and Nadachowski, 2006), were pushed into refugia. These refugia are likely to have been situated in the Iberian Peninsula, Italy or possibly Eastern Europe and Asia. The dominance of Lut 1 suggests that this haplotype was pushed into fixation during the declines and when populations spread back across Europe this remained the dominant haplotype. Isolated areas, such as Scotland, show what is likely to be isolated mutational events resulting in low levels of nucleotide diversity in those countries (Cassens et al., 2000) as opposed to the monomorphism seen in others (Mucci et al., 1999; Pérez-Haro et al., 2005). Higher levels of nucleotide diversity seen in Russia and Belarus ($\pi = 0.0037$, Ferrando et al., 2004) indicates that this area was unlikely to have been the refugia whose bottleneck caused the fixation of Lut 1, making the Iberian Peninsula and Italy more likely candidates. However, the presence of Lut 3 in both Eastern Europe and Germany suggests that some limited spread may have occurred from this direction.

The spread of haplotype Lut 3 is particularly interesting. In Eastern Germany, as in many areas of Europe, severe declines in otter numbers occurred during the 1920s to 1950s. It appears that Lut 3 was present in the south eastern corner of Germany during this period of bottlenecking. It may have been present at low levels before this period or

it may have entered the population during this period, either through a mutational event, a natural migration event or through a human induced translocation of individuals. As the population recovered from the bottlenecking event to become one of Europe's otter strongholds Lut 3 population became the more dominant haplotype within the region (see Table 1.2).

Ferrando et al. (2004) sampled one otter from the country of Belarus on the eastern borders of Europe and found it to be Lut 3, suggesting that this haplotype may have originated from Eastern Europe and or Asia, although more work should be carried out before firm conclusion can be drawn. In addition Lut 3 has been identified in captive populations from both France (n 2) and England (n 4) (Mucci et al., 1999; Pérez-Haro et al., 2005), suggesting that animals used in these captive breeding programs are likely to have been sourced from the wild German populations, known to be back up to healthy populations size by the beginning of the captive breeding programs in the 1980s.

Table 1.2 The haplotypes of otters from various countries from around Europe. Data originally from Mucci et al., 1999, Cassens et al., 2000, Ferrando et al., 2004 and Pérez-Haro et al., 2005.

Country	Lut 1	Lut 2	Lut 3	Lut 4	Lut 5	Lut 6	H2	H3	H5	H6	H7	n
Shetland	4											(4)
Scotland	17			2								(19)
England	1											(1)
Wales							1					(1)
Finland	25							1				(26)
Norway	4											(4)
Sweden	5											(5)
Denmark	30											(30)
Netherlands	5											(5)
Eastern Germany	33		42		1							(76)
Belarus			1									(1)
Poland	1											(1)
Russia	2								1	1	1	(5)
Austria and Hungry	24	1										(25)
Portugal and Spain	82											(82)
French captive			2									(2)
English captive			4			1*						(5)
Totals	233	1	49	2	1	1	1	1	1	1	1	292

* The 4 otters sampled were all descendant from one female, therefore treated as one for the purposes of this study.

Other regions of the control region appear to be more variable, although no phylogenetic or phylogeographic relationships have ever been drawn from data concerning anything other than the 5´ section of the control region (Mucci et al., 1999; Pérez-Haro et al., 2005). A potentially informative minisatellite region has been identified in a stretch known as the CSB domain, however its ability to form secondary structures has tended to complicate its use for analysis (Ferrando et al., 2004; Ketmaier and Bernardini, 2005).

During RFLP analysis of the control region just one haplotype was observed in samples from four countries from across Europe: Scotland, Germany, Austria and Hungry. Variation only increased when the cytochrome b region was added during a secondary analysis of German samples, suggesting that the cytochrome b region may be a more variable region in the mitochondrial DNA of Eurasian otters (Effenberger and Suchentrunk, 1999).

Higher levels of genetic diversity observed in studies using markers such as microsatellites suggest that the low variability seen in the control region of mitochondrial DNA may represent a conservative pattern of molecular evolution in this region of DNA (Mucci et al., 1999 – look up Hansen and Loeschcle 1996 for explanation)

1.1.8iv) Microsatellite Assessment of Otter Populations in Europe

Across Europe populations of otters have been affected by the declines to a greater or lesser extent, as is evident from both demographic data and the population genetic structure. In a study of populations from around Europe Denmark and Spain proved to be genetically distinct, whilst other countries studied showed varying degrees of genetic admixture (Randi et al., 2003).

In the case of Denmark this genetic differentiation is likely to have been caused by the severe declines between the 1950s and 1980s, at the height of which the population is thought to have been restricted mainly to the northern part of the western island of Jutland (Pertoldi et al., 2001). The Danish otter population now suffers from some of

the lowest levels of allelic diversity (average of 2.9 alleles) and heterozygosity (*He* = 0.45) seen in Europe (Randi et al., 2003). Genetic analysis of contemporary and historic Danish otter populations show differences in allele frequencies and genetic distances (F_{ST} values) that indicate variation over time, however the genetic assignment of historic samples to present day populations from the same location suggests these differences may be due to differences in the geographic range sampled (Pertoldi et al., 2001).

For the Danish samples both contemporary and historic sample sets suggested that severe population declines had occurred, down to approximately 1-3% of the original population size. Both sample sets placed the bottleneck at around 2000-3000 years ago (Pertoldi et al., 2001), which would place it at around the time of major human population expansion in Europe (reference needed). However it should be noted that the stepwise mutation model used is not always accurate when used with microsatellite data (reference needed), therefore it should be considered that the signature seen may represent, or partially represent, earlier declines caused by the glacial age 10,000-13,000 years ago.

Similar bottleneck events have been detected for countries around Europe, placed at 2000-2600 years for the United Kingdom, Sweden, France, and again for Denmark (Randi et al., 2003). Bottleneck events were placed further back in time for Spain and Germany at 4700-4900 years ago, again drawing into question the accuracy of these methods of detection and questioning the true time of major declines in Europe.

As with the Danish populations, the Spanish population appears to be genetically distinct from other populations in Europe. High levels of genetic diversity in the Spanish otter population suggest that the reason for this genetic distinctiveness is likely to be the use of the Iberian Peninsula as a refugia for otter populations during the last glaciation period (Randi et al., 2003).

1.1.8v) Summary - Otter Populations in Europe

By the 1990s the otter population was absent from much of Central and western Germany, but numbers in Eastern Germany were recovering fast. Studies of mitochondrial haplotypes indicate that, although still low, haplotype diversity is higher in populations of otter from Eastern Germany than in many regions across Europe (Cassens et al., 2000; Pérez-Haro et al., 2005). Microsatellite analysis too indicates that German otter populations are more diverse than populations in other European countries with Eastern German populaiotns expressing high allelic diversity (average of 6.0 alleles) and high levels of heterozygosity ($He = 0.74$), suggesting that more than one refigia may have influenced the German otter population (Randi et al., 2003)

1.2 Introduction to Population Genetics

1.2.1 What is Population Genetics?

Population genetics is the study of between and within population dynamics, using genetic markers as a tool for statistical analysis. Depending on the genetic markers and statistical analysis used, the technique can be used to study the natural history of populations, as well as the effects of recent population changes (Estoup *et al.*, 1995; Goodman *et al.*, 2001; Walker *et al.*, 2001). The natural and human induced splits in populations, metapopulation structures and migration patterns can all be studied using population genetic techniques (von Segesser *et al.*, 1999; Van de Zande *et al.*, 2000; Hale *et al.*, 2001; Kyle & Strobeck, 2001; Beveridge & Simmons, 2006). In addition, the present genetic structure of a population can be used to infer recent changes in population size and structure over time (Ehrich & Stenseth, 2001; Goodman *et al.*, 2001), and if combined with historic samples, can be used to detect more drastic population changes over calculable time scales (Walker *et al.*, 2001; Wang *et al.*, 2002; Coyer *et al.*, 2003). It is because of this wealth of possible information, which would otherwise often be hidden within the populations, that as a tool for detecting population changes and effects, genetic analysis can prove to be an invaluable aid for conservation and population management (Gutiérrez-Espeleta *et al.*, 2000; Maudet *et al.*, 2002).

1.2.2 Microsatellite Genotypes

1.2.2i) Microsatellite Loci

Microsatellite loci, also known as short tandem repeats (STRs), are comprised of repeats of a short sequence motif, the length of the microsatellite being dictated by the number of repeats. For example, a microsatellite of notation $CAGA_{(12)}$ would comprise of the sequence motif CAGA repeated twelve times in sequence. The microsatellite loci used in this study are found in the nuclear DNA. Microsatellite loci do occur in other sources of DNA, such as the mitochondrial DNA, but for population studies such as this it is necessary to use markers found on the nuclear DNA, which is passed down maternally and paternally in equal measures.

Another prerequisite for microsatellite loci used in population genetic study is for them to be situated in neutral genetic material, i.e. not within genes. This neutrality means that they are passed on randomly in a population, without being affected by selection. This helps us to view what is going on in a population because the effects that we see at microsatellite loci should be as a result of changing population dynamics, rather than as a result of selective events within the population.

Another property of short sequence repeats, such as microsatellite loci, is that they have a relatively high mutation rate (Kelly *et al.*, 1991; Van de Zande *et al.*, 2000) and tend to mutate in an organised fashion, with one or more repeat sequences being gained or lost during the majority of mutational events (Jeffreys *et al.*, 1988). Rates of mutation for microsatellite loci can reach $x10^{-4}$ to $x10^{-3}$ mutations per locus per gamete per generation (Weber & Wong, 1993; Thuillet *et al.*, 2005), far exceed those of other genetic markers such as allozymes and RFLPs (Slatkin, 1995; Jarne & Lagoda, 1996), where mutation rates are more likely to fall into the range found across the genome of approximately $x10^{-8}$ mutations per nucleotide site per generation (Drake *et al.*, 1998; Nachman & Crowell, 2000). Mutation rates are higher at microsatellite loci partly because the loci used for population studies are chosen for their position in a neutral stretch of DNA, for which DNA repair systems are less stringent (Webster *et al.*, 2004), and partly because the repeating nature of the microsatellite loci itself opens it up to the mutational processes of unequal crossing-over and slipped strand mis-pairing (Levinson & Gutman, 1987: Jeffreys *et al.*, 1988; Stephan, 1989; Kelly *et al.*, 1991; Jarne & Lagoda, 1996). The mutation rates seen for microsatellite loci are high enough to result in allelic polymorphism in populations, but are rarely high enough to that a mutational event is witnessed during the course of a population study.

1.2.2ii) Microsatellite Genotypes

Due to the nature of microsatellite loci, being constructed of repeats of short sequences, mutations usually cause the formation of a copy one or more repeat sequence units longer or shorter than the original (Jarne & Lagoda, 1996; Yamada *et al.*, 2002). These variations in length of microsatellite are known as alleles. Mammals, being diploid organisms, contain two alleles per microsatellite locus inherited from each of their two parents. An individual can contain two alleles of the same length, homozygous, or two alleles of differing length, heterozygous, depending on what they inherited from their parents. Inheritance of alleles will in theory be random because parental survival, fecundity and mate choice will be based on genes and not on the basis of neutral markers such as microsatellite loci. Alleles are passed on in a simple mendelian inheritance fashion, providing an equal possibility for each of the four allelic compositions possible per loci for each parental pairing (Figure 1.7).

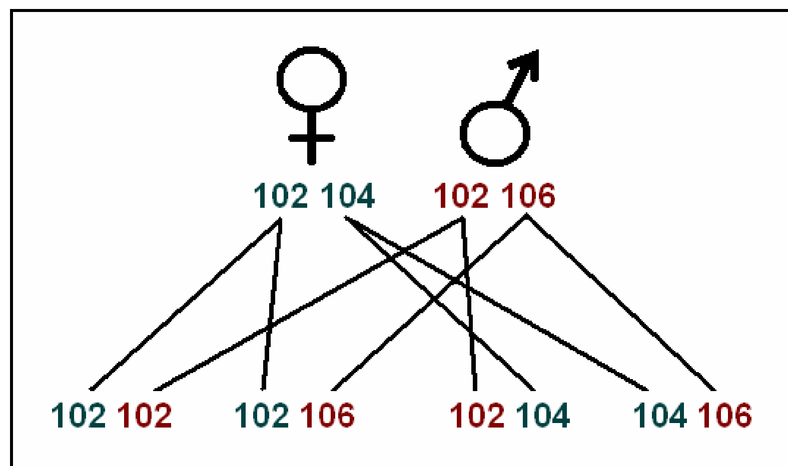

Figure 1.7 Mendelian inheritance of microsatellite alleles provides equal weighting to each of the four possible combinations of allelic inheritance in the offspring of a mated pair.

A genotype is essentially an amalgamation of the allelic information from several microsatellite loci. The end result will consist of the sizes of the two alleles, per microsatellite loci studies. In theory, any time that an individual's microsatellite allele sizes are viewed for one or more loci it is known as a genotype. Although for a genotype to be useful for genetic analysis purposes it is generally preferable to have as many loci genotyped as is practical and cost effective (Ehrich & Stenseth, 2001).

1.2.3 Checking the Suitability of a Microsatellite Loci

When assessing the viability of a microsatellite locus for use in population genetic studies the first check should be the number of alleles expressed by a locus. If only one allele is expressed a locus is said to be monomorphic. In this case the inclusion of this locus in statistical analysis will provide no benefit to the project because the locus does not provide any useful information (von Segesser *et al.*, 1999; Wang & Schreiber, 2001; Maudet *et al.*, 2002). The exception to this is if the locus is polymorphic, i.e. expresses two or more alleles, in other populations being compared to the first in analysis, although even in this case a locus which is polymorphic in all populations being studied would be preferable if available. The more polymorphic a locus is the more informative it will be (Bernatchez & Duchesne, 2000; Gutiérrez-Espeleta *et al.*, 2000), and so if a choice over which loci to use is possible, as may be likely at the beginning of a project, then the most polymorphic loci should be chosen for study (Ehrich & Stenseth, 2000; Maudet *et al.*, 2002). Larger mammalian species generally have less polymorphic loci than more mobile (relative to size), short lived and more fecund groups such as small mammals, birds, fish and insect (Table 1.3). For mammals, this low allelic diversity, combined with the fact that many wild mammalian species have been little studied meaning there are often few identified microsatellite primers, means that there is often little choice but to use loci with low numbers of alleles.

Table 1.3 Examples of the variation in the numbers of alleles found in species of different taxon.

Species	Alleles per locus	Reference
Wolverines *Gulo gulo*	2 - 5	Walker *et al.*, 2001
Alpine ibex *Capra ibex*	2 - 6	Maudet *et al.*, 2002
Bighorn sheep *Ovis canadensis*	2 - 10	Gutiérrez-Espeleta *et al.*, 2000
Barbary macaques *Macaca sylvanus*	4 - 12	von Segesser *et al.*, 1999
Root vole *Microtus oeconomus*	4 - 22	Van de Zander *et al.*, 2000
Apostlebirds *Struthidea cinerea*	4 - 27	Woxvold *et al.*, 2006
Chum salmon *Oncorhynchus keta*	3 - 32	Chen *et al.*, 2005
Dawson's burrowing bee *Amegilla dawsoni*	4 - 38	Beveridge & Simmons, 2006
Túngara frogs *Physalaemus pustulosus*	12 - 42	Lampert *et al.*, 2003

As discussed in previous sections, microsatellite loci are situated in neutral genetic material (Maudet *et al.*, 2002), i.e. not within genes. This neutrality means that they should be passed on randomly in a population, without being affected by selection. The exception to this being if a microsatellite locus is situated so close to a gene that it tends, more often than not to be included with the gene during recombination events, i.e. when DNA is being recombined in gametic cells ready to be passed on to the next generation. In this case the locus is said to be linked to the gene and is likely to be selected for or against along with the gene in question. In addition to this, if situated close together in the genome, loci can be linked to each other and similarly get passed on together if recombination is not sufficient to separate them in the usual random pattern (Sacks *et al.*, 2004). For use in population genetic studies microsatellite loci need to be neutral and unlinked to each other or to genes; loci are checked for this using tests for Hardy-Weinberg equilibrium and linkage disequilibrium.

1.2.3i) Single Locus Deviations from HWE

If there are no selective pressures upon a population, and the processes of passage of allelic composition from one generation to the next is completely random, then we would expect this to be reflected in the levels of heterozygosity observed within the population. The levels of heterozygosity we would expect to see can be predicted given the allele frequencies found in the population (Carr, 2002). If the expected levels of heterozygosity, calculated from the allele frequencies, match the levels of heterozygosity actually observed in the population, then the population is said to be in Hardy-Weinberg equilibrium (HWE).

By calculating Hardy-Weinberg probabilities for each individual locus across each population, it is possible to identify if there is a potential selective pressure which may cause a locus to bias future statistical analyses. If a locus is out of HWE it is a sign that it may be linked to a gene, and therefore be under the influence of selection. If a locus shows a deficit in heterozygosity this is a sign that the locus is probably expressing null alleles (alleles which are not picked up in PCR reaction) and if the heterozygosity deficit persists across several populations the locus should be dropped from further statistical analysis (Lampert *et al.*, 2003; Baliraine *et al.*, 2004).

Hardy-Weinberg tests assume random sampling from a large population, and that members of the sample are unrelated (Robertson & Hill, 1984; Goodman *et al.*, 2001). Obviously this is not the case with our samples, as with many such studies, and so the results should be viewed with caution. As stated above, in larger studies, any single locus out of HWE in several populations (see section *1.2.4iv, Deviations from Hardy-Weinberg* for situations when several loci are out of HWE in the same population) should be discarded from future analysis (Maudet *et al.*, 2002; Lampert *et al.*, 2003; Achmann *et al.*, 2004). In a study utilising small sample sizes then more caution should be taken before loci are discarded as deviations from HWE can be heightened by the affects of small sample size (Baliraine *et al.*, 2004), and the fact that the samples are taken from effectively small populations means that the chances of there being related individuals in the sample is heightened. The more loci being analysed the greater the statistical power of detection (Bernatchez & Duchesne, 2000). Therefore, the influence of removing a locus may have a greater effect on the results of further genetic analysis than the inclusion of a locus for which the occurrence of deviation from HWE has been exaggerated. Therefore, a judgement should be made based on the extent of deviation from HWE and the reduction this will cause to the overall numbers of loci available for analysis.

1.2.3ii) Linkage Disequilibrium

If two microsatellite loci sit close to each other in the genome this will alter the rate at which recombination rates separate the alleles of the two loci (Sacks *et al.*, 2004). In this situation we would find a higher than expected level of concordance between the alleles expressed at the two loci. For example, if a significantly higher proportion, than would be expected given the allele frequencies, of individuals in which allele i was expressed as one of the alleles at locus A, also expressed allele j at locus B, it would be a sign that the two loci were linked.

Linkage between loci has the potential to alter the results of statistical analysis of population genotypic data. The linked loci will, at least to a certain extent, be acting as one in their behaviour, but will be counted as two separate loci within the analysis. With a limited number of loci, linkage between two can mean a significant bias in the results. The fact that they have been picked up as being significantly linked will suggest that the affect that they are likely to have upon any future analysis will also be significant. Therefore, for any pair of loci expressing significant linkage, particularly if the linkage is strong and persists across several populations, it should be considered that at least one of the loci be dropped from use in future statistical analysis.

1.2.4 Genetic Analysis of Populations

The microsatellite genotypes of a sample of individuals from the same population can be used to gain information about that population, such as the level of genetic diversity expressed. Analysing the genotypes of sample sets of individuals from several populations, or subpopulations will allow for comparisons and links between populations to be drawn. Analysis of how closely linked two populations are genetically can be compared to geographic and demographic information to help to understand better what is going on in and between the populations. There are various parameters and statistical analysis of microsatellite genotypes which can be studied to help us understand what is going on within and between populations, all of which provide slightly different information and need to be analysed and understood in a particular way.

1.2.4i) Allelic Richness

The more genetically diverse a population is the greater the level of polymorphism, a measure of the number of alleles, per microsatellite loci (Wang *et al.*, 2002). In other words a genetically diverse population will contain many alleles per microsatellite, whilst a less diverse population will contain significantly fewer alleles per locus.

When comparing two or more populations, rather than comparing counts of the number of alleles in each population it is more accurate to compare allelic richness. Allelic richness is a measure of the number of alleles expressed in populations standardised to take into consideration any variation there may be due to variation in the number of samples collected from the different populations (Baliraine *et al.*, 2004; Beveridge & Simmons, 2006). It is a measure of the number of alleles based on a sample number equal to that found in the population with the smallest sample number. For example, if fifteen individuals from one population are compared to twenty-five individuals in a second population, then the number of alleles in the second population is adjusted to represent that which would be likely if only fifteen individuals were surveyed, given the genotypes seen for the twenty-five.

1.2.4ii) Allele Frequencies

The frequency of an allele is effectively the proportion of alleles in that population made up of that allele type. For example, if we take a hypothetical population made up of five individuals, three of which are homozygous for allele x (i.e. both alleles are x) and two of which are heterozygous for alleles x and y , then the allele frequency of allele x would be 0.8 ($8 \div 10$) and the allele frequency of y would be 0.2 ($2 \div 10$).

Allele frequencies are an easily observable measure of the genetic diversity of a population (O'Brien *et al.*, 1987). The greater the number of alleles and the more even the allele frequencies are the more diverse a population is likely to be. The fewer alleles there are, and the more one or two allele lengths appear to dominate, the less diverse and more homogenous that locus will be within the population. If the same occurs over several loci then it is a sign that there are likely to be low levels of genetic diversity in the population as a whole.

1.2.4iii) Observed and Expected Heterozygosity

One way of looking at the diversity and homogeneity of a population is to view the amount of heterozygosity (Seppä & Laurila, 1999; Gutiérrez-Espeleta *et al.*, 2000). The levels of heterozygosity observed in a population, averaged across all loci, can act at a measure of the genetic diversity (Carr, 2002). A more diverse population will contain greater numbers of alleles at more even frequencies, therefore increasing the instances of heterozygosity.

In a population that is not under any selective pressures, levels of heterozygosity would be expected to be reflected in the numbers and frequencies of alleles. So at a locus with two alleles of approximately equal frequencies, we would expect approximately half of the individuals in the population to be heterozygous at that locus. However, if there is an imbalance in the process of allele selection, then this is likely to affect the levels of heterozygosity observed in the population. Therefore, if there are imbalances in the populations caused by selective pressures, such as non-random mating or high migration rates, these can be detected by comparing levels of expected heterozygosity (He) to the actual levels of heterozygosity (observed heterozygosity, Ho) found in the population (Beveridge & Simmons, 2006).

1.2.4iv) Deviations from Hardy-Weinberg

Hardy-Weinberg probabilities are essentially a more comprehensive method of measuring the difference between observed and expected heterozygosity. Rather than viewing the difference between the observed (Ho) and expected (He) heterozygosities, the Hardy-Weinberg test provides a probability that the difference between the two measurements is a true representation of a deviation from HWE. In other words, the Hardy-Weinberg probabilities provide a level of significance to the fact that the observed heterozygosities are different from the expected, and as a consequence provide evidence to suggest a significant cause for the effect.

On an individual locus scale deviations from HWE can be a sign of linkage to a gene (see section *1.2.3i, Single Locus Deviations from HWE*). However, if deviations are observed for several loci within a population it is a sign of population level effects (Seppä & Laurila, 1999; von Segesser *et al.*, 1999). HWE assumes that there are no

forces acting to change the allele frequencies. For a population to be in HWE there must be:

- No mutation – rare event to witness with microsatellite loci (Ehrich & Stenseth, 2001)
- No migration – migration into the population must not be significant enough to alter allele frequencies, or migration may be so common that population being studied is not genetically distinct enough from surrounding populations for migration to have an effect
- No selection – loci must not be linked to genes which are under selection
- Random mating within the population – no unaccounted for subdivision within the population (Ehrich & Stenseth, 2001; Sacks *et al.*, 2004) and no significant levels of inbreeding (von Segesser *et al.*, 1999)
- No genetic drift – generally levels of genetic drift are too slow to have an effect, however if the process is sped up for some reason then this will effect gene frequencies.

Any departure from HWE would indicate that one or more of the above factors is acting upon the population.

1.2.5 *F*-Statistics

The idea of using *F*-statistics to assess population structure was first introduced in by Wright in 1951 (Weir & Cockerham, 1984), and the formulas used to carry out the statistics were then updated and modified by Nei in the 1970s (Nei, 1973, 1977). Since that time the precise and correct method of calculating F_{ST}, F_{IT} and F_{IS} given various population parameters and dynamics has been debated (Robertson & Hill, 1984; Weir & Cockerham, 1984). However, all measurements of *F*-statistics are essentially based on the excess of homozygotes above the Hardy-Weinberg expectations of the population (Lampert *et al.*, 2003); F_{IT} being relative to the entire population, in this case the Cornish, Itchen and Dorset populations combined, F_{IS} being relative to the subpopulation, and F_{ST} being a measure of genetic difference between subpopulations (Robertson & Hill, 1984). The two measurements we are interested in for this study are F_{ST} and F_{IS} - F_{ST} being a measure of genetic differentiation between two different subpopulations within the whole (Chen *et al.*, 2005) and F_{IS} being a measure of levels of inbreeding within a subpopulation (Seppä & Laurila, 1999).

1.2.5i) Genetic Differentiation (F_{ST})

Genetic differentiation, F_{ST}, is a measure of the genetic drift between populations (Slatkin, 1995; Gutiérrez-Espeleta *et al.*, 2000). In other words, since they split how much have the allele frequencies of populations A and B drifted so that the populations no longer resemble each other genetically. The calculation works on the assumption that since separation the populations have been maintained under identical conditions, so that the samples from the two populations differ because of the genetic sampling between generations (Weir & Cockerham, 1984) - genetic sampling being the alleles present in the offspring of individuals which successfully mated. Remembering that microsatellite loci chosen for study are neutral, and therefore alleles are passed on to the next generation in a random, non-selective process.

Neighbouring populations are often still linked, with migration events still occurring between otherwise distinct populations. As it is likely that levels of migration would be expected to be lower between more geographically distant populations then it would follow that more geographically distant populations should be more genetically distinct

from each other (von Segesser *et al.*, 1999), a phenomenon known as isolation by distance (IBD) (Seppä & Laurila, 1999; Coyer *et al.*, 2003; Woxvold *et al.*, 2006). Therefore F_{ST} can be interpreted as a measure of the time since population divergence, as a measure of the effective migration rate, or as a measure of geographical separation (Slatkin, 1995; Goodman *et al.*, 2001; Walker *et al.*, 2001). However, the true cause of genetic differentiation is likely to be a combination of these factors (Seppä & Laurila, 1999; Lampert *et al.*, 2003) and this should always be taken into consideration whenever values of F_{ST} are being interpreted against real-life population dynamics.

1.2.5ii) Inbreeding Coefficients (F_{IS})

A population where relatively high numbers of offspring are born to pairs of related individuals is said to be inbred. Related individuals share a greater number of alleles than unrelated individuals and so the offspring of related individuals are more likely to inherit two alleles of the same size and are therefore likely to express high levels of homogeneity across loci (Keller & Waller, 2002). In a population where levels of inbreeding are high we would expect to see lower levels of heterozygosity than would be predicted given the allele frequencies of the population. The inbreeding coefficient, F_{IS}, is effectively a measure of heterozygosity deficit, following the assumption that a population with lower than expected levels of heterozygosity is more likely to be inbred (Van de Zande *et al.*, 2000).

1.2.6 Construction of a Neighbour-Joining Tree

Phylogenetic trees are more commonly used to look at the evolutionary history of species (Baliraine *et al.*, 2004) or to look at historic links between geographically separate populations – also known as phylogeography (Rowe *et al.*, 1998). However, trees can be used to infer evolutionary relationships between individuals. The method of carrying out allele-sharing neighbour-joining trees has been used in several studies using genetic differences between individuals to look at levels of within and between population variation (Rengmark *et al.*, 2006), as well as to look at relationships between species and breeds of wild and domestic animals (Edwards *et al.*, 2000; Tapio *et al.*, 2005).

Phylogenetic trees are commonly constructed using information provided by the DNA sequence variation of markers such as the mitochondrial control region. However, it is possible to use the information provided by microsatellite genotypes to construct a tree using the levels of allele sharing between genotypes as a measure of the genetic distance between them. A tree is then constructed using the neighbour-joining methodology developed by Saitou and Nei (1987).

Neighbour-joining trees are constructed using the principles of minimum evolution or maximum parsimony (Saitou & Nei, 1987), in other words the simplest route of evolution is taken as the correct one. The tree is created by using the genetic distances to calculate the relationships between operational taxonomic units (OTUs). OTUs can be populations (Kyle & Strobeck, 2001), groups of individuals within a population, or, as in this study, individual animals can represent separate OTUs (Estoup *et al.*, 1995; Edwards *et al.*, 2000; Tapio *et al.*, 2005; Rengmark *et al.*, 2006).

The neighbour-joining method starts with all relationships being equal and all OUTs positioned around a central node (Saitou & Nei, 1987). Branch lengths are calculated using Fitch and Margoliash's (1967) method, seen below as a calculation for the branch lengths between OUTs 1 and 2 (Saitou and Nei, 1987).

$$L_1 = (D_{12} + D_{1Z} - D_{2Z}) / 2$$

$$L_2 = (D_{12} + D_{2Z} - D_{1Z}) / 2$$

Where: z = a group of OUTs including all but OUTs 1 and 2

D = allele sharing distances

L = branch length

Pairs of OUTs are separated out into their own interior branches, with the pair that provides the smallest sum of branch lengths separated out first. This pair of OUTs is then treated as a single OUT in its own right during the next calculation of branch lengths. This process of adding branches is continued until all interior branches are found (Saitou & Nei, 1987).

1.2.7 Relatedness between Individuals

1.2.7i) Relatedness

Calculations for the measurement of genetic relatedness were originally designed for use in the investigation of altruistic behaviour (Queller & Goodnight, 1989). The calculations were used to help prove that individuals exhibiting altruistic behaviour were in fact related to the individual their behaviour was benefiting, and therefore that the behaviour was not truly altruistic.

The measurement of genetic relatedness can be used to look at levels of relatedness between groups as well as between individuals. The measure can be used to help study group behaviour and dispersal behaviour by looking at levels of same-sex and between-sex relatedness for individuals and groups of individuals (Taylor *et al.*, 1997; Kameyama *et al.*, 2002; Coltman *et al.*, 2003; Lampert *et al.*, 2003; Fuller *et al.*, 2005; Woxvold *et al.*, 2006). The technique can also be used to help to define family relationships, for example in instances where an observed relationship does not match the genetic reality (Lunn *et al.*, 2000). However, being designed to prove genetic relatedness between individuals for which assumed relationships are already known, caution must be taken when attempting to transfer the method to a more general situation of looking at pairwise relatedness in a population we know little about in terms of likely family relations.

The measurement of relatedness between individuals works on the basis that related individuals will show a greater proportion of shared alleles than the background rate of the population. Parents and their offspring share 50% of their DNA and will therefore have a much higher percentage of alleles in common with each other than with an unrelated member of the population. Similarly, full siblings will share approximately 50% of their DNA. For each locus an offspring inherits one of two possible alleles from each parent, and so it is pure probability that 50% of the time an individual inherits the same allele from its parent as a sibling does.

Measured levels of pairwise relatedness should distribute normally around the value of zero, with zero representing the average level of relatedness. This means that pairs with values below zero are considered to indicate below average levels of relatedness, whilst pairs with positive values of relatedness are considered to show above average levels of

relatedness. However it is only pairs with the highest values of relatedness for which true genetic relationships can be assumed. The exact cut-off point is debatable, however in this study we have taken it to be between 0.4 and 05.

1.2.7ii) Parentage

Parentage assignment is used to assign parentage to an offspring or to identify a parent-offspring bond. It is more commonly used in situations where at least one of the parents is known and therefore can be genotyped; for example, in situations where offspring are found with their mother and knowledge of the paternity of the offspring is desired (Morin *et al.*, 1993; Utami *et al.*, 2002). In these situations the genotype of the known parent can be used to eliminate alleles from the offspring. In other words, if the individual in question contains one allele in common with its mother then it must have inherited its second allele from its father, and therefore the true paternal individual must contain an allele to match.

The technique becomes much less accurate in situations where neither parent is known because the known parent cannot be used as a reference to eliminate any of the possible alleles. The method is also less accurate when used in an open system (i.e. when animals can move into and out of the system being tested freely), particularly if not all the animals in the system have been genotyped. Therefore the technique must be used with caution under these circumstances and the results of analysis can only be used as guidance and not as true parentage assignment.

1.2.8 Mitochondrial Control Region Haplotypes

1.2.8i) Mitochondrial DNA

Mitochondria are organelles that exist as subunits in the plasma of animal cells. It is thought that mitochondria were once free-living, independent prokaryotic organisms, defined as a species in their own right. It is thought that mitochondria moved into and formed a symbiotic relationship with the Eukaryotic cells of early animal life; the mitochondria converted food sources into adenosine triphosphate (ATP), a more useful energy source for the cell, and the Eukaryotic cell provided a safe and stable environment for the mitochondria to survive in.

One of the reasons for the theory of mitochondria once having been a separate organism is that each mitochondria contains a ring of DNA. Both organisms have now become dependant on each other and one would not be able to survive without the other. In fact it has even been suggested that a DNA war has been waged, with gene transfer and subsequent responsibility for certain survival needs being swapped between the two DNA sources so both the mitochondria and the cell are locked into fulfilling their roles in order to survive.

In birds and mammals, where sexual reproduction is the norm, mitochondria are maternally inherited. The larger gametes of the females contain far higher numbers of mitochondria and it is from this cell that the offspring develop, meaning that the mitochondria are always passed from mother to offspring.

Most mammal and bird cells contain one complete copy of nuclear DNA (i.e. two sets of chromosomal DNA), exceptions being red blood cells which have no nuclease and consequently no DNA and gametes which for obvious reasons contain half the DNA content of somatic cells. The number of mitochondria and conversely the number of mitochondrial DNA strands varies depending on the cell type, but generally cells contain much greater numbers of mitochondrial DNA copies and in tissues with high energy requirements, such as muscle this difference in DNA copy number can be very large. This difference in copy number per cell means that it is therefore much easier to obtain copies of mitochondrial DNA and this is one of the main reasons why, particularly in early studies of genetic structure, mitochondrial DNA is often chosen above nuclear.

1.2.8ii) Mitochondrial Control Region

The mitochondrial control region, also sometimes referred to as the mitochondrial d-loop, is a stretch of DNA that makes up part of the mitochondrial loop. Often considered a relatively variable region of DNA, the control region is one of the regions of DNA commonly used in phylogenetic studies (Nyakaana & Arctander, 1999; Vernesi *et al.*, 2002; Valdiosera *et al.*, 2007) where differences in the sequences, known as haplotypes, are used to look at differences between populations.

1.3 Introduction to Polybrominated Diphenyl Ethers (PBDEs)

1.3.1 Structure and Use

1.3.1i) PBDE Structure and congeners

Polybrominated Diphenyl Ethers (PBDEs) are similar in structure to PCBs in that they are constructed of two hydrocarbon rings. However, unlike PCBs the two rings are joined through an oxygen molecule, forming an ether (Figure 1.8).

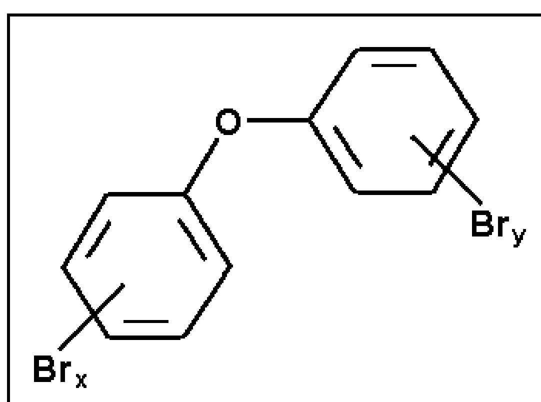

Figure 1.8 Basic structure of polybrominated diphenyl ethers (PBDEs)

The basic chemical formula for PBDEs is $C_{12}H_{(9-0)}Br_{(1-10)}O$ and, as with PCBs, the number and location of bromine molecules around the two hydrocarbon rings dictates the congener. The result is that there are 209 possible PBDE congener combinations, ranging from mono-BDEs with just one bromine molecule to deca-BDE (also known as BDE-209) with ten bromine molecules filling each of the ten available carbon bonds.

1.3.1ii) Industrial Mixtures and Their Uses

The United Kingdom has particularly stringent fire safety requirements. This stringency apparently pays off as, in the UK alone it has been predicted that since 1988 over 3000 lives have been saved through the use of flame retardants (BSEF, 2008).

PBDEs have been used as additives to flame retard materials since the 1970s (Vethaak *et al.*, 2002). Over the years they have been used in a wide variety of materials, including electrics/electronics, textiles (not clothing), building materials and household appliances (Hooper *et al.*, 2004; BSEF, 2006). PBDEs have been used widely in the casings and internal components of TVs and computer equipment, as well as for car components, including fabrics, plastics and electronic components (Hooper *et al.*, 2004; BSEF, 2006). PBDEs have also been used widely in household and office furnishings, such as sofas and chairs, in foam under-lays for carpeting and even in mattresses (Alcock *et al.*, 2003; BSEF, 2007).

There are four main commercial mix's of PBDEs, TetraBDE, PentaBDE, OctaBDE and DecaBDE, composed of mixtures of different congeners and different congener sizes (Table 1.4)(Vethaak *et al.*, 2002). Due to the chemistry of BDE structures certain congeners are naturally more easily produced than others, and the individual congeners most commonly produced are BDE-47 (TetraBDE), BDE-99 and BDE-100 (PentaBDE), BDE-153 and BDE-154 (HexaBDE), BDE-183 (OctaBDE) and BDE-209 (DecaBDE). Therefore it is these congeners which make up the bulk of the material in the commercial PBDE mixtures produced.

Table 1.4 Composition of commercial PBDEs. Data taken from Darnerud *et al.* (2001), originally from IPCS (1994)

Congener group	Commercial product			
	TetraBDE (%)	PentaBDE (%)	OctaBDE (%)	DecaBDE (%)
Unknown	7.6			
TriBDE		0-1		
TetraBDE	41-41.7	24-38		
PentaBDE	44.4-45	50-62		
HexaBDE	6-7	4-8	10-12	
HeptaBDE			43-44	
OctaBDE			31-35	
NonaBDE			9-11	0.3-3
DecaBDE			0-1	97-98

PBDEs make good flame retardants because of the ability of halogen atoms, released during the thermal decomposition of the PBDE structure, to chemically reduce and retard the development of fire (Darnerud *et al.*, 2001). In addition, commercial PBDEs tend to be relatively resistant to physical, chemical and biological degradation due to

properties such as a high boiling temperature, a low vapour pressure at room temperature and a low solubility in water (Darnerud *et al.*, 2001; Martin *et al.*, 2004).

PBDEs are only one type of brominated flame retardant and until recently they made up approximately one third of the global brominated flame retardant market (Meerts , 2000; Darnerud *et al.*, 2001). There is often great difficulty in working out precisely what a countries usage of PBDEs is, particularly as many products will be used in manufacture without proper knowledge of the components of that product being known to the user. However, an estimate 100,000 tonnes of pentaBDE has been manufactured globally since the 1970s, of which approximately 15000 tonnes have been manufactured and/or used within Europe, approximately 20% (3000 tonnes) of that in the UK (Alcock *et al.*, 2003).

During the 1990s, as concerns over the safety of the penta and octaBDE commercial mixtures increased, so demands for these products decreased significantly (Alcock *et al.*, 2003). In contrast deca-BDE was, at the time thought to be safe to use, and so production of this mixture became relatively high. During the 1990s global PBDE production was approximately 40,000 tonnes per year, of which approximately 10% was pentaBDE, 15% octaBDE and 75% decaBDE (Darnerud *et al.*, 2001). During 1999 alone global demand for decaBDE was estimated to be 54,800 tonnes, approximately 14% (7672 tonnes) of which being used or produced in Europe (Alcock *et al.*, 2003).

1.3.1iii) Bans on Use of PBDEs

The industrial mixture TetraBDE was the first commercial PBDE mix to be banned from use (Darnerud *et al.*, 2001). The PBDE technical mixtures pentaBDE and octaBDE were banned from manufacture, use or sale within the European Union in 2003 (The European Parliament and the Council of the European Union, 2003), although the ban did not fully come into place until the summer of 2006 (BSEF, 2008). A recommended reduction in the use of decaBDE was passes along with the ban for penta and octaBDE and a full ban came into place in 2008.

1.3.2 Levels in the Environment

PBDEs are generally used as an additive flame retardant, which means that they tend to leach out and escape from the product more easily than more reactive flame retardants (Darnerud *et al.*, 2001, Alcock *et al.*, 2003). Martin *et al.* (2004) predicted that under worst case scenarios between the years of 1997 and 2004 between 6 and 12 x10^8 kg of PBDEs could have been released into the environment via electronic waste products alone. Similar in structure to PCBs, PBDEs tend to be transported and distributed through environmental systems in a similar manor (Strandberg *et al.*, 2001; Martin *et al.*, 2004). PBDEs are persistent environmental contaminants, meaning it takes a long time for them to be lost from a system (Li *et al.*, 2006). They are also capable of undergoing long-range atmospheric transport and distribution meaning that, like PCBs, PBDEs can be deposited in areas of the global environment distant from original sources (de Wit *et al.*, 2006). They have low water solubility and a high binding affinity and so tend to adhere to particular matter. For this reason they often migrate into sediment at the bottom of rivers, lakes, estuaries and seas. Smaller congeners, such as the tetra- to hexa-BDEs, tend to be more mobile than larger, heavier congeners, such as the nona- and Deca-BDEs and can be transported large distances around the globe (de Wit *et al.*, 2006). PBDEs are lipophilic and have a tendency to resist biological degradation and so, in particular the smaller congeners, tend to build up in biota.

Concentrations of ∑BDEs tend to be found at lower concentrations than ∑PCBs, with ∑PCBs often up to 100 to 500 times higher than observed ∑BDEs (de Wit *et al.*, 2006; Jaspers *et al.*, 2006). However, this is in part due to the higher number of PCB congeners released into the environment. Therefore, on an individual congener basis the most concentrated BDE congeners are of significance when compared to concentrations of PCB congeners. The main BDE congeners reported in the environment are BDE-47 (TetraBDE), BDE-99 and BDE-100 (PentaBDE), BDE-153 and BDE-154 (HexaBDE), BDE-209 (DecaBDE), and to a lesser degree BDE-183 (HeptaBDE) (Vethaak *et al.*, 2002), mirroring the congeners most commonly produced in industry (Manchester-Neesvig *et al.*, 2001).

1.3.2i) Air

Air samples tend to reflect industrial PBDE usage in the surrounding areas or in source areas. The congeners that tend to be present include BDE-47, BDE-99, BDE-100, BDE-153 and BDE-154, reflecting leaching from the pentaBDE industrial mixture (de Wit *et al.*, 2006). The congeners BDE-47 and BDE-99 tend to be found in particularly high concentrations. In a study of air samples collected from four sampling locations situated close to the Great Lakes in North America Strandberg *et al.* (2001) found that BDE-47 made up 50-65% of the congener composition, whilst BDE-99 made up 35-40%, the concentrations of these two BDE congeners being approximately equal to concentrations of the seven most abundant PCB congeners (PCB-28, -52, -101, -118, -153, -153 and -183). In addition, concentrations of PBDEs were significantly higher in air samples collected at the Chicago site as opposed to the other more rural locations ($p < 0.01$)(Strandberg *et al.*, 2001). Larger PBDE congeners tend to be less volatile, as a result BDE209 is generally only found in air samples at trace levels (Strandberg *et al.*, 2001)

1.3.2ii) Sewage Sludge

Due to their hydrophobic nature, PBDEs entering sewage treatment systems tend to adhere to particulate matter which is often then deposited in the sewage sludge that is produced during the treatment process (Langford *et al.*, 2007). Monitoring the concentrations which build up in this sludge is important, particularly as sewage sludge is often applied to land as a fertiliser (Knoth *et al.*, 2007)

De Wit *et al.* (2007) investigated PBDE concentrations in sewage sludge samples from 50 Swedish sewage treatment plants (STPs). They found BDE-209 to be the most common congener, at a mean concentration of 120 $ng.g^{-1}$ dry weight. The congeners BDE-99 and BDE-47 were also found at relatively high concentrations (mean concentrations of 60 $ng.g^{-1}$ dry weight and 49 $ng.g^{-1}$ dry weight respectively), suggesting that, despite pentaBDE being discontinued from use in Sweden in the 1990s, residues of the mixture were still leaching out into the sewage system from diffuse sources (de Wit *et al.*, 2007).

In a similar study of sewage sludge from eleven sewage treatment plants in Germany Knoth *et al.* (2007) also found BDE-209 to be the dominant congener at a range of 97.1 to 2217 $ng.g^{-1}$ d.m. (median 256 $ng.g^{-1}$ d.m.), as opposed to 12.5 to 288 $ng.g^{-1}$ d.m. (median 108 $ng.g^{-1}$ d.m.) recorded for the remaining ∑BDE (BDEs-28, -47, -99, 153, -154, -183). Again BDE-99 was the second most dominant congener, followed by BDE-47, suggesting that both deca and pentaBDE industrial mixtures are leaching into the sewage system, despite all BDE mixtures being voluntarily phased out in Germany during the late 1980s (Knoth *et al.*, 2007)

Concentrations in sewage sludge will often reflect local point sources, such as textile or polystyrene production factories, and will vary according to the size of the residential and industrial areas feeding them (de Wit *et al.*, 2007). Across Europe concentrations of BDE-209 in sewage sludge vary from below the detection limit to 2217 $ng.g^{-1}$ d.m. (Knoth *et al.*, 2007), with an outlying high of 18032 $ng.g^{-1}$ d.m. found in Spain in 2002 (Fabrellas *et al.*, 2004). Total tetra- to hepta-BDE concentrations range from 0.3 $ng.g^{-1}$ d.m. in the Netherlands in 1999 (de Boer *et al.*, 2003) to 327 $ng.g^{-1}$ d.m. found in Germany in 2001 (Kuch *et al.*, 2001; Knoth *et al.*, 2007).

In edition to high concentrations in sewage sludge, concentrations of BDE-209 can also be high in the effluent being discharged from sewage treatment plants (de Boer *et al.*, 2003). Certain types of treatment methods results in high proportions of particulate matter in the effluent being discharged and therefore high concentrations of BDE congeners, such as BDE-209 associated with this particulate component (de Boer *et al.*, 2003).

1.3.2iii) Sediments

Because of their insoluble nature PBDEs, in particular the larger congeners are not found in particularly high concentrations in water samples taken from river, estuarine or marine waters (Darnerud *et al.*, 2001). However, they do leach out into these environments and rather than remain in the water body, due to their high binding affinity they tend to adhere to particular matter (de Boer *et al.*, 2003), meaning that they are often found at relatively high concentrations in the sediment at the bottom of aquatic systems. Larger congeners, which are generally less mobile because of their size and

are less volatile than lower congeners, tend to be found at relatively high concentrations in sediment samples (Darnerud *et al.*, 2001; de Boer *et al.*, 2003). As the main component of the industrial mixture decaBDE, BDE-209 is often the most dominant congener found within sediment samples (Sawal *et al.*, 2004). In riverine and marine sediment samples from seven sites on the Drammens River and four sites from Drammensfjord in Norway 80 – 99% of the ∑BDE content was composed of BDE-209 (Schlabach *et al.*, 2004). Li *et al.* (2006) measured PBDE concentrations in a total of 199 sedimet samples collected from 16 different locations in the Great Lakes in North America, where concentrations of ∑BDE (BDE-28, -47, -66, -85, -99, -100, -153,- 154 and -183) ranged from 0.5 to 6.7 $ng.g^{-1}$ dry weight, whilst BDE-209 concentrations ranged from <4.0 to >240 $ng.g^{-1}$ dry weight (average of 50 $ng.g^{-1}$). Although the general trend is for BDE-209 to dominate in sediment samples, the ratios of congeners are sometimes altered in situations where point sources, such as a landfill site or a factory, introduce high levels of congeners consistent with the Penta-BDE or Octa-BDE mixture (Sawal *et al.*, 2004).

The actual concentrations of PBDEs found in sediment can vary widely from location to location, as pointed out by Sawal *et al.* (2004) with a table of values from around Europe (reproduced in Table 1.5). Concentrations can also vary widely in sediment samples taken from locations within the same country and will often reflect areas of high industrial activity and high usage of PBDE mixtures (de Boer *et al.*, 2003). In addition, PBDE concentrations in sediments samples are increasing over time, both in terms of lower BDE congeners indicative of the pentaBDE mixture and higher BDE congeners such as BDE209 (Li *et al.*, 2006).

Table 1.5 Concentrations of BDE-209 in sediments from around Europe Note the variations in the unit of measurement (data obtained from Sawal *et al.*, 2007).

Location	Sediment concentration of BDE-209	Reference
Germany	0.5 to 17.4 $ng.g^{-1}$ dry wt or 10 to 230 $ng.g^{-1}$ OC	Sawal *et al.* (2004)
River Rhine and Lobith, Germany/Holland	84 $ng.g^{-1}$ dry wt	de Boer *et al.* (2003)
Holland	470-990 $ng.g^{-1}$ dry wt	de Boer *et al.* (2001)
River Tees, UK	up to 1400 $ng.g^{-1}$ OC	Allchin *et al.* (2001)
Britain and Ireland	up to 104,700 $ng.g^{-1}$ OC	de Boer *et al.* (2001)

OC = normalised to the organic carbon (OC) content

1.3.2iv) Aquatic Invertebrates and Fish

The congener BDE-47 generally dominates the congener profiles of aquatic wildlife. In Marine fish BDE-47 can make up 90-95% of the ∑BDE congener concentration (de Wit *et al.*, 2006). In a study of brown trout (*Salmo trutta*, n = 217) from Switzerland, Hartmann *et al.* (2007) report that BDE-47 was the only congener quantifiable in many of the liver and bile samples analysed. In a study of three fish species, eel (*Anguilla anguilla*), carp (*Cyprinus carpio*) and gibel carp (*Cyprinus auratus gibel carpio*), from freshwater systems in Belgium, BDE-47 was found to represent 63-82% of the ∑BDE congener profile (Covaci *et al.*, 2004).

In aquatic invertebrate and fish species BDE-47 is generally expressed as the most dominant congener, with BDE-100 found at lower but usually still significant concentrations (Boon *et al.*, 2002; Ikonomou *et al.*, 2002; Covaci *et al.*, 2004). Comparative concentrations of BDE-99 vary. Occasionally this congener is found at concentrations paralleling those of BDE-47, as in freshwater zebra muscles (*Dreissena polymorpha*) from Holland or Belgium (de Boer *et al.*, 2003; Covaci *et al.*, 2004). More commonly concentrations of BDE-99 are significantly lower than BDE-47 but approximately equal to those of BDE-100. For example, in mussels (*Mytilus galloprovincialis*) from the coast of Portugal (Gama *et al.*, 2006), dungeness crab (*Cancer magister*, n = 6 composites of 2-6 organisms) and English sole (*Pleuronectes vetulus*, n = 14 composites of 1-13 organisms) from coastal British Columbia in Canada (Ikonomou *et al.*, 2002), lake trout (*Salvelinus namaycush*) from North American (Lake Ontario, Log BDE-99 = 4.48, Log BDE-100 = 4.44; Lake Michigan, Log BDE-99 = 4.33, Log BDE-100 = 4.29; Whittle *et al.*, 2004), and trout (*Salmo trutta*) and perch (*Perca fluviatilis*) from Drammensfjord in Norway, where concentrations of BDE-100 ranged from 9-22% of ∑BDE (across six local fish species) and in trout and perch BDE-99 ranged from 14-31% of ∑BDE (Schlabach *et al.*, 2004). In some instances concentrations of BDE-99 are much lower than those of BDE-100, indicating metabolism or preferential uptake/excretion of BDE-99 in those species (Covaci *et al.*, 2004). Examples include eel (*Anguilla anguilla*), carp (*Cyprinus carpio*) and gibel carp (*Cyprinus auratus gibel carpio*) from freshwater systems in Belgium (Covaci *et al.*, 2004) and orfe (*Leuciscus idus*), cod (*Gadus morhua*), flounder (*Platichthys flesus*) and eel (*Anguilla anguilla*) from Drammensfjord in Norway, in which BDE-99

concentrations ranged from 0.3-5% of ∑BDE whereas BDE-100 concentrations fell within the range of 9-22% of ∑BDE (Schlabach *et al.*, 2004).

There is some evidence to suggest that PBDEs are capable of biomagnifying up the aquatic food chain (Gama *et al.*, 2006) and this can result in elevated concentrations in predatory fish. For example, ∑BDE concentrations in polar cod (*Boreogadus saida*) from Svalbard in Norway were found to range from 10-15 ng.g^{-1} lipid wt, whereas concentration in three species of pelagic zooplankton (*Calanus glacicals, Thysanoessa inermis* and *Paratemisto libellula*) approximated 1 ng.g^{-1} lipid wt (Jenssen *et al.*, 2004).

Table 1.6 Concentrations (ng.g^{-1} ww) of ∑BDE found in a predatory fish species, lake trout, and three common prey species (Whittle *et al.*, 2007).

	Lake trout, *Salvelinus namaycush*	Smelt, *Osmerus mordax*	Slimy sculpin, *Cottus cognatus*	Alewife, *Alosa pseudoharengus*
Lake Michigan	148.23	10.02	5.37	30.40
Lake Ontario	181.32	17.77	19.76	26.75

Table 1.7 Concentrations of BDE-47 in species of invertebrate and fish from a North Sea food web (Boon *et al.*, 2002).

Species	mean	median	range
Sea star, *Asterias rubens*	22	19	(3.4 – 56)
Hermit crab, *Pagurus bernhardus*	38	29	(8.6 – 118)
Whelk, *Baccinum undatum*	10	5.5	(2.6 – 30)
Cod, *Gadus morhua*			
liver	133	99	(63 – 307)
fillet	43	34	(26 – 74)
Whiting, *Merlangius merlangus*			
liver	70	74	(7.6 – 132)
fillet	26	28	(7.1 – 40)

In a study of a food web in two North American lakes Whittle *et al.* (2007) found biomagnification factors ranging from 3.7 to 21.9 (Table 1.6). However, Boon (2002) found what they considered to be very little evidence of biomagnification between species of North Sea invertebrate and Sea fish species (Table 1.7). They consider that the differences observed between the species are likely to be due to the differences in

volume to surface area ratios between the species, with the smaller species having a greater relative surface area, and in particular a greater gill surface area for the removal of contaminants (Boon *et al.*, 2002).

There is very little evidence than invertebrates are capable of bioaccumulating large congeners, such as BDE209. Klosterhaus *et al.* (2007) found that marine polychaete worms (*Nereis virens*) only accumulated BDE-209 when exposed to a spiked sample and not when exposed to field sediments, even when the samples contained naturally high concentrations of BDE-209. Even from spiked samples bioaccumulation was low (Bioaccumulation factor <0.05). They also found that worms exposed to a spiked sample containing both penta and decaBDe would preferentially accumulate penta congeners over BDE-209 suggesting that under natural conditions polychaete worms are unlikely to bioaccumulate BDE-209 (Klosterhaus *et al.*, 2007).

This apparent lack of bioaccumulation potential has meant that until recently it was believed that highly brominated congeners, such as BDE-209 were unlikely to cause a problem in biota. As a consequence many previous studies of invertebrate and fish species have not attempted to measure BDE-209 (Ikonomou *et al.*, 2002; Hartmann *et al.*, 2007). However, in those studies which have measured BDE-209 concentrations in invertebrates and fish the congener is often only found at trace concentrations (Boon *et al.*, 2002), and in some species , such as the freshwater zebra mussel (*Dreissena polymorphia*), this low BDE-209 concentration is most likely due to particulate matter present in the gut (de Boer *et al.*, 2003). In sediments these larger congeners tend to be found at higher concentrations than the lower congeners. The greater availability but lower uptake of the higher congeners suggests that bioaccumulation of the higher congeners is relatively low (Darnerud *et al.*, 2001).

1.3.2v) Marine Mammals

As with marine fish, BDE-47 is often the most dominant congener found in marine mammal species (Boon *et al.*, 2002; She *et al.*, 2002), followed by BDE-99 and BDE153 (de Wit *et al.*, 2006). For example, in ringed seals (*Phoca hispida*, n = 19) from east coast of Greenland the congener BDE-47 accounts for an average of 82% and

77% of ∑BDE concentrations for males and females respectively (Vorkamp *et al.*, 2004).

Concentrations of PBDEs can be relatively high in marine mammals. For example, concentrations of ∑BDE in harbour porpoise (*Phocoena phocoena*, n = 5) from coastal British Columbia ranged from 350 to 2300 ng.g^{-1} lipid wt (Ikonomou *et al.*, 2002). Some of the highest concentrations found in any marine mammals were also found in water close to the North American continent, where concentrations of PBDEs in the liver tissue from 80 female Southern sea otters (*Enhydra lutris nereis*) from California ranged from 10 to 26,800 ng.g^{-1} lipid wt (mean 2200 ±3700 ng.g^{-1} lipid wt; Kannan *et al.*, 2007), and concentrations in 26 male Californian sea lions (*Zalophus californianus*) ranged from 570 to 24,240 ng.g^{-1} lipid wt (Stapleton *et al.*, 2006). In sea lions BDE-47 was the dominant congener at approximately 55% of ∑BDE, followed by BDE-100. In many of the sea lions BDE-99 was found at lower concentrations than BDE-100, which Stapleton *et al.* (2006) suggest is a sign of metabolism of the BDE-99 congener. The congener BDE-209 was measured but not detected in any of the samples analysed (Stapleton *et al.*, 2006).

In a study of blubber samples collected from eight species of marine mammals from Asian waters, Kajiwara *et al.* (2006) found concentrations ranging from 6.0 ng.g^{-1} lipid wt in a spinner dolphin (*Stenella longirostris*) sample from the coast of India to 6000 ng.g^{-1} lipid wt in a humpback dolphin (*Sousa chinesis*) from the coast of Hong Kong. BDE-47 dominated the congener profiles of many of the species studied. However some species, such as the Dall's porpoise (*Phocoenoides dalli*, n = 10), finless porpoise (*Neophocaena phocaenoides*, n = 5) and melon-headed whale (*Peponocephala electra*, n = 5) (all from Japanese waters), contained relatively high concentrations of the congener BDE-154, and to a lesser extent BDE-153. The congener BDE-209 was measured but was not found in any of the samples analysed (Kajiwara *et al.* 2006).

In terms of how these mammals build up these high concentrations, it is believed that PBDEs are capable of biomagnifying up the food chain and therefore higher relative concentrations are to be observed in top predators such as marine mammals. Jenssen *et al.* (2004) observed significant levels of biomagnification between polar cod (*Boreogadus saida*) and ringed seals (*Phoca hispida*) in Svalbard, Norway, with average ∑BDE concentrations of 10-15 ng.g^{-1} lipid wt in cod and 50-100 ng.g^{-1} lipid wt

in seals. However polar bear (*Ursus maritimus*), the species at the top of this paricular food chain, contained average ∑BDE concentrations of just 15-30 ng.g^{-1} lipid wt. The fact that polar bear contain relatively low concentrations compared to those found in ringed seals or polar cod (both prey species of the bear), combined with the fact that the polar bears congener pattern is dominated by BDE-153 as well as BDE-47(de Wit *et al.*, 2006), suggests that the species is capable of metabolising several BDE congeners (Jenssen *et al.*, 2004).

Table 1.8 Concentrations (ng.g^{-1} ww) of BDE-47 in species of fish and mammals, representing different trophic levels of the North Sea food web (Boon *et al.*, 2002).

Species	mean	median	range
Cod, *Gadus morhua*			
liver	133	99	(63 – 307)
fillet	43	34	(26 – 74)
Whiting, *Merlangius merlangus*			
liver	70	74	(7.6 – 132)
fillet	26	28	(7.1 – 40)
Harbour porpoise, *Phocoena phocoena*			
liver	1331	720	1.2-4877
blubber	864	796	245-1312
Harbour seal, *Phoca vitulina*			
liver	1328	368	95-5065
blubber	1236	210	57-9248

Table 1.9 Concentrations of BDE-47 and BDE-99 in a food web from Svalbard, Norway (Wolkers *et al.*, 2006a).

Species	Congener concentrations as geometric means (ng.g^{-1} lipid wt)	
	BDE-47	BDE-99
White whales, *Delphinapterus leucas* (n=4)	68.6	3.1
Narwhales, *Monodon monoceros* (n=3)	172.9	22.1
Polar cod, *Boreogadus saida*	0.066	0.012
Halibut, *Reinhardtius hippoglossoides*	0.162	0.009

Significant levels of biomagnification where observed between fish and mammal species representing different trophic levels of the North Sea food web (Table1.8; Boon

et al., 2002). Wolkers *et al.* (2006a) also found evidence of biomagnification between two species of fish and marine mammal sampled from the coast of Svalbard in Norway (Table 1.9). Schooling fish such as polar cod make up an important part of the diet of both species of whale, whilst narwhale are also known to take a large proportion of deepwater prey items such as halibut (Wolkers *et al.*, 2006a).

1.3.2vi) Birds

Birds have been recognised as useful indicators of local contamination due to their ability to accumulate contaminants, including PBDEs (Lam *et al.*, 2007). The dominant congener in many fish eating bird species is BDE-47 (Table 1.10; Vorkamp *et al.*, 2004; de Wit *et al.*, 2006; Braune *et al.*, 2007). As this congener is also found in high concentrations in many freshwater and marine species, this would suggest transfer through the food chain into aquatic feeding birds (Vorkamp *et al.*, 2004; Jaspers *et al.*, 2006). Other dominant congeners include BDE-99, -100, -153 and -154, indicative of congeners originating from the pentaBDE industrial mixture. In terrestrial feeding birds the congeners BDE99 and BDE153 are often the most dominant congeners, with BDEs-47, 100 and 183 also found at relevant concentrations (Table1.10; de Wit *et al.*, 2006). The differing congener patters found in aquatic and terrestrial birds are likely to occur due to differing exposure routes, such as differences in the diets of species. It highlights the need to be aware of the differences between the two systems in order to fully understand the exposure hazards for biota inhabiting different habitat systems.

Concentrations of BDE-209 are still relatively low in many species of bird (de Boer *et al.*, 2007; Gauthier *et al.*, 2007). However, there is evidence to suggest that birds feeding in terrestrial environments may be more exposed to higher BDE congeners, such as BDE209 (Law *et al.*, 2003; Jaspers *et al.*, 2006). In addition, there are isolated cases of aquatic species expressing particularly high concentrations of BDE-209; for example, Chinese pond heron (*Ardeola bacchus*; n = 5; range 3.1 to 290 $ng.g^{-1}$ lipid wt; Lam *et al.*, 2007), and glaucous gulls (*Larus hyperboreus*; range 23 to 53 $ng.g^{-1}$ lipid wt; Herzke *et al.*, 2003). There is evidence that the blood plasma (n = 49) and egg yolks (n = 31) of glaucous gulls in the Norwegian Arctic contain octa-BDE (BDE-196, 197, 201, 202, 203 and 205) and nona-BDE (BDE-206, 207 and 208) congeners, breakdown products of the BDE-209 congener (Verreault *et al.*, 2007).

Table 1.10 BDE congener composition and BDE209 concentrations in aquatic and terrestrial bird species.

Species	Diet / lifestyle and sample type	Location /sample numbers	Congener pattern – most dominant to least	BDE-209	Reference
Common Buzzard, *Buteo buteo*	Terrestrial predator, liver	Belgium n=16	BDE153, BDE47, BDE183 and BDE99, then BDE154, BDE100	ND	Jaspers *et al.* (2006)
Kestrel, *Falco tinnunculus*	Terrestrial predator, liver	Belgium n=3	BDE153, BDE99, BDE183, BDE100, then BDE47 and BDE154	* (1) 85 $ng.g^{-1}$ lipid wt	Jaspers *et al.* (2006)
Sparrowhawk, *Accipiter nisus*	Terrestrial predator, liver	Belgium n=5	BDE99, BDE47, BDE100, BDE153, BDE183, BDE154	* (2) 52 $ng.g^{-1}$ lipid wt	Jaspers *et al.* (2006)
Long-eared owl, *Asio otus*	Terrestrial predator, liver	Belgium n=6	BDE153, BDE99, BDE47, BDE183, BDE100	* (1) 66 $ng.g^{-1}$ lipid wt	Jaspers *et al.* (2006)
Barn owl, *Tyto alba*	Terrestrial predator, liver	Belgium n=7	BDE99 and BDE153, then BDE47, BDE100, BDE183	* (3) 59 $ng.g^{-1}$ lipid wt	Jaspers *et al.* (2006)
Peregrin falcon *Falco peregrinus*	Terrestrial predator, eggs	Sweden	BDE153, BDE99, BDE100, BDE154, then BDE47 and BDE183	28-430 $ng.g^{-1}$ lipid wt	Sellström *et al.* (2001)
Little owl *Athene noctua*	Terrestrial predator, eggs	Belgium n=40	BDE99, BDE153, BDE47, BDE183, BDE154, BDE100	<8-17 $ng.g^{-1}$ lipid wt	Jaspers *et al.* (2004)
Grey heron, *Ardea cinerea*	Fish eating, liver	Belgium n=9	BDE47, BDE100, BDE154, BDE153, BDE99	ND	Jaspers *et al.* (2006)
Great crested grebe, *Podiceps cristatus*	Fish eating, liver	Belgium n=3	BDE47, BDE100, BDE99, then BDE153 and BDE154	ND	Jaspers *et al.* (2006)
Little egret, *Egretta garzetta*	Fish eating, eggs	China n=5	BDE47, BDE154, BDE100, BDE153, BDE99	<0.5-3.8 $ng.g^{-1}$ lipid wt	Lam *et al.* (2007)
Black crowned night heron, *Nycticorax nycticorax*	Fish eating, eggs	China n=5	BDE47, BDE154, then BDE100 and BDE153, then BDE99, BDE183	<0.5-59 $ng.g^{-1}$ lipid wt	Lam *et al.* (2007)
Chinese pond heron, *Ardeola bacchus*	Fish eating, eggs	China n=5	BDE209, BDE47, then BDE153 and BDE154, then BDE99 and BDE100	3.1-290 $ng.g^{-1}$ lipid wt	Lam *et al.* (2007)
Cattle egret, *Bubulcus ibis*	Fish eating, eggs	China n=5	BDE153, BDE183, BDE209, BDE47, BDE154, BDE100, BDE197	<0.5-75 $ng.g^{-1}$ lipid wt	Lam *et al.* (2007)
Ivory gull *Pagophila eburnea*	Marine bird, eggs	Arctic	BDE47, BDE99, BDE28, BDE100 and BDE138, then BDE153	<0.01 $ng.g^{-1}$ wet wt	Braune *et al.* (2007)
European shag, *Phalacrocorax aristotelis*	Marine bird, hatchlings	Norway n=30	BDE100, BDE47, BDE153, BDE99	N/A	Murvoll *et al.* (2006)

* The numbers in parenthesis show the number of individuals in which BDE209 was at measurable concentrations. Concentrations therefore are averages from these individuals only

1.3.2vii) Terrestrial Mammals

Many consider that the highest concentrations of PBDEs are likely to be found in marine biota, and as a consequence little research has been carried out to study concentrations in terrestrial mammals. As a rule concentrations of PBDEs do tend to be comparatively low in terrestrial mammals (Darnerud *et al.*, 2001), however new research suggests that there is more to be learnt about the concentrations and behaviour of PBDEs in terrestrial biota (Voorspoels *et al.*, 2006; Kinisue *et al.*, 2008).

Herbivorous mammals express relatively low concentrations of PBDEs (Law *et al.*, 2003; de Wit *et al.*, 2006). For example, in reindeer (*Rangifer tarandus*) and moose (*Alces alces*) from Sweden ∑3BDE concentrations ranged from 0.5-1.7 $ng.g^{-1}$ lipid wt (Law *et al.*, 2003; original data from Sellström *et al.*, 1999). The congeners expressed by herbivores tend to reflect those found in industrial mixtures, with BDE-47 and BDE-99 being dominant congeners in the few herbivorous terrestrial species studied (de Wit *et al.*, 2006). As with birds, more predatory species of terrestrial mammals often contain higher concentrations of BDE-153 (Mariussen *et al.*, 2008).

In a study of racoon dogs (*Nyctereutes procyonoides*, n = 39) from Japan concentrations of PBDEs, although still much lower than concentrations of many other contaminants, were found to be significant at a range of 0.36 to 250 $ng.g^{-1}$ lipid wt (Kunisue *et al.*, 2008). Average ∑BDE concentrations ranged from 18 $ng.g^{-1}$ lipid wt in the region of Osaka (n = 8) to 34 $ng.g^{-1}$ lipid wt in the region of Kanagawa (n = 10) (Kunisue *et al.*, 2008) – still comparatively low when compared to concentrations found in marine mammals (see section *1.3.2v, Marine Mammals*). The striking finding was that BDE-209 was found to be the dominant congener (Figure 1.9). In Kanagawa for example, average BDE-209 concentrations (19 $ng.g^{-1}$ lipid wt) were approximately four times that of the next highest congener BDE-153 (3.8 $ng.g^{-1}$ lipid wt). The congener BDE-209 has been found to have a half-life within biota of just a few days (Thomas *et al.*, 2005), suggesting that racoon dogs must have been exposed to a recent source of BDE-209 in order to build up the concentrations observed (Kunisue *et al.*, 2008).

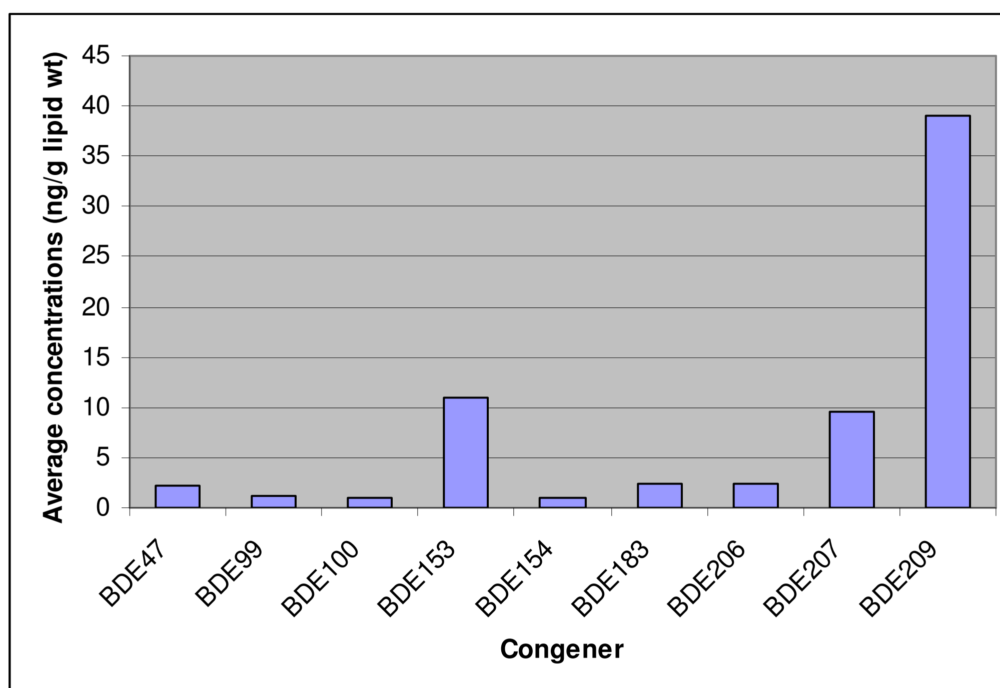

Figure 1.9 The congener profile of racoon dogs from Kanagawa, Japan (Kunisue *et al.*, 2006).

In a study of another terrestrial predator Voorspoels *et al.* (2006) found that BDE-209 also dominated the congener profile of red fox (*Vulpes vulpes*, n = 33) from Belgium. Concentrations of tri- to hepta-BDEs ranged from 0.61 to 115 $ng.g^{-1}$ lipid wt (median, 2.4 $ng.g^{-1}$ lipid wt), whereas BDE-209 concentrations ranged from <9.1 to 760 $ng.g^{-1}$ lipid wt (median, <9.1 $ng.g^{-1}$ lipid wt). In the 40% of fox liver samples which expressed measurable concentrations of BDE-209 the congener made up approximately 70% of the congener profile. Concentrations of the congener BDE-153 were also relatively high, contributing approximately 19% of the congener profile. The low tissue burden of lower congeners, given the foxes position as a top predator and given the known concentrations in prey species, indicates that foxes may be metabolising these lower congeners (Voorspoels *et al.*, 2006).

1.3.2viii) Summary – PBDEs in the Environment

PBDE concentrations observed in biota are still far lower than those of PCBs and certain organochlorines (Jaspers *et al.*, 2006; Kannan *et al.*, 2007), but they are becoming increasingly significant, particularly for individual congeners, such as BDE-47 which can be found in particularly high concentrations, and BDE-209 which is still to be fully understood in terms of its bioavailability and metabolic breakdown products.

Congener profiles in biota tend to resemble those present in the pentaBDE industrial mixture, with BDE-47 often the most dominant congener (Jenssen *et al.*, 2004), followed by BDE-99 and BDE-100 (Manchester-Neesvig *et al.*, 2001; de Wit *et al.*, 2006). The congeners BDE-153 and -154 are also often present at relatively low concentrations (Ikonomou *et al.*, 2002; de Wit *et al.*, 2006) indicating the influence of octaBDE industrial mixtures. However, congener profiles rarely mirror exactly those of industrial mixtures (She *et al.*, 2002), suggesting transformation of commercial BDE mixtures, either by physical or chemical breakdown in the environment, or by preferential uptake or by metabolism in biota (Ikonomou *et al.*, 2002; de Boer *et al.*, 2003; Jaspers *et al.*, 2004; Stapleton *et al.*, 2006). Evidence of this breakdown can be found by looking at the congener profiles of biota. The Dungeness crab (*Cancer magister*, 6 composits of 2-6 animals) contains a high proportion of BDE-49 (~20% of ∑BDE), suggesting a unique metabolic pathway in this species producing this congener from the breakdown of a larger congener (Ikonomou *et al.*, 2002; Ikonomou *et al.*, 2004). The eggs of glaucous gulls in the Norwegian Arctic contain octa- and nona-BDE congeners, breakdown products of the BDE-209 congener (Verreault *et al.*, 2007).

Concentrations of PBDEs have been steadily increasing over time, both in the environment and in biota which inhabit that environment. Concentrations of BDE-47 and BDE-99 doubled between the years of 1997 and 2001 in char (*Salvelinus alpinus*) sampled from Resolute Lake in the Canadian Arctic (de Wit *et al.*, 2006). She *et al.* (2002) found significant increases in concentrations of ∑BDE over time in harbour seals (*Phoca vitulina*) collected from the San Fransisco Bay area in North America between 1989 and 1998 ($r^2 = 0.833$, $p<0.001$), with concentrations doubling every 1.8 years.
In addition, concentrations of PBDEs the eggs of Ivory gulls have shown an increase over time, with samples collected in 1976 (∑BDE 18.3 ±2.3 $ng.g^{-1}$ lipid wt), 1987

(∑BDE 26.0 ±4.3 ng.g^{-1} lipid wt) and 2004 (∑BDE 44.5 ng.g^{-1} lipid wt) (Braune *et al.*, 2007).

There is also some evidence to suggest that reductions in production and use of PBDEs in recent year is already having an impact upon concentrations in biota. In Herring gulls from the Laurentian Great Lakes of North America an initial trend of increase in concentrations of PBDEs between 1982 and 2000 appears to have been followed by a decrease in concentrations in 2004 (Gauthier *et al.*, 2007). In another example, Johansson *et al.* (2006) found a varying time trend in concentrations of ∑BDE (BDE-47, -99 and -100) in blue mussels (*Mytilus edulis* or *Mytilus galloprovincialis*) from the River Seine estuary in Northern France. Initially concentrations rose from 2.71 ng.g^{-1} dw in 1982 to 9.88 ng.g^{-1} dw in 1993-1995, with concentrations doubling approximately every 4.6 years and rising. However, after 1995, with the exception of certain high points attributable to flooding and dredging events in 1999-2000, concentrations were observed to decreased, with concentrations halving approximately every 24.6 years. A similar, if not more pronounced time trend was observed for the higher congeners ∑BDE (BDE-153, -154 and -183), with concentrations doubling approximately every 5.6 years, from 0.5 ng.g^{-1} dw in 1981 to 1.13 ng.g^{-1} dw in 1991, and then declining after 1991, halving approximately once every 9.8 years.

BDE-209 is found only at trace levels in species of marine wildlife. The congener tends to only be found in species of terrestrial wildlife, and even then in species at high trophic levels, such as birds of prey (Table 1.9, section *1.3.2vi, Birds*), or mammalian predators/scavengers (Voorspoels *et al.*, 2006; Kunisue *et al.*, 2008).

1.3.3 Breakdown of PBDEs

Very little is understood about the breakdown and metabolism of PBDEs, either in the environment or in biota itself. One of the most integral and best studied environmental PBDE breakdown mechanisms is photochemical breakdown (Eriksson *et al.*, 2004; Fang *et al.*, 2008), however, this mechanism relies on exposure of the compounds to UV radiation and so is not relevant once compounds are stored within sediments and soils, etc. (Raff & Hites, 2007). It has been suggested that the metabolism of PBDEs is an important mechanism for the elimination of PBDEs from biota. More study is

needed to understand this process better, particularly as certain metabolic processes have the potential to lead to the production of more toxic compounds depending on the breakdown mechanism triggered (Meerts *et al.*, 2000, 2001). The mechanisms of metabolism are not fully understood. It is possible that the metabolism of PBDEs results in an alteration of the basic structure, resulting in compounds such as hydroxylated or methoxylated polybrominated dyphenyl ethers (OH-PBDEs and MeO-PBDEs; Marsh *et al.*, 2005). Alternatively, metabolism may simply result in the loss of bromines to form lower BDE congeners (Ikonomou *et al.*, 2004; Law *et al.*, 2006).

1.3.3i) Methoxylated PBDEs (MeO-PBDEs)

Methoxylated PBDEs (MeO-PBDEs) have been found in several marine species, including Polar and Arctic cod, beluga and pilot whales, polar bear and Californian sea lions (de Wit *et al.*, 2006; Stapleton *et al.*, 2006) and it has been suggested these MeO-BDEs compounds may form as a metabolic breakdown product of BDE congeners. However, there is evidence to suggest that these MeO-BDEs are in fact naturally occurring compounds and that the metabolic breakdown of PBDEs is not a significant factor in the concentrations of MeO-BDEs observed in biota (Marsh *et al.*, 2005). For example, in a study of Californian sea lion (*Zalophus californianus*) no significant correlation was observed between concentrations of 6-MeO-BDE-47 and BDE-47 ($p< 0.01$), suggesting that the 6-MeO-BDE-47 concentrations originated from a natural source and not as a result of metabolism of BDE-47 in the sea lions (Stapleton *et al.*, 2006).

1.3.3ii) Debromination of BDE-209

There is inconclusive evidence to show whether low BDE-209 concentrations are due to low bioaccumulation or metabolism (Boon *et al.*, 2002). The evidence that is available suggests that both factors are likely to play a part and that the extent to which each factor plays a part may be species specific.

The low or undetectable BDE-209 concentrations observed in many fish species (Boon *et al.*, 2002) suggests that fish only bioaccumulate low concentrations of this compound or that metabolism of BDE-209 is common in fish. A dietary exposure of carp (*Cyprinus carpi*) resulted in a low proportion (<1%) of BDE-209 being absorbed by the fish (Stapleton *et al.*, 2004). In addition, the majority of the BDE-209 which was

absorbed was converted to lower congeners via debromination, resulting in undetectable concentrations of BDE-209 but significant concentrations of seven penta- to octa-BDE congeners (Stapleton *et al.*, 2004).

Marine mammals also show low concentrations of BDE-209 in the wild (Kajiwara *et al.*, 2006; Stapleton *et al.*, 2006). However this may be due to low levels of dietary exposure. In a BDE-209 exposure experiment three captive grey seals (*Halichoerus grypus*) were fed a supplement of 2µg of BDE-209 per day for a month (Thomas *et al.*, 2005). A range of PCBs, OCs and PBDEs were analysed in the diet, faecal matter, blood and blubber biopsies of the animals, collected at points before, throughout and after the exposures in order to assess levels of input, output and bioaccumulation of the various compounds, with a focus on BDE-209. The study showed high levels (89%) of absorption of BDE-209 into the body of the seals, but also showed a short half-life of just 8.5 to 13 days for BDE-209 in the blood (Thomas *et al.*, 2005). Increasing concentrations of BDE-209 in the blubber suggests that this lipophilic compound is much more stable when stored in the adipose tissue (Thomas *et al.*, 2005).

This disparity in rate of accumulation of BDE-209 suggests that the compound is processed in different ways by different species. Sweeping statements about the bioaccumulation potential of the compound and the level of biological degradation once absorbed are unlikely to hold true from one species to the next. More research is needed to ascertain the cause of this disparity between species and to assess individual species potential susciptability to this particular compound.

1.3.4 Toxicity

1.3.4i) Thyroid Hormone Disruption and Related Affects

The structure of the thyroid hormone thyroxine (T_4) is similar to that of PBDEs (Figure 1.10). Both compounds consist of two hydrocarbon rings joined through an oxygen molecule and both structures are surrounded by halogens, bromine in the case of PBDEs and iodine in the case of T_4. The main difference being that T_4 has two extra groups, one off of each hydrocarbon ring (Figure 1.10).

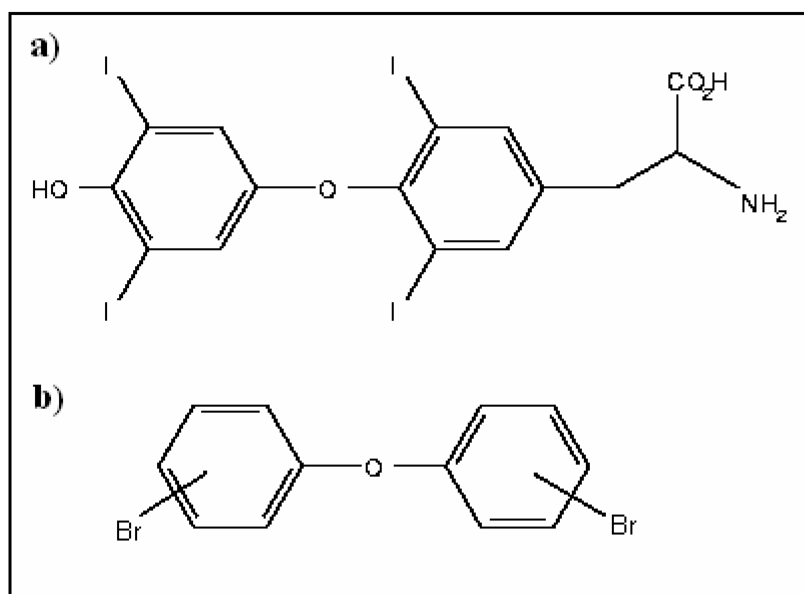

Figure 1.10 Structures of a) Thyroxine and b) PBDEs.

PBDEs have been shown to have significant affects on rats and mice exposed to pentaBDE (Bromkal70-5DE and DE-71) and octaBDE (DE-79) industrial mixtures (Zhou *et al.*, 2001), as well as to the single congener BDE-47 (Hallgren *et al.*, 2001; Hallgren & Darnerud, 2002). In exposed animals T_4 levels, both plasma free T_4 (FT4) and to a lesser degree total T_4 (TT4), were significantly reduced (Zhou *et al.*, 2001; Hallgren *et al.*, 2001; Hallgren & Darnerud, 2002). In exposed animals PBDEs induced the activity of the microsaomal phase I enzymes ethoxy (EROD), methoxy (MROD) and pentoxy resorufin O-dealkylase (PROD) (Zhou *et al.*, 2001; Hallgren *et al.*, 2001; Hallgren & Darnerud, 2002). In addition reduced vitamin A (retinol) levels (Hallgren *et al.*, 2001), increased liver somatic indexes (Darnerud *et al.*, 2001; Zhou *et al.*, 2001) and decreases in the amount of T_4 binding to the thyroid hormone-binding transport protein TTR have been observed (Hallgren & Darnerud, 2002), suggesting a range of interlinked effects.

Meerts *et al.* (2000) found that PBDEs were only capable of competing with T_4-TTR binding after metabolic conversion by microsomes, during which hydroxylation (i.e. addition of an OH group) of the PBDE structure occurs. The potency of PBDEs in competing with T_4 on TTR was found to be both congener and metabolic enzyme specific, with more highly brominated congeners generally being less potent than lower congeners (Meerts *et al.*, 2000).

It should be noted that affects seen for PBDE exposure were generally lower in potency than those observed for animals exposed to PCB mixtures and congeners (Hallgren *et al.*, 2001; Hallgren & Darnerud, 2002). Also of note are the significant additive effects observed in animals exposed to both BDE-47 and the PCB mixture Arochlor 1254 (Hallgren & Darnerud, 2002).

1.3.4ii) Neurological Disruption

Thyroid hormones play an important role in brain development (Hallgren *et al.*, 2001). Exposure of mice dams to BDE-99 affected several neurobehavioural parameters in the offspring (Branchi *et al.*, 2002; Branchi *et al.*, 2005). Similarly, neonate mice exposed to BDE-99 express behavioural abnormalities later in life consistent with neurotoxic effects of exposure (Viberg *et al.*, 2004). Other PBDE congeners, including BDE-209, can also induce neurobehavioural affects in rodents exposed during early stages of development (Viberg *et al.*, 2003).

It is not fully understood whether the alterations to neurobehavioural development induced by PBDEs are the result of thyroid hormone disruption (Branchi *et al.*, 2005) or some other developmental mechanism (Viberg *et al.*, 2004).

1.3.4iii) Other Potential Effects

The exact mechanisms behind the effects of exposures are often not fully understood (Van der Ven *et al.*, 2008). However, in addition to thyroid hormone disruption and neurological effects, there are a host of other possible effects of PBDE exposure observed. Some of which, such as the inhibition of metamorphosis in tadpoles (Balch *et al.*, 2006), probably result from the disruption of the thyroid hormone system. However, some effects of PBDE exposure appear to be independent of this thyroid system effect.

Male mice exposed to high doses (500mg/kg/day and 1500mg/kg/day) of BDE-209 post-weaning expressed significantly reduced sperm mitochondrial membrane potential (MMP) and sperm lateral head amplitude (Tseng *et al.*, 2006). They also expressed increases in sperm H_2O_2, suggesting that BDE-209 is capable of significantly altering some reproductive parameters in developing mice (Tseng *et al.*, 2006).

1.3.4iv) Observations in Populations in the Wild

Toxic effects of PBDEs have been observed in studies of wildlife species from several trophic levels. Langford *et al.* (2007) observed a reduction in the population diversity of a bacterial community exposed to PBDE loadings, suggesting that PBDEs are toxic to some bacterial species. Environmental PBDE exposure has been shown to be capable of inducing metabolic enzyme activity, oxidative stress in the liver, and signs of neurotoxicity in the brain of barbel from a river in Spain (Raldúa , 2008).

In top predator species, PBDEs have been shown to be capable of altering T_4 and retinol (vitamin A) concentrations in Kestrels (*Falco sparverius*) exposed to environmentally relevant concentrations (Fernie *et al.* , 2005). In addition, correlations have been found between concentrations of PBDEs and concentrations of T_4 and T_3 in wild grey seal pups (Hall *et al.*, 2003), suggesting that the concentrations of PBDEs observed in wild populations are concentrated enough to cause significant biological effects.

1.4 Introduction to the Project

The overall aim of the project was to investigate techniques used to monitor otter populations in England and Wales with a view to better understanding the size and health of present day populations and possible future threats to otters. The study was aimed at increasing our knowledge of local otter populations, the genetic impacts of recent population declines and human intervention, as well as the potential for organic pollutants, in particular PBDEs, to impact upon otter populations. The overall hypothesis for the project was that:

H_0 There is no evidence that human activities have impacted upon the genetic diversity of English and Welsh otter populations, or that organic pollutants, including PBDEs, are likely to impact upon the health of otter populations in the future.

H_1 The genetic diversity of English and Welsh otter populations has been significantly altered by human activities, and concentrations of organic pollutants, including PBDEs, in present day otter populations are such that they should be considered of concern in terms of the future health of English and Welsh otter populations.

This overall hypothesis was split into three main areas of study to allow a greater depth of analysis.

1.4.1 Obtaining Population Information on a Local Scale

The first area of study was aimed at improving our knowledge of local otter populations, in terms of population sizes and the ways in which individual otters use particular locations in the study area. The study area was the River Camel catchment in Cornwall, where numbers of road kill and sightings suggest that there is a healthy otter population present (Crawford, 2003; Simpson, 1998, 2007; Kate Stokes, pers. comm.). The census was carried out using the non-invasive census technique of microsatellite genotyping DNA extracted from spraint samples, which also provided the dates and locations of sample collections essential for the analysis of basic behavioural parameters. The main hypothesis for this section of study was as follows:

H_0 The microsatellite genotyping of DNA from spraint material is not an efficient censusing technique and does not significantly increase our understanding of local otter populations.

H_1 Significant increases in our understanding of the numbers of otters and the way in which otters are using a particular geographical location can be achieved by using the microsatellite genotyping of DNA extracted from spraint material.

1.4.1i) Spraint Samples Genotyping Census

Part of the strategy to help conserve a species is to know how many individuals of that species inhabit an area. Censuses based on field signs, such as spraint and tracks, provide useful information about which rivers are being inhabited by otters (Erlinge, 1967), and over the years have provided useful information about the wider expansion of otter populations in England (Crawford, 2003). Although such methods can provide useful information about which areas are being inhabited, they provide much more sketchy information about the health of the population present. For example, it is possible to say that there are X number of active sprainting sites spread along a river, but it is much harder to prove whether it is the same otter using multiple sprainting sites or if several otters are using the various sites, or a subset therein. The use of footprint size can shed some light on the numbers of otters present (Erlinge, 1967); an adult male has a much larger pad mark than either a female or a juvenile, and a cub has a smaller pad mark still. However, clear pad marks are a relatively rare find and this technique is generally more suited to locations with colder conditions, where pad marks are commonly imprinted and preserved in the snow (Erlinge, 1967; Arrendal *et al.*, 2007). A further drawback of this method is that it is highly likely that various individuals are indistinguishable, particularly as the tracks of adult females and juvenile males tend to be very similar.

Genetically typing spraint samples, in this case via microsatellite genotypes, will provide a far more effective method of identifying the individual that produced the spraint. By counting the number of genotypes obtained from a set of spraint samples it is possible to get a count of the number of otters that have used the area surveyed over the time period of the spraint sample collection (Dallas *et al.*, 2003; Hung *et al.*, 2004). The method has drawbacks; in particular, it provides only a minimum count and it

should be accepted that there may still be individuals unaccounted for at the end of the sampling period (Frantz *et al.*, 2003). Although data on dates and locations of spraint collections can provide some idea of the regularity of the identification of individuals, it can be difficult to distinguish between resident and transient individuals – a common occurrence in species such as otter which are territorial and are known, particularly in males, to disperse between catchments (Erlinge, 1967). Despite these downfalls, the technique still provides far more in-depth information regarding the number of individuals using a particular area (Arrendal *et al.*, 2007), in this case the river system of study.

1.4.1ii) Improving Techniques for Genotyping Otter Spraint

Success rates for extraction and genotyping faecal material can be low due to the low DNA content and the effects of bacterial, chemical and physical breakdown that act upon the DNA content of faecal matter, both in the digestive system and once the faecal matter has left the body. Success rates for genotyping otter spraint vary, but can be as low as 20% of samples collected being successfully genotyped (Coxon *et al.*, 1999; Dallas *et al.*, 2000). This variation in success rates has been attributed to a number of factors, including the weather the samples were collected in (principally the temperature; Hájková *et al.*, 2006), the storage technique used, and the time between collection and extraction of DNA (Hájková *et al.*, 2006; Lampa *et al.*, 2008).

A further problem frequently encountered when genotyping faecal material is that of quality and reproducibility of the genotypes obtained. In a spraint genotyping study carried out to find out how many otters were using the River Itchen catchment in 1997-98, a total of 13 different genotypes were identified, indicating that at least 13 otters were using the catchment (Coxon *et al.*, 1999; Dallas *et al.*, 2000). In a second study of the same river system, carried out just eighteen months after the first, a total of 35 genotypes were obtained from spraint samples (Chanin, 2004). Given what is known about the natural ranges of otters (Erlinge, 1967) and resource limitations, this second estimate of population size would be considered too large to be an accurate representation of an otter population using a 40km stretch of river. This would suggest that work needs to be done to identify the faults in the data and that in future greater care needs to be taken when genotyping such difficult and error prone samples (Creel *et al.*, 2003).

One of the key aims of this project was to improve on techniques used to genotype otters via their spraint material by increasing confidence in the accuracy of genotypes obtained and by investigating ways to improve the low success rates, and in consequence lower the costs of any future such study.

1.4.2 The River Itchen Case Study

This section of the project was an investigation of the genetic consequences of the severe national decline in otters numbers witnessed in the 1950s to 1980s, and also the genetic effects of the captive breeding and release programme set up as a consequence. The otter population on the River Itchen in Hampshire, due to its particular demographic history, was used as a case study population and the resulting overall hypotheses were:

H_0 There is no genetic signature identifiable in the River Itchen otter population indicative of either a population bottleneck resulting from a severe historic population decline, or of the introduction of novel foreign genetic material introduced via the captive release programme.

H_1 The River Itchen otter population shows significant genetic distinction from other local otter populations, in terms of genetic distance, diversity and composition, in a pattern which is indicative of population bottlenecking, the introduction of novel genetic material via the release of captive bred individuals, or a combination of both of these demographic factors.

1.4.2i) The River Itchen

The River Itchen is located on the south coast of England with its estuary situated above the Isle of Wight. The river is a prime chalk stream, and as such is capable of supporting healthy salmon and trout populations (Graham Roberts, personal comm.). This readily available supply of fish, combined with a healthy well maintained river system, makes the River Itchen an ideal habitat for otters. Indeed throughout the declines a small number of otters have remained on the river, with around half the sites surveyed showing signs of otter activity in the 1977-79 and 1984-86 otter surveys

(Crawford, 2003). However this small population, probably representing just a handful of individuals, was virtually isolated, with very few of the surrounding rivers from the Southern Region to the east, Dorset Region to the west or Thames Valley Region to the north showing any signs of significant otter activity until the 1991-1994 surveys (Crawford, 2003). The Otter Trust released four captive bred individuals onto the River Itchen catchment in 1993-94 (Jefferies *et al.*, 2000; in Crawford, 2003). Thought to be one male and three females, it is known that at least one female died without producing offspring (Graham Roberts, pers. comm.). However, it is assumed that the other three released otters integrated into the native population and may well have bred. It is unknown whether further, unrecorded releases were also made within the area.

In summary, the River Itchen population is likely to have suffered from the effects of bottlenecking during the period of low population numbers experienced at the height of otter decline in the UK. Individuals introduced as releases from the captive breeding programme are likely to have introduced new genetic material and therefore to have increased the genetic diversity found in the present day River Itchen population. However, if the otters used in the captive breeding programme were from a source too genetically distant from the River Itchen population this may have resulted in a lowering of fitness in the resulting population as a result of the effects of outbreeding depression.

1.4.2ii) Comparative Populations - Microsatellite Genotyping

The assessment of the likelihood of the genetic composition observed in the River Itchen population having resulted from a particular demographic history was carried out via comparisons to other UK populations. To this end, the genetic diversity and composition of the River Itchen population, calculated using microsatellite genotyping, was compared to that of otter populations in eastern Cornwall and southern Dorset.

The population in eastern Cornwall was chosen as a representative of a relatively healthy population known to have suffered a significantly less severe reduction in numbers during the period of national decline than did the River Itchen population. Indeed the otter population in the south west of England, of which eastern Cornwall sits at the heart, maintained the highest level of otter occupancy (23.5%; Lenton *et al.*, 1980) of any geographical location in England (Crawford, 2003). In addition, there are

not thought to have been any captive bred otters released in Cornwall. Therefore, the eastern Cornish population should represent a population which is unlikely to have suffered from the effects of severe population bottlenecking, or to contain genetic information introduced via the release of captive bred otters. Although a certain amount of difference in genetic composition will result from the effects of the geographical distance between the two populations, if the levels of genetic differentiation (F_{ST}) between the eastern Cornish population and the River Itchen population is particularly high this is likely to represent differences in the demographic histories of the two populations. Therefore, a particularly high level of genetic differentiation - higher than would be expected given the geographical separation of the two populations - would indicate a demographic cause for this increased genetic drift.

The second population used for comparisons using microsatellite genotyping was the southern Dorset otter population. This population has a similar demographic history to the River Itchen population, with a severe drop in numbers and effective isolation of remnant populations during the period of national declines, followed by the release of several captive bred otters into the population (Crawford, 2003). One major difference between the Dorset population and the River Itchen population is that Dorset is situated geographically further east. Therefore, as the otter population in the south west of England grew, the leading edge of expansion reached the Dorset otter population sooner than the River Itchen. This means that the Dorset otter population is likely to show increased genetic diversity resulting from a merging of otters from the south west of England with the original Dorset population. In contrast, the River Itchen population, which has only recently been reached by the expansion of the south west otter population, if at all, is less likely to express the genetic signature of such a recent merge. The result is that the Dorset otter population is likely to contain a genetic composition showing similarities to the south west otter population (i.e. Cornwall) due to immigration from this area, as well as to the River Itchen population, which has shared a similar demographic history.

1.4.2iii) Mitochondrial Haplotypes

A study of haplotypes from the first ~300bp of the mitochondrial control region was used to assess the impact that the release of captive bred otters is likely to have had upon the River Itchen otter population. This section of DNA has been used previously

to haplotype otters from around Europe, with a suitable identifiable pattern of occurrence consistent with a realistic and specific demographic pattern (see section *1.1.7iii, Mitochondrial Haplotype Assessment of Otters in Europe*). In this study the marker was used to identify otters which were likely to be descendant from captive bred otters released by The Otter Trust. The major drawback of using mitochondrial haplotypes is that the mitochondrial DNA is passed down the matriline, and so if captive released males also bred in the population of interest then the total genetic input from captive bred otters will have been underestimated using this method. However, it can still provide a definitive answer as to whether any descendants of female captive-bred otters exist in the present-day population, provided the signature has not been lost via the occurrence of solely male cubs surviving to breed.

1.4.3 PBDEs in Otters from England and Wales

It is thought that one of the main causes of the decline in otter numbers observed in the 1950s to 1980s was the release of compounds such as PCBs and dieldrin into the environment (see section *1.1.3 Otter Declines and Chlorinated Hydrocarbons*). The third main aim of this project was to measure concentrations of a further hallogenated organic pollutant - PBDEs - in the liver tissue of otters from England and Wales. The overall hypothesis for this section of study was that:

H_0 The liver tissue of otters does not contain significant concentrations of any of the PBDE congeners analysed

H_1 The otter liver tissues analysed contain significant concentrations of PBDEs, particularly of the BDE congeners most commonly observed in high concentrations in aquatic top predators, at concentrations approaching those observed for other organic pollutants in otter liver tissue, as well as concentrations of PBDEs observed in other aquatic predators.

H_2 The otter liver tissue samples analysed contain significant concentrations of PBDEs, but in a different congener profile to that expected for an aquatic top predator.

1.4.3i) PBDEs in Otters

PBDEs have been used over recent decades to flame retard a large range of man-made materials. However, due to the nature in which they are bound to the materials, they have a tendency to leach out into the surrounding environment. PBDEs are environmentally persistent and highly mobile (Strandberg *et al.*, 2001; Martin *et al.*, 2004), often being found in locations geographically distant from their source (de Wit *et al.*, 2006). As a result, in recent years they have been found in increasing concentrations in many species of wildlife (Boon *et al.*, 2002; Covaci *et al.*, 2004; Jenssen *et al.*, 2004; Jaspers *et al.*, 2006). As a top aquatic predator otters are likely to accumulate comparatively high concentrations of PBDEs. Therefore, they are probably one of the most important species to study in terms of monitoring PBDE contamination in UK freshwater and coastal ecosystems.

In exposure experiments PBDEs have been shown to elicit similar toxicological responses as PCBs, although generally greater exposure is required to induce the same level of toxicological response (Hallgren *et al.*, 2001; Hallgren & Darnerud, 2002). This indicates that PBDEs have the potential to impact upon otter populations if concentrations become high enough. This is particularly true if exposure to PBDEs acts as an additive toxicological pressure in animals already suffering the effects of exposure to compounds such as PCBs.

1.4.3ii) Comparisons with PCBs and OCs

A suite of PCB congeners, DDT breakdown products and other selected organic pollutants have previously been measured in otter liver tissue samples collected from across England and Wales (Simpson, 1998, 2007; Chadwick, 2007). Many of the samples analysed in these previous studies have been extracted and analysed for PBDE content in this study, where the samples were reanalysed for many of the PCB and DDT compounds in order to create a more accurate comparisons with measures of PBDE content. In this way possible biases arising from variations in extraction technique or laboratory conditions were negated.

Comparisons of PBDE concentrations with those of PCBs and DDTs provided an indication of the significance of the PBDE concentrations observed in relation to those of other organic pollutants. There is evidence to suggest that concentrations of PCBs

and other organo chlorines found in otter liver tissue have decreased in concentration over the last few decades (Simpson, 1998; Chadwick, 2007), as may be expected given that most of the compounds analysed have been out of use for several decades. However, despite this apparent decrease in concentration, many of the compounds are still present in relatively high concentrations (Simpson, 2007). It is therefore advisable to consider the concentrations of all organic pollutants together when trying to assess their potential impact upon populations.

2 Sample Collection and Molecular Methods

2.1 Sample Collection

2.1.1 Tissue Collection for Genetic Work

The Environment Agency, aided by local organisations, such as the Wildlife Trusts, and members of the general public, have been collecting otter carcasses from the south west of England since 1988 (Simpson, 1998) and from across England and Wales since 1992 (Chadwick, 2007). Carcasses have been collected primarily for the collection of liver tissue for the analysis of levels of PCBs and organochlorines (Simpson, 1998; Simpson, 2000; Chadwick, 2007). In addition *post mortems* also reveal information about the general health of the otters collected, with age, sex, general body condition and cause of mortality all noted where possible (Philcox *et al.*,1999; Madsen *et al.* 1999; Hauer *et al.* 2002b; Chadwick, 2007) and over the years a range of pathological and histological analysis have been carried out on a range of tissue samples (Simpson, 1998; Simpson, 2007).

Recent *post mortem* examinations – those concerning samples used in this study – were carried out in one of two locations. A degree of overlap occurred in terms of the sample collection sites and the destination of their *post mortem*, however, the majority of samples from the south west of England were examined at the Wildlife Veterinary Investigation Centre (WildlifeVIC) in Cornwall (Simpson, 2007) and the majority of samples from the north, east and midlands of England, as well as samples from Wales were processed by the Cardiff University Otter Project (CUOP) (Chadwick, 2007).

As part of *post mortem* examinations, from those animals not too badly damaged or decomposed, samples of specific organs, such as liver and kidneys, are removed and stored appropriately (Simpson, 1998). In the case of samples of kidney, which were collected during certain time periods for the specific purpose of genetic analysis, the samples were stored frozen. At WildlifeVIC large proportions of kidney samples were dissected into 1cm^2 pieces and stored frozen in tubes. At Cardiff the amount of tissue collected for genetic analysis was generally far less but still more than sufficient in quantity. The tissue samples, either kidney or liver, were often submerged in ethanol before being frozen. Both are suitable storage methods, the additional use of ethanol providing easier transportation of samples which will no longer require caution over the

risk of defrosting, however the storage of ethanol in freezers creates a health and safety hazard with the possibility of sparks within electrical equipment igniting the ethanol.

2.1.2 Spraint Collection

Spraint collection was carried out in a similar manor to Coxon *et al.* (1999). Collections were carried out by teams of volunteers, trained and co-ordinated by Graham Roberts and Kate Stokes from the Hampshire and Isle of White and Cornwall Wildlife Trusts respectively. Suitable collection sites on the River Itchen had been located during previous otter study (Coxon *et al.* 1999) and further sites were located by the team during the course of collection. On the River Camel, where no previous spraint DNA study had been carried out, suitable sites had to be located – a task carried out by collection volunteers located close to the river site who therefore had a good knowledge of the river system. Sites were chosen for ease of access and for the level of previous known otter activity at the site. This maximised the number of samples collected given the limitation of volunteer availability and time constraints, however it did compromise on the coverage of the catchment in terms of end results. These gaps in coverage are likely to result in areas where little or no information about the extent of otter habitation is known and will mean that commenting on the Rivers coverage as a whole will not be realistic without acknowledgement of this fact.

The surface of the lining of the mammalian gut is similar to the surface of the skin in that it regularly sheds dead and dying surface cells in order to replace them with new. It is the DNA from these shed cells that was targeted in the spraint material analysed in this study. Once these cells have been incorporated in the spraint they are no longer part of a living tissue and as such loose the protection of the defence and DNA repair systems present in living cell tissue (Lindahl, 1993; Handt *et al.*, 1994). Once the spraint has been excreted from the body it becomes a target for bacterial action, which breaks down any cellular material not fully digested during passage through the otters' digestive system, including cells shed by the otter. Other environmental factors, such as ultra-violet radiation, enzyme activity and hydrolytic and/or oxidative damage can also act to break down cellular and genetic material within the spraint (Seutin *et al.*, 1991; Lindahl, 1993; Handt *et al.*, 1994). It is therefore vital, if we are to be able to obtain

DNA from samples, that spraint material is fresh when collected and the volunteers were asked to collect only the spraint they were confident had been laid down during the last few hours.

Volunteers were encouraged to collect once a month over a dedicated weekend, although volunteers also collected *ad lib* during the study period. Spraint samples collected were transferred directly into a tube of IMS using natural implements such as twigs and leaves so as not to transfer contamination from one collected sample to the next. Tubes were labelled with relevant information that would enable the sample to be tracked (largely left to the discretion of the volunteers). Although records were not consistently kept, volunteers were encouraged to fill in data sheets regarding the samples collected – an example of which can be seen in Figure 2.1. This not only provided us with dates and locations for individual samples but also provided us with information about the field conditions the spraint samples were subjected too and allowed us to predict what effects these conditions may have upon success of DNA extractions.

SURVEYORS NAME:

<table>
<tr><td colspan="2">SAMPLE TYPE COLLECTED
Spraint/Anal Jelly/None</td><td>SITE NAME + CODE</td><td>WATERCOURSE</td></tr>
<tr><td>TUBE NO</td><td>DATE

TIME</td><td>GRID REF</td><td>TRACK SIZE (mm)</td></tr>
<tr><td colspan="2">SUBSTRATE UNDER SAMPLE
Rock/Gravel/Sand/Mud/Other (describe)</td><td colspan="2">DEGREE OF EXPOSURE OF SAMPLE
e.g. Under bridge/Trees/Open and in Sun/Shade/dew-covered</td></tr>
<tr><td colspan="2">WEATHER CONDITIONS ON DAY OF SURVEY</td><td colspan="2">WEATHER CONDITIONS IN PREVIOUS WEEK</td></tr>
<tr><td colspan="4">COMMENTS – add any information that may be relevant to the condition of the sample collected or other signs of otter activity</td></tr>
</table>

Figure 2.1 Layout of field data collection form used for the River Camel collections

Close contact was maintained between the volunteers and the laboratory, with regular meetings and emails to discuss collection techniques, collection success and progress in the laboratory. This enabled the volunteers and myself to remain confident in the

methods of collection being carried out and maintained motivation and enthusiasm in the volunteers throughout the study. As discussed later, the methods of data handling used enabled easy access to, and manipulation of data enabling easy construction of reports regarding the success of individual collections, as well as the success river wide, providing collection volunteers with a more personal level of information regarding the otters found using their collection sites.

2.1.3 Tissue Collection for Chemical Analysis

As with the kidney tissue samples collected for genetic analysis, liver tissue samples for chemical analysis were collected as part of *post mortem* examinations. Liver tissue samples were originally collected for analysis of PCB and selected organochlorines carried out by Environment Agency laboratories. In many cases duplicate samples were taken and the second (and sometimes third) set of samples were stored frozen by the *post mortem* teams. It was these duplicate samples that became available for analysis for PBDE content.

Samples were stored wrapped in foil and placed in individual plastic bags before freezing to minimise the risk of contamination, either from the environment, from other samples or, in the case of the foil wrapping, from the plastic bags themselves. During sample preparation the outer surface of the liver tissue was removed so as to again reduce the possible effects of surface contamination (see section *5.2.1, Extraction and Clean-up*)

2.2 DNA Extraction

2.2.1 Extracting DNA from Tissue

Three different extraction techniques were attempted in order to find the most effective method of extraction from the tissue samples used. The three methods attempted being an ammonium acetate extraction protocol (Bruford *et al.*, 1998), a salt extraction technique (Aljanabi and Martinez 1997) and a method using a phenyl chloroform mix (Taggart *et al.* 1992). All three methods use a digestive solution and the enzyme protinase K to digest cellular material, before the ammonium acetate, salt (NaCl) or phenol chloroform are used to separate out the DNA and ethanol is used to precipitate and extract the DNA.

2.2.1i) Ammonium Acetate Extraction Method

1) Add 200µl of digestive solution to ~5mm^2 of chopped tissue

    Digestive solution recipe:

    10ml 1M Tris (pH8)
    172ml ddH_2O
    8ml 0.5 EDTA (pH8)
    1.37ml NaCl
    Autoclave
    10ml 20% SDS (pH8)

2) Add 5µl of protenase K solution
3) Incubate overnight at 37ºC
4) Add 400µl of 4M ammonium acetate
5) Vortex several times over a period of 2 to 3 hours
6) Centrifuge for 10mins at 15,000rpm
7) Transfer supernatant to a fresh tube
8) Add 1ml of 100% ethanol and invert tubes several times to precipitate DNA
9) Centrifuge for 10mins at 15,000rpm
10) Pour of ethanol, taking care not to loose the pellet
11) Add 500µl of 70%ethanol and invert tubes several times to wash pellet
12) Cetrifuge for 5mins at 15,000rpm
13) Pour off ethanol and stand tubes inverted in clean tissue to dry
14) Once fully dry resuspend pellet in 100µl ddH_2O

2.2.1ii) Salt Extraction Method

1) Add 400µl of digestive solution to ~5mm^2 of chopped tissue
2) Add 8µl of protenase K solution
3) Incubate overnight at 37ºC
4) Add 300µl of 6M NaCl
5) Vortex for 30secs
6) Spin for 30mins at 10,000g
7) Transfer supernatant to a fresh tube
8) Add an equal volume of isopropanol and mix well
9) Incubate sample at -20ºC for 1 hour
10) Cetrifuge for 20mins, at 10,000g and at 4ºC
11) Wash pellet with 70% ethanol
12) Dry pellet and re-suspend in 300-500µl ddH_2O

2.2.1iii) Phenol Chloroform Extraction Method

1) Add 500µl of digestive solution to ~5mm^2 of chopped tissue
2) Add 5-10µl of protenase K solution
3) Incubate overnight at 37ºC
4) Add 500µl of phenol:chloroform:isoamyl alcohol (25:24:1)
5) Gently mix for 10mins
6) Centrifuge for 3mins at 12,000g/13,000rpm
7) Transfer top aqueous phase to fresh tube
8) Repeat steps 4 to 7 halving mixing time to 5mins
9) Add 400µl of 100% ethanol
10) Invert tube several times to mix then place on ice for ~10mins
11) Centrifuge for 10mins at 13,000rpm
12) Pour off ethanol and stand tube inverted on tissue to dry
13) Re-suspend pellet in 200µl of ddH_2O

Note: Washing with 70% ethanol often resulted in the loss of the DNA pellet as the DNA became re-suspended in the liquid. Therefore the 70% ethanol wash was dropped with the awareness that some PCR inhibition may result due to ethanol remaining in the sample elution, and some protein matter may remain.

2.2.1iv) Comparisons of Extraction techniques

For the extraction of DNA from tissue samples it was imperative that extractions were reliable but cost-effective. In order to quickly compare the strength and purity of DNA produced during the use of different extraction protocols the DNA produced was run on 0.8% agarose gels. In initial comparisons ammonium acetate and salt extractions were found to perform poorly in comparison to the phenol chloroform extraction technique (see Figures 2.2 and 2.3).

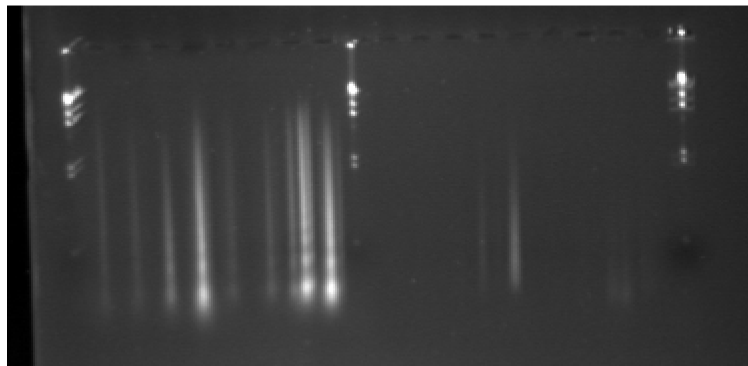

Figure 2.2 Agarose gel comparing results of extractions using the phenol chloroform method, rows 2 to 9, and the salt extraction method, rows 11 to18. Also shown are the results of extractions using varying sizes of tissue, with rows 2, 6, 11 and 15 using ~1mm^3, rows 3, 7, 12 and 16 using ~3mm^3, rows 4, 8, 13 and 17 using ~5mm^3 and rows 5, 9, 14 and 18 using ~7mm^3

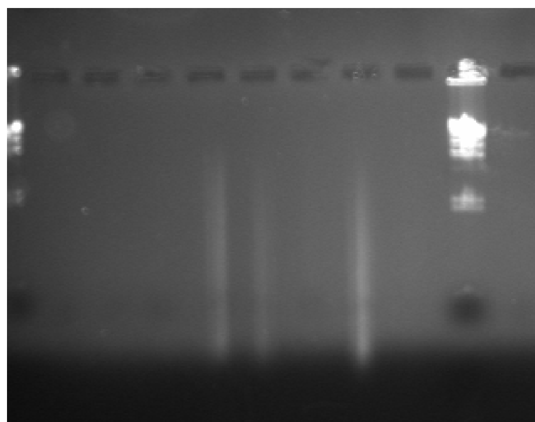

Figure 2.3 Results of ammonium acetate extractions.

Although they appear to give a much cleaner product (see Figure 2.4), QIAGEN blood and tissue DNA extraction kits did not produce the quantities of DNA obtained during phenol chloroform extractions. Table 2.1 summarises DNA concentrations as measured on a uv-vis recording spectrophotometer (UV-240IPC, Shimadzu), comparing concentrations produced during different extraction methods carried out using the same set of tissue samples. A graph of the results (Figure 2.5) clearly shows that phenol chloroform extraction is the most effective extraction for these samples. The results also indicate that similar concentrations of DNA are produced during the first and second elutions (elutions a and b) from the extraction kits (see Table 2.1 and Figure2.5). Being the method that reliably produced the highest concentrations of DNA, phenol chloroform was the method chosen for extraction of DNA from tissue samples.

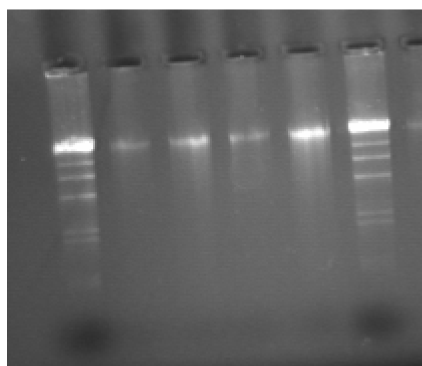

Figure 2.4 Results of extractions using blood and tissue DNA extraction kits

Table 2.1 DNA concentrations (ng.μl^{-1}) per sample, as produced by three different extraction techniques. The QIAGEN kits produced two different elutions which are shown here as elution a and elution b

Sample	QIAGEN elution a	QIAGEN elution b	Salt	Phenol chloroform
M638	100	90	225	1000
M639	350	250	750	2400
M640	90	76	850	550
M642	150	110	1850	2350
M647	112	500	950	1750
M650	90	90	550	950
M654	90	75	75	350
M658	115	95	425	475

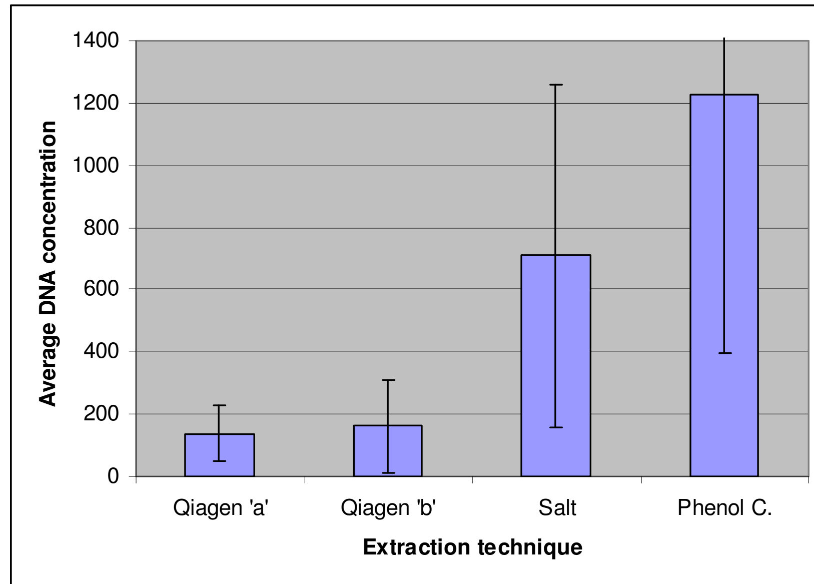

Figure 2.5 Average DNA concentrations (ng.µl^{-1}) produced during different extraction techniques. For QIAGEN extractions DNA concnetrations from the two elutions, a and b, are compared

2.2.2 Extracting DNA from Spraint

Silica based DNA purification has long proved to be the most successful method for extracting DNA from samples of low DNA content (Höss & Pääbo, 1993; Hoff-Olsen *et al.*, 1999), and is particularly useful for samples such as faecal samples due to the methods ability to remove potential PCR inhibitors as part of the extraction process (Handt *et al.*, 1994; Taberlet *et al.*, 1996). The QIAGEN stool DNA extraction kits used in this study are a modern commercial kit version of the silica based method, an extraction technique which has been shown to more effective than many other extraction methods for faecal DNA studies (Lampa *et al.*, 2008). A cheaper CTAB/phenol chloroform method (see Coxon *et al.*, 1999 for methodology) was also tested. Spraint samples (n = 8) were extracted using both methods of extraction and the results of PCR reactions were compared. No results were obtained from the CTAB/phenol chloroform extractions and PCRs, even from samples which produced results using the QIAGEN kit method

In order to try and reduce the possible effects of PCR inhibitors present in the spraint material the spraint itself was not used in the extraction process (Hung *et al.*, 2004). Instead, the majority of IMS was poured off of the spraint and just the last ~1-4ml of liquid, containing what appears to be light cellular medium, was collected in a 2ml tube. This was then spun down to pellet the solid material before the IMS was poured away. This process was repeated with any further liquid remaining with the spraint sample before the spraint was re-immersed in 100% ethanol. The pellet of material collected in the 2ml tube was then extracted as by the QIAGEN stool extraction kit protocol.

2.3 Microsatellite Primer Optimisation and Development

2.3.1 The Basic Optimisation Process

Individual primers have individual optimum PCR conditions and these optimums often vary from lab to lab as a result of minor changes in factors such as the PCR machines and reagents being used or the laboratory conditions and methodologies themselves (Esposito *et al.*, 1998). It is therefore necessary to carry out investigations into what these optimum conditions are for each primer before the study can be carried out successfully. This is particularly so in studies utilising low DNA and/or poor quality DNA yields, such as those obtained from spraint samples, as used in this study. With studies utilising DNA extracted from faecal material there is the added possibility that PCR inhibitors produced in the digestive tract remain in the DNA extract (Deuter *et al.*, 1995). A more stringently optimised PCR protocol should work to minimise the effects of any inhibitors present.

Table 2.2 PCR reaction reagents and volumes, as used in PCR reactions in the final study

Reagent	Final concentration	Volume added	
		Bioline Taq	Sigma Red Taq
DNA	NA	1µl	1µl
Buffer	10mM Tris-HCl, 50mM KCl	1µl	1µl
$MgCl_2$	1.5-2.5mM	0.3-0.5µl	0.16-0.56µl *
Primer	0.20-0.25mM	0.75-1µl **	0.75-1µl **
Taq	1U	0.25µl	0.5µl
Reaction volume then made up to 10µl using double distilled of HPLC water			

** of a 10pmol.µl^{-1} working stock solution

* 1.1mM of $MgCl_2$ added to reaction buffer by manufacturer, therefore lab stock supply of $MgCl_2$ used to make up final concentrations for reactions

The recipe and range of concentrations of reagents used in PCR reactions is given in Table 2.2. The reagents most in need of optimisation are $MgCl_2$ and primer. Primer, as the molecule that binds to the DNA strand and initiates replication of the sequence of interest, is essential for the successful acquisition of strands from the loci of interest. However, too much primer can be detrimental to the reaction process (see Figure 2.6), and can result in the production of large volumes of primer dimmer, (Ruano *et al.*, 1989) resulting in a strong primer flare which will compete with fragment signals further down the run, i.e. with microsatellite fragment analysis readings.

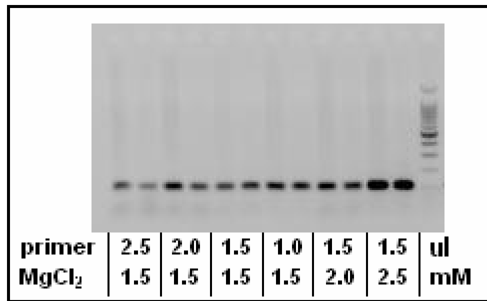

Figure 2.6 The inverted image of a gel showing, for the primer Lut7171b differing strengths of PCR products relative to differing primer and $MgCl_2$ concentrations

In PCR reactions $MgCl_2$ aids the binding of the primer to the template DNA, therefore too little $MgCl_2$ and primer binding can be week, resulting in a faint or non-existent product (see Figure 2.6). Conversely, if the $MgCl_2$ concentration is too high then the primer is encouraged to bind to sites for which it is not a perfect match, replication of the site of interest, in this case the microsatellite loci, is reduced and the number of artefacts produced due to binding to other areas of DNA increases, often producing spurious bands in the gel or sequencer run produced.

Another important part of the optimisation of a primer is to find the correct annealing temperature. A PCR reaction is cycled through steps of differing temperatures to encourage the reactions to cycle through the various steps of the process. An example of a full PCR reaction cycle can be seen in Figure 2.7. In the first step, of the process as a whole, and of each cycle within, the temperature is raised to 94ºC to encourage the DNA to denature and loose its double helix structure. The central step is the annealing step, during which the primer binds to the DNA strands and the reaction is set up. During the final step the temperature is raised up again to 72ºC. This is the temperature at which the taq polymerase is designed to work best and the enzyme works to extend from the primer and create a second copy of the section of DNA of interest. The PCR reaction will cycle through these various temperatures, in theory doubling the number of copied DNA strands each cycle, for a set number of cycles before a final extension period at 72ºC. Working in opposite directions along the DNA the forward and reverse primers work together so that the PCR reaction results in the formation of many DNA strands of a set length (Figure 2.8).

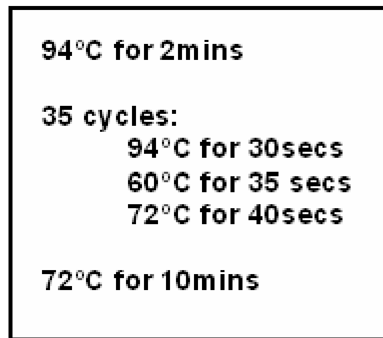

Figure 2.7 An example of a PCR cycle used for a primer with an annealing temperature of 60°C when processing tissue samples

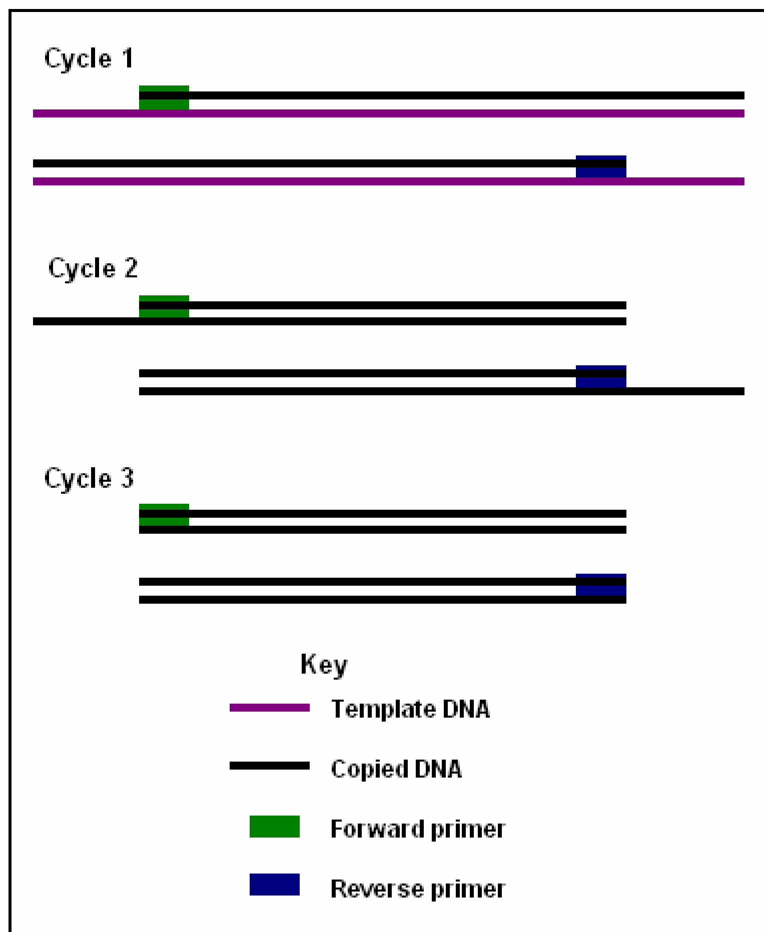

Figure 2.8 The principles of PCR involve the forward and reverse primers working in reverse directions copying from template and later copies of DNA strands. The result is an exponential number of copies of the DNA strand of interest

Each primer will have an optimum annealing temperature, which, as with the reagent concentrations may vary between labs. These annealing temperatures generally fall between 48ºC and 60ºC and so running a temperature gradient, with the temperatures graduated across the PCR block, is the ideal way to effectively identify the correct annealing temperature for a primer (see Figures 2.9 and 2.10). A step down PCR cycle - where the annealing temperature starts high in the first few cycles and is gradually reduced throughout the run - was tested for some of the primers and found not to significantly or detectably improve PCR product yields (results not shown).

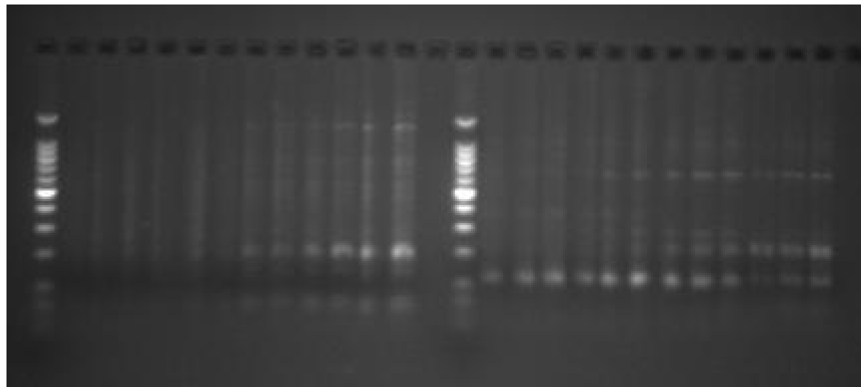

Figure 2.9 Temperature gradients for primers Lut701 to the left and Lut715 to the right of the 100bp ladder. Temperatures range from left to right from 48ºC to 60ºC. The figure clearly shows that Lut701 produces the best products at temperatures close to 60ºC, whereas Lut715 barely works at this annealing temperature and produces the brightest band, and therefore the greatest product at around 52 - 54ºC

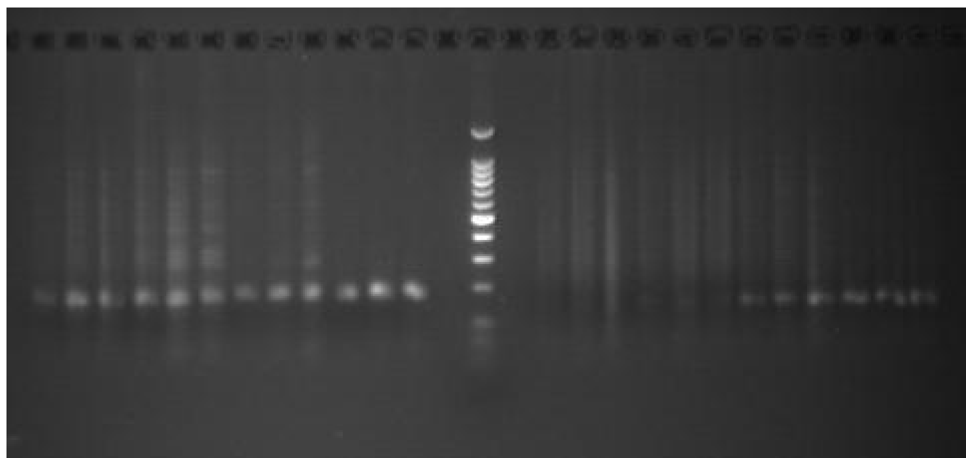

Figure 2.10 Some primers appear to produce products over a vast range of annealing temperatures, with only an improvement in band quality to indicate the most appropriate annealing temperature to use, as seen here with the results from Lut832 on the left of the gel. Conversely, some primers will only produce products at or close to their ideal annealing temperature, as seen here with Lut833 on the right of the picture

Bovine serum agent (BSA) and the detergent DMSO have both been shown to increase PCR success rate and clean-up the product produced (Bachmann *et al.*, 1990), however adding further reagents only serves to increase the risks of contamination. In cases where primers were not producing results with DNA extracted from tissue to a standard which would be considered reasonable for use with low quantity yields, testing a new primer was considered preferable to testing the effectiveness of either BSA or DMSO.

Before further testing was carried out and labelled primers ordered, PCRs were carried out using the reverse or forward primer alone in order to be certain that the products produced were as a result of both primers working together and not one of the primers complementing with itself. None of the primers were ordered pigtailed, a process designed to reduce the occurrence of adenylation (Brownstein *et al.*, 1996); however this does not seem to have effected the final results with none of the loci showing signs of variation due to variation in rates of adenylation.

2.3.2 Silver Staining as a Tool to Aid the Primer Optimisation Process

Silver staining gels are no longer suitable for accurate sizing of allele fragments, which should be carried out using a more sophisticated and accurate system such as the capillary array sequencer used later on in this study. However, the silver staining of polyacrylamide gels was used to gage allele size ranges and help asses which primers were likely to work best and produce the neatest, most easily interpreted product sizes. This meant that a more informed decision could be made over regarding primers were most suitable for use in the more expensive process of sequencer system analysis, used to genotype the samples. It also meant that labelled primers could be ordered in a range of dyes that would enable the construction of multiplexes (see section *2.4.3, Multiplexing Microsatellite Loci*) where three or four microsatellite products would be run from the same sequencer well.

2.3.2i) Silver Staining Methodology

The samples were run in batches down polyacrylamide electrophoresis gels with size stardards placed strategically across the sample set. The silver staining technique used was based on the basic silver staining protocol and was adapted in the Exeter laboratories by Ann Smithson and Lucie Evans.

Requirements:

0.1% CTAB
1M MaOH
25% ammonia solution
40% formaldehyde
3% glycerol
$AgNO_3$
Sodium carbonate

Essential Information:

Developer must be ice-cold before adding it to the gel
Don't make up the stain until the last second before adding it to the gel and protect it from the light if possible as the silver compound degrades in light
To get clear bands quickly, ensure that the gel is moved from stain to developer within 10 seconds
Always use UHP water to make solutions up
Use the agitator for soaking but agitate staining and developer by hand
Add ammonia to the stain slowly to prevent addition of too much – this will result in a brown gel
Fully dissolve the chemicals before use (dissolve CTAB for 1 hour in a hot oven) – undissolved sodium carbonate causes black speckles on a gel
Don't pour any chemical directly onto the gel
Change trays and rinse them between solutions
Recycle silver by placing in the waste container, adding 10g NaCl to precipitate silver, leaving overnight and tipping away the clear liquid.

Protocol:

1) Disassemble gel, remove top plate using a plastic spatula or ruler, and place lower plate with gel in tray. Take care not to crack corners of plates
2) Rinse gel in H_2O for 3 minutes
3) Soak gel for 30 minutes in 1 litre 0.1% CTAB (1g made up to 1 litre) with agitation
4) Dip gel in H_2O. Soak gel for 30 minutes in 1 litre 0.3% ammonia (9mls 35% ammonia made up to 1 litre) with agitation
5) Make developer by dissolving 20g sodium carbonate in 1 litre water with intense stirring. Chill until use
6) Make stain by dissolving 1.2g $AgNO_3$ in 500mls H_2O/ Add 2ml 1M NaOH – the solution will turn cloudy brown. Titrate with ammonia solution (i.e. add 35% ammonia solution drop by drop with continuous swirling until the solution turns clear). Add two further drops of ammonia solution.
7) Rinse gel in H_2O. Add the stain to the gel and agitate for 20 minutes by hand
8) Add 525µl formaldehyde to chilled developer solution (see step 5)
9) Dip gel briefly in H_2O and place immediately in half of the developer. Agitate until the first bands are visible
10) Tip off developer and add the other half. Agitate until all bands are clear. Do not overdevelop or the gel will turn cloudy black
11) Fix by adding 3% glycerol (30mls in 1 litre) for 30 minutes
12) Air dry the gel overnight and transfer information to acetate

2.3.2ii) Interpretation of the Gels

The gels, viewed on the transfers as a reverse of the samples loaded, provide rough allele sizes and ranges. Although it is not possible to accurately size alleles, by comparing at allele banding from several tissue samples against size standards we can gain a limited but informative allele size range and gain an idea of the allelic variation we are likely to gain from the microsatellite loci. From the gel shown in Figure 2.11 it can be seen that the primers Lut833 and Lut 782 both appear to produce clean allelic products with an amount, although in the case of Lut782 perhaps not a great amount, of allelic variation. It can also be seen that re-designed primers Lut833b and Lut717b (see section 2.3.3iii. Re-Designing Primers) produced stutter bands above the true allelic product. These may disappear with further primer optimisation, but similarly they may cause problems with analysis of future sequencer runs if the stutter persists. In Figure 2.12, results from the primer Mvis57 show a lot of stutter, probably owing to the fact that this primer was originally designed for use in a different species (see section 2.3.3ii. Cross-Species Amplification). Other cross species primers produced good clear results, with allelic variation and the minimum of stutter, for example Mer95 and Mvis75 shown in Figure 2.12. Also shown in Figure 2.12 are the results from the primer Lut715 which shows high amounts of allelic variation but at a range of around 210bp to 260bp which is too large for use in a spraint DNA study where products of this size are unlikely to amplify from the low DNA yields.

With greater band separation and clarity than agarose gels, silver staining of polyacrylamide gels provided information about allele ranges and variation that could then be used to make more informed decisions regarding the ordering of labelled primers for use in multiplexed sequencer runs and future sample analysis.

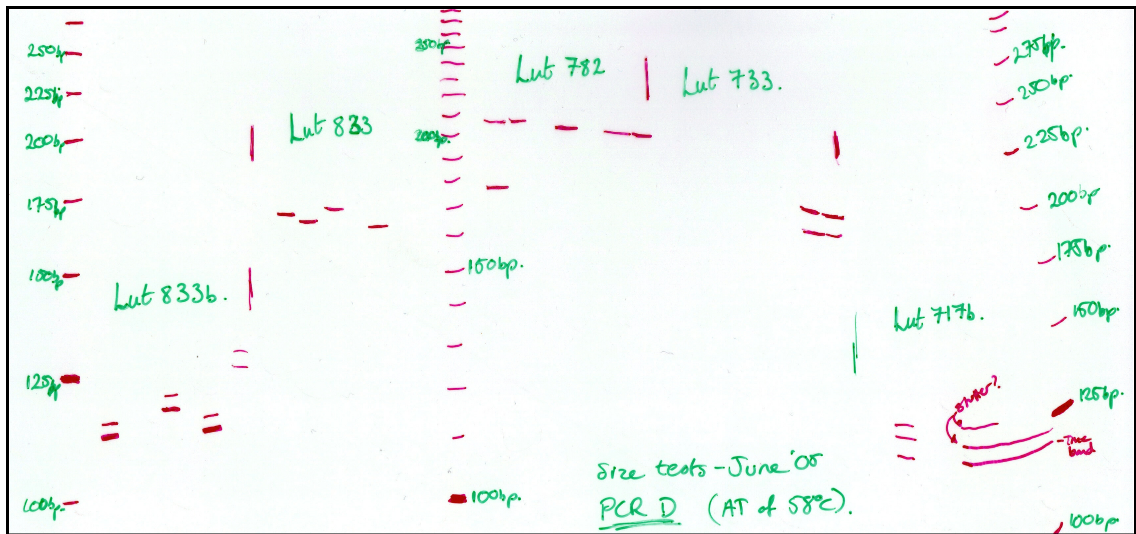

Figure 2.11 A transfer of one of the silver staining gels carried out to test the otter primers before ordering labelled primers for use with the more expensive sequencer analysis

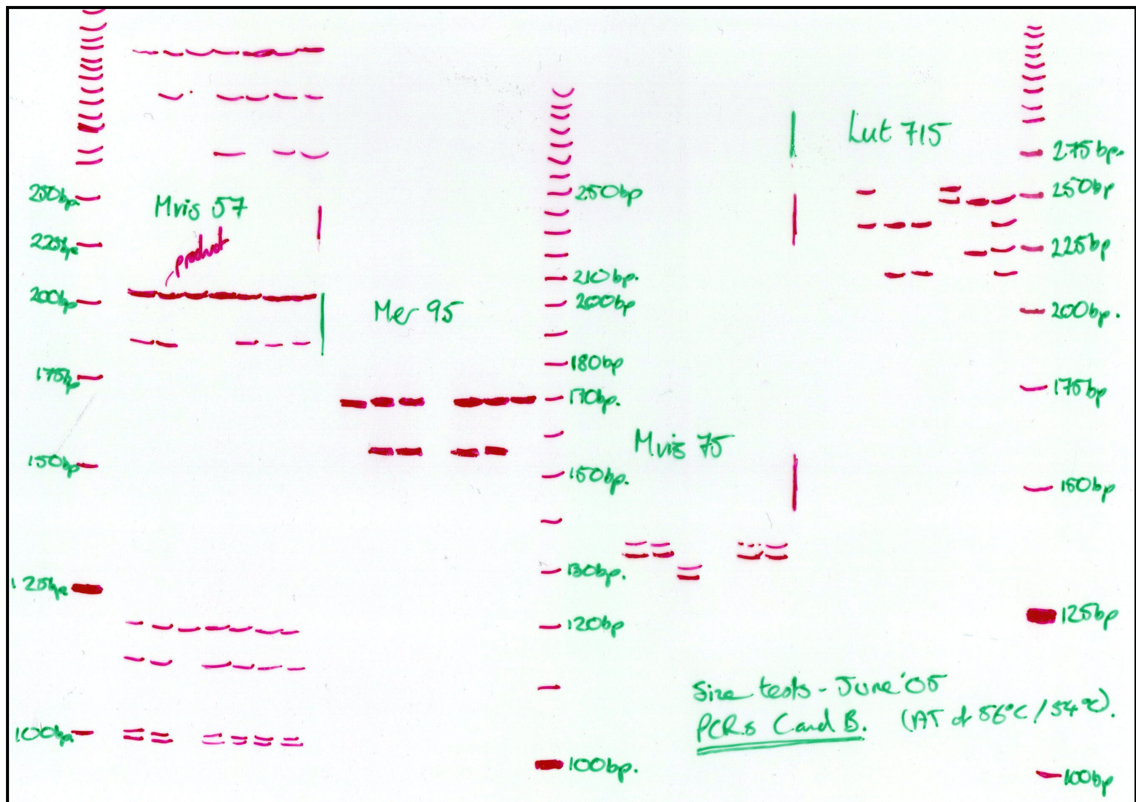

Figure 2.12 A transfer showing results from four primers tested using the silver staining process

2.3.3 Microsatellite Loci

2.3.3i) Otter Primers

The majority of previous studies of otter microsatellite analysis used the suite of microsatellite primers identified by Dallas and Piertney in 1998 (Dallas *et al.*, 1999; Pertoldi *et al.*, 2001; Randi *et al.*, 2003; Hung *et al.*, 2004; Hájková *et al.*, 2006; Arrendal *et al.*, 2007), therefore they were an obvious choice for inclusion in the first suite of microsatellite primers to be tested (Table 2.3). During this study a second set of Eurasian otter primers were published (Huang *et al.* 2005)(Table 2.3), providing a further suite of primers and allowing a better selection in the choice of primers used.

2.3.3ii) Cross-Species Amplification

Cross-species amplification is the use of primers designed for use in one species to amplify DNA fragments from another species (Slate *et al.*, 1998; Williamson *et al.*, 2002; Galan *et al.*, 2003). The method works best when species are closely related in evolutionary terms because mutational changes between the species are less drastic (Slate *et al.*, 1998; Galan *et al.*, 2003). Cross-species amplification has previously been successful across various species of mustelid (Davis & Strobeck, 1998; Kyle & Strobeck, 2001; Beheler *et al.*, 2005). In particular Blundell *et al.* (2002) successfully used several European otter primers (Dallas & Piertney, 1998) to amplify microsatellite loci from North American river otter (*Lontra canadensis*). In this study cross-species amplification was attempted using primers designed for use in other mustelid, including wolverines (*Gulo gulo*), American mink (*Mustela vison*) ervine (*Mustela erminea*) and North American river otters (*Lontra canadensis*) (see Table 2.4).

Table 2.3 Eurasian otter (*Lutra lutra*) primers tested and used in this study. Only the most informative and reliable primers were used in the spraint study

Microsatellite loci	Repeat unit	Amplified otter DNA	Used in study	Reference
Lut435	$(CA)_{29}$	**Yes**	**Yes**	Dallas & Piertney (1998)
Lut453	$(CA)_{26}$			Dallas & Piertney (1998)
Lut457 [R]	$(CA)_{26}$	**Yes**		Dallas & Piertney (1998)
Lut615 [R]	$(CA)_{27}$			Dallas & Piertney (1998)
Lut701 [R]	$(GATA)_{11}GAA(GATA)_{2}GAA(GATA)_{4}$	**Yes**	**Yes**	Dallas & Piertney (1998)
Lut715	$(GATA)_{6}GAT(GATA)_{7}GAT(GATA)_{5}$	**Yes**		Dallas & Piertney (1998)
Lut717 [R]	$(GATA)_{6}GAT(GATA)_{7}GAT(GATA)_{5}$	**Yes**	**Yes**	Dallas & Piertney (1998)
Lut733 [N]	$(GATA)_{4}GAT(GATA)_{12}$	**Yes**		Dallas & Piertney (1998)
Lut782 [N]	$(GATA)_{6}GAT(GATA)_{10}$	**Yes**		Dallas & Piertney (1998)
Lut818	$(GATA)_{11}$	**Yes**		Dallas & Piertney (1998)
Lut832 [N]	$(GATA)_{11}$	**Yes**	**Yes**	Dallas & Piertney (1998)
Lut833	$(GATA)_{15}$	**Yes**		Dallas & Piertney (1998)
Lut902	NA			Dallas *et al.* (1999)
04OT02 [M]	$(GAAA)_{16}$	**Yes**		Huang *et al.* (2005)
04OT04 [R]	$(GAAA)_{16}$	**Yes**	**Yes**	Huang *et al.* (2005)
04OT05	$(GAAA)_{14}$	**Yes**		Huang *et al.* (2005)
04OT07	$(GAAA)_{12}GAAGG(GAAA)_{9}$	**Yes**	**Yes**	Huang *et al.* (2005)
04OT14	$(GAAA)_{13}$	**Yes**	**Yes**	Huang *et al.* (2005)
04OT17	$(GAAA)_{13}$	**Yes**	**Yes**	Huang *et al.* (2005)
04OT19 [R]	$(GAAA)_{12}$	**Yes**	**Yes**	Huang *et al.* (2005)
04OT22	$(GAAA)_{16}$	**Yes**		Huang *et al.* (2005)

[R] Primer re-designed to shorten the allele fragments produced, see section 2.3.3iii)

[N] Nested PCR technique tested using this primer, see section 2.3.3iv)

[M] Preliminary results suggest loci is monomorphic in at least one of the populations being studied

Table 2.4 List of the primers from related species tested and used in this study

Microsatellite loci	Species designed for	Repeat unit	Amplified otter DNA	Used in spraint study	Reference
Gg25	*Gulo gulo*	$(CA)_{16}$			Walker *et al.* (2001)
Gg443	*Gulo gulo*	$(CA)_{14}$			Walker *et al.* (2001)
Gg452	*Gulo gulo*	$(CA)_{14}$			Walker *et al.* (2001)
Mvi057	*Mustela vison*		**Yes**		O'Connell *et al.* (1996)
Mvi087	*Mustela vison*		**Yes**		O'Connell *et al.* (1996)
Mvis075 [M]	*Mustela vison*	$(CA)_{12}$	**Yes**	**Yes**	Fleming *et al.* (1999)
Mer082	*Mustela erminea*	$(CA)_{9}$			Fleming *et al.* (1999)
Mer095	*Mustela erminea*	$(CA)_{13}$	**Yes**		Fleming *et al.* (1999)
Mel07	*Meles meles*	$(GT)_{21}$			Bijlsma *et al.*(2000)
RIO11	*Lontra canadensis*	$(AC)_{14}$	**Yes**	**Yes**	Beheler *et al.* (2005)
RIO13 [R]	*Lontra canadensis*	$(GT)_{20}$	**Yes**		Beheler *et al.* (2005)
RIO16 [R]	*Lontra canadensis*	$(GT)_{14}$			Beheler *et al.* (2005)
RIO18	*Lontra canadensis*	$(CT)_{6}(CTAT)_{14}$	**Yes**	**Yes**	Beheler *et al.* (2005)

[R] Primer re-designed to shorten the allele fragments produced, see section 2.3.3iii)

[M] Preliminary results suggest loci is monomorphic in at least one of the populations being studied

2.3.3iii) Re-designing Primers

The degraded and fragmented state of the DNA extracted from spraint means that allele sizes of around 220bp and above are often not viable (Frantzen *et al.*, 1998). Microsatellite loci which produce larger product sizes may also be prone to higher levels of allelic dropout (Arrendal *et al.*, 2007; Pearson's correlation, $r = 0.76$, $p = 0.045$), increasing the chances of mistyping a genotype. Many of the available primers are known from previous study to produce products of 200bp or greater (Dallas & Piertney, 1998) and so these primes were redesigned to create smaller product sizes (Figure 2.13)

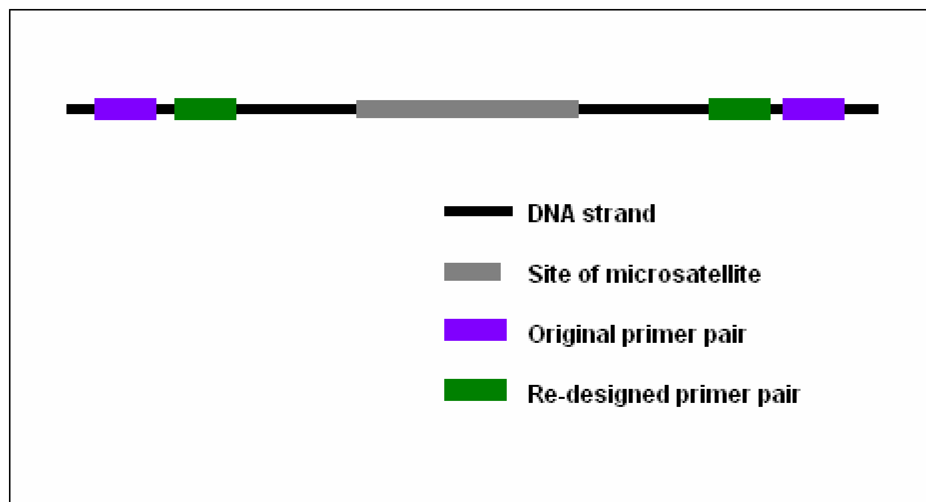

Figure 2.13 Re-designed primers sit closer to the site of the microsatellite, thus reducing the size of the fragments produced

The computer program chosen to redesign the primers was Primer3 (Rozen & Skaletsky, 2000), a copy of which is available over the internet at: http://frodo.wi.mit.edu/

Basic preferences were entered into the parameters, including preference for high GC content and aversion to long stretches of single base repetition. The sequence of DNA containing the microsatellite and the repeat motif of the microsatellite must be identified to the program, and the original primer sites are identified. The program is then asked to provide a list of potential primer pairs, cite the predicted length of sequence produced, and provide a measure of confidence in the annealing properties of the primer sequences. The resulting primer sequences chosen for testing and use in this study are given in Table 2.5.

Table 2.5 Table of re-resigned primers

Microsatellite loci	Re-designed primer sequence 5'-3'	Amplified otter DNA	Used in spraint study
Lut457b	F- ATG GCT TTA TGG CTT TCA CC R- TGT CCA CAC ATG GCC TTT C	**Yes**	
Lut615b	F- GTT GAA CTG GGC CTG TCT G R- TTT GCC CTT TGCT TCT GC		
Lut701b	F- TCC TTC CAT CCT TCC TTC CT *	**Yes**	**Yes**
Lut717b	F- GGG TCA AGG AGA TAC CAA GTA TG R- GGG TAA GGA CTG GAC GTT TG	**Yes**	**Yes**
Lut833b	R- GAG AGG GAG GGT GAA TGT CC *	**Yes**	
04OT04b	F- GGG TGG AGG TGT TAA GCA AA R- TGG GAG GCA GCA TGA TTA GT	**Yes**	**Yes**
04OT19b	F- CAC GGT GTC TGG TGT GAA AC R- CTC TGC AGC CTT TGC TTT TT	**Yes**	**Yes**
RIO13b	F- GCA CAT GGG CTT TTA TGA AGA R- TGT CCT GGG AGA GAG AGG AG	**Yes**	
RIO16b	F- CCC GTG GTC ACT TTA CCT GT R- TTT ATT GGG CAT GGA AGC A	**Yes**	

* Original primer used as complement (Dallas & Piertney, 1998)

Dallas *et al.* (1999) themselves chose to re-design primers Lut457 and Lut615 to shorten the products produced. Their re-designed primers, Lut457 (F 5´ GGTTTATGGCTTTATGGCTTTC 3´, R 5´ CCACACATGGCCTTTCTTC 3´) and Lut615 (F 5´ TCTTGCAAGTGTAACTAATAGTACAAGAC 3´, R 5´ TTTTCATTCATCACTCATCACATAC 3´), did amplify DNA extracted from spraint, however ours, of a different design, failed to do so (Table 7).

2.3.3iv) Nested PCR

The technique of nested PCR is usually applied in studies of low DNA yield products, such as (Esposito *et al.*, 1998), where copies of template DNA are scarce. The nested PCR method has been successfully used to genotype faecal samples; including those of brown bear (*Ursus arctos*) (Bellemain & Taberlet, 2004), and even human stool samples (Deuter *et al.*, 1995).

In the nested PCR set up an initial pre-PCR of around ten PCR cycles is carried out using an outer pair of primers (Figure 2.14). This targets the area of DNA of interest and increases the number of copies of this region. A normal length PCR is then carried out using the inner pair of primers and the pre-PCR mix, now containing extra copies of the region of interest, as template DNA. The method works by homing in on the region of interest during the pre-PCR in order to increase the numbers of template DNA available and ultimately the success rate of the main PCR reaction.

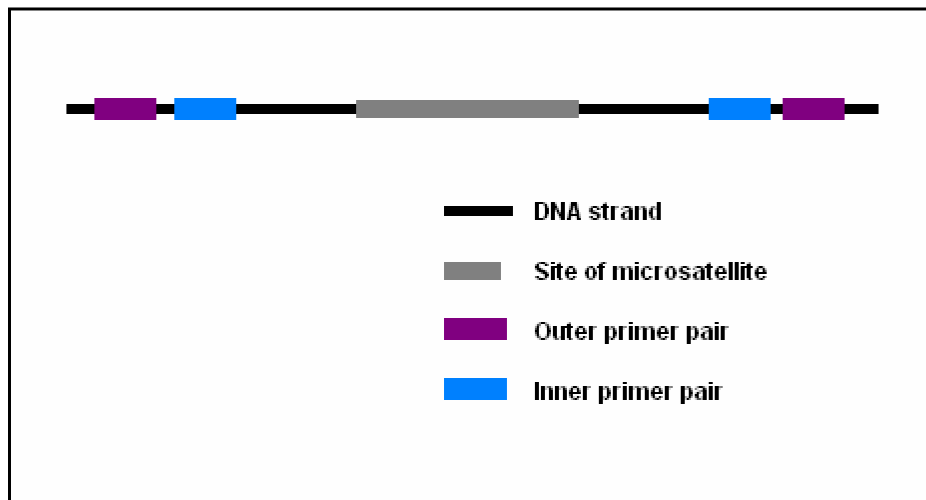

Figure 2.14 Nested PCR uses the outer primer pair in a short initial PCR cycle followed by a more standard PCR cycle using the inner primer pair

In this study nested PCR was attempted for three of the microsatellite loci for which product sizes tended to be on the larger size in the hope of improving PCR success – in particular with the failing Itchen samples in mind. Primers were identified using the Primer3 program described in section *2.3.3iii. Re-Designing Primers*.

All three nested PCR reactions worked and produced good results using DNA extracted from tissue samples. With spraint samples the technique also worked very well with the peaks produced often being very strong despite the large size of product. One major

drawback of the nested PCR approach is that it increases the potential for contamination. With two PCR set-ups and reactions instead of just one, and the transfer of materials from the first PCR reaction to the second the chances for contamination events are far greater. This was particularly evident with the results from Lut733 where contamination rates were high for the nested PCR technique (see Table 2.8 and section *2.4.4i, Contamination*).

Although the results from the nested PCRs were good the risks from contamination events were considered too high and only the results from Lut832, the majority of which were obtained using the non-nested approach, were used in the final spraint genotype analysis. Lut733 and Lut782 were dropped in favour of more productive loci.

2.3.3v) The Final Set

The final suite of primers used for genotyping spraint and tissue samples includes nine microsatellite primers designed for use in otters, four of which have been re-designed to produce smaller product sizes, and two primers originally designed for use in North American river otters (*Lontra canadensis*). The majority of microsatellite loci producing allelic products over 200bp were dropped, the exception to this being loci 04OT07 which routinely produced bands which were neat and easily identifiable, if a little weak. Loci producing large product sizes were dropped primarily due to the greater likelihood of allelic dropout with larger product sizes when using unreliable DNA sources such as those obtained from faecal samples (Paetkau & Strobeck, 1995; Arrendal *et al.*, 2007).

Previous studies of otter populations using genotypes of spraint material have used between six (Hájková *et al.*, 2006) and nine loci (Dallas *et al.*, 2003; Lampa *et al.*, 2008). The use of genotypes obtained from tissue samples to calculate measures of Probability of Identity (PI) has shown that nine loci (plus the SRY gene) are sufficient to accurately genotype spraint samples, with the likelihood of two samples from different but related individuals expressing the same genotype (PI_{sibs}) being between 2.33×10^{-3} (Arrendal *et al.*, 2007) and $5.6\text{-}6.8 \times 10^{-3}$ (Dallas *et al.*, 2003). These figures would suggest that the eleven loci analysed here are excessive. However, the accuracy of identification of individuals via genotypes relies too on the levels of polymorphism expressed by the loci, as many of the loci used in this study express relatively low levels

of polymorphism it is desirable to use increased numbers of loci. In addition, even given the apparently low likelihood of two individuals expressing the same genotype at nine microsatellite loci the phenomenon has been observed by Coxon *et al.*, (1999), who observed identical genotypes in both the carcass of an otter and in spraint samples collected both before and after the otter's death.

Table 2.6 List of microsatellite loci analysed as part of the genotyping of spraint and tissue samples. Note that allele size ranges include alleles from both the River Camel and the River Itchen population

Microsatellite loci	Number of alleles	Allele size range
Lut435	2	125-139
Lut701b	4	147-159
Lut717b	4	98-118
Lut832	5	181-197
04OT04b	4	164-196
04OT07	4	206-226
04OT14	4	118-142
04OT17	3	143-159
04OT19b	3	180-188
RIO11	3	150-156
RIO18	2	181-185

In summary, although our use of eleven microsatellite loci may be considered by some to be excessive, given the low levels of polymorphism observed for the loci used it is advisable to use as many loci as are available in order to increase the chances of always being able to distinguish between individuals, particularly in situations where there are likely to be closely related individuals sampled.

The suite of primer pairs used for the analysis of spraint are all otter specific (or specific to a related otter species not present in this country) and therefore are unlikely to amplify bands of a similar size from prey species. The most likely species for the primers to amplify are the relatively closely related American mink (*Mustela vison*) and polecat (*Mustela putorius*) (Slate *et al.*, 1998; Kyle & Strobeck, 2001; Galan *et al.*, 2003), the scat from which do sometimes get mistaken for otter spraint (Hansen & Jacobsen, 1999). However, even with these species the mistaken identity should be easily observable in the results as the allele sizes of the different species are unlikely to match the allele sizes expected to be seen from otter samples (Davis & Strobeck, 1998; Beheler *et al.*, 2005).

2.4 Genotyping Spraint

2.4.1 Introduction to Spraint Genotyping

Work with DNA extracted from spraint was carried out using a multi-tube approach designed to provide a high level of confidence in the results obtained. The idea of the multi-tube approach is to carry out multiple PCR reactions for each of the microsatellite loci for each spraint sample analysed (Taberlet *et al.*, 1996). The aim is to increase confidence in the results obtained by increasing the number of times each allele is observed (Taberlet *et al.*, 1996). The method should act to reduce the effects of allelic dropout and contamination events.

2.4.2 PCR Reactions

For DNA elution obtained from spraint samples, which typically consist of much lower DNA concentrations, the number of cycles per PCR was increased to forty-five. In theory every PCR cycle increases the number of copies of the DNA stretch of interest by two fold. Therefore a reaction starting with only one or two copies of DNA, as is possible with DNA extracted from spraint, will take longer, i.e. a greater number of cycles, to reach the same concentration obtained in a PCR with a much larger concentration of DNA template, as obtained from tissue samples. Increasing the number of PCR cycles increases the end product concentrations in instances where initial template DNA concentrations are low (Esposito *et al.*, 1998), and the number of PCR cycles used should be tailored to the initial starting concentration of DNA.

2.4.3 Multiplexing Microsatellite Loci

For the analysis of samples PCR products were run through a Beckman Coulter 8 well sequencer on a fragment analysis setting. It is typical in microsatellite genotyping studies to design fragment analysis sequencer runs so that PCR products from several microsatellite loci can be run down the same well in what is known as a multiplex, thereby saving in terms of time and cost. In order to do this the design of the multiplex must be such that each PCR product can be read accurately and must not interfere with the products from any other loci. With the availability of several dyes and knowledge

of the expected allelic ranges it is often possible to multiplex several primers in the same run, significantly saving on the running costs of sequencer runs.

Unfortunately, during the spraint analysis side of this study it was only possible to multiplex two microsatellite products at once. This was due in part to the fact that many of the microsatellite loci produced artefacts in various areas of the run that would be likely to interfere with similarly coloured/dyed allelic products from other loci. Artefacts can be caused by the amplification of DNA not associated with the microsatellite but sharing a similar primer site - a more commonly seen occurrence when dealing with fragmented DNA where the primer site may sit close to a break in the DNA forming a shorter than normal by-product. False alleles, explained in more detail in section 2.4.4iii, False Alleles, also have the ability to interfere with the reading of the allele sizes of another microsatellite if they happen to be of a similar size and dye colour.

For the spraint samples greater volumes of product are required to be loaded into the sequencing wells, therefore the maximum recommended loading volume is reached with only two or three products loaded, as opposed to the six or seven possible for samples with greater PCR success, such as tissue samples. As more PCR products are added the more they interfere and compete with each other, and the strongest products can overwhelm products from weaker PCRs or those using weaker dyes. In addition high concentrations of primer remaining after PCR reaction can affect the successful reading of allele sizes, with strong primer flare having the potential to out-compete weaker products further down the run. Given these factors it was considered that multiplexes for spraint samples should consist of products from two primers only, each labelled with a different dye. Although no-where near as cost effective as larger multiplexes, the multiplexing of two primer products still effectively cut the costs of fragment analysis by half.

2.4.4 Negative PCR Events

2.4.4i) Contamination

The low concentration of DNA used and the increase in numbers of PCR cycles to compensate mean that instances of contamination are more likely to show up in the results of spraint DNA studies (Creel *et al.*, 2003). Several steps were taken to reduce the number of instances of contamination in this study. In order to try and reduce cross-contamination all DNA extractions and PCRs from spraint material were carried out in a separate laboratory to that where tissue samples were stored and extracted and where DNA from tissue was analysed (Frantzen *et al.*, 1998). This second laboratory was also the location where post-PCR work was carried out. The separation of lab work into two different locations was used to reduce instances of cross contamination from sources with high concentrations of DNA to sources with low but important sources of DNA (Kwok & Higuchi, 1989; Handt *et al.*, 1994). This is particularly important for post-PCR samples as these will contain concentrates of the section of DNA of interest, in this case the microsatellite lDNA fragment, which could produce a false positive for the particular allele it represents if it contaminated the PCR reaction of a different sample.

Further efforts to reduce contamination include the use of 10% bleach, which will break down DNA strands it contacts with, and 100% ethanol to clean surfaces and equipment on a regular basis. The main lab bench was cleaned every day before lab work was started and communal benches, such as the bench housing the PCR machines, were cleaned approximately once a month. Laboratory equipment, such as pipettes and racks, and machinery, such as centrifuges and PCR machines, were cleaned using 10% bleach followed by a wash with 100% ethanol, with a frequency to roughly match the frequency of usage. Pipette tips, microcentrifuge tubes and solution bottles were autoclaved before use to remove any bacterial agents (Kwok & Higuchi, 1989). Any laboratory equipment being moved from the high concentration laboratory to the spraint work laboratory was also subjected to approximately 15 minutes under ultra violet light during transfer. An awareness of possible transfer on the person was taken into consideration with the use of separate laboratory coats and sets of disposable gloves in the separate laboratories used (Kwok & Higuchi, 1989; Handt *et al.*, 1994).

In this way contamination from PCR products and tissue DNA was kept to a minimum when performing DNA extractions and PCRs for spraint samples. Negative PCRs,

using HPLC or double distilled water in replacement of DNA extract, were used to assess rates of contamination. Table 2.8 shows the percentage of contamination events seen with each primer.

Table 2.7 Results for the negatives run alongside the spraint DNA PCRs, given per primer and as a percentage

Primer	No. of negatives run	Showed signs of minor contamination	Considered positive	% Contaminated (i.e. % positive)
Lut435	18	0	0	0%
Lut701b	12	0	1	8.3%
Lut717b	12	0	0	0%
Lut733	4	0	3	75.0%
Lut782	8	0	0	0%
Lut832	14	0	3	21.4%
04OT04b	2	0	0	0%
04OT07	4	0	0	0%
04OT14	12	2	1	8.3%
04OT17	18	1	1	5.6%
04OT19	6	0	0	0%
RIO11	4	0	0	0%
RIO13b	2	0	0	0%
RIO18	4	0	0	0%
Mvis75	8	0	0	0%
Total	**128**	**3**	**9**	**7%**

The multi-tube approach allowed us to detect with relative confidence the instances of contamination in the PCRs of spraint samples. Table 2.9 shows the results for sample CAM469 and loci Lut832. Only one of the nine PCRs carried out resulted in a product 197bp long, suggesting that this allele has occurred as a result of a contamination event.

Table 2.8 Allele sizes for the nine PCR and sequencer runs carried out for the sample CAM469 and the microsatellite loci Lut832

PCR	PCR well	Sequencer run	Sequencer well	Allele 1 (bp)	Allele 2 (bp)
04/09/07A	5E	09.04.07	D1	189	197
05/09/07A	7E	09.05.07	I5	189	189
05/09/07A	7F	09.05.07	I6	189	189
05/09/07A	7G	09.05.07	I7	189	189
05/09/07A	7H	09.05.07	I8	189	189
06/09/07D	1A	09.05.07	V1	189	189
06/09/07D	1B	09.05.07	V2	189	189
06/09/07D	1C	09.05.07	V3	189	189
06/09/07D	1D	09.05.07	V4	189	189

2.4.4ii) Allelic Dropout

In instances where low quantities of low quality DNA are being used it is common for only one of the two alleles to be amplified (Taberlet *et al.*, 1996; Creel *et al.*, 2003), a phenomenon known as allelic dropout. If the results from such a reaction were to be taken then it would appear that the individual was a homozygote for that microsatellite locus, when in reality they may not be. Allelic dropout such as this is commonly seen in studies of otter spraint genotyping (Hájková *et al.*, 2006, mean dropout rate = 18.5% (SD = 0.22)). Dropout rates vary greatly between microsatellite loci (Hung *et al.*, 2004, 29-62%; Hájková *et al.*, 2006, 0-85%). Hájková *et al.* (2006) found that levels of allelic dropout and PCR success rates were highly correlated (n = 239, r = -0.803, p < 0.001), suggesting that the variables are both reliant on sample quality in a similar manor.

For this study the principles of allelic dropout can be clearly illustrated by looking at an example of results. In Table 2.10 the results are given for spraint sample CAM012 for the primer 04OT17. The results from PCR 22.03.06A run in sequencer well 4F found allele 143bp but failed to amplify the allele 147bp. In sequencer well 4G the results are reversed, with the allele 143bp failing to amplify whilst the allele 147bp is clearly present. In order to increase certainty that 143bp and 147bp are both true alleles for

sample CAM012 a further two PCR and sequencer runs were carried out. Taking the results as a total it can be seen that the spraint sample CAM012 expresses both the alleles 143bp and 147bp for the microsatellite loci 04OT17, but, despite products being produced in all of the first three PCRs, it takes extra PCRs to reach the three repeats per allele necessary to call each allele size with confidence (see section *2.4.5, Spraint Genotyping Methods*).

Table 2.9 PCR results and allele sizes for sample CAM012 and microsatellite 04OT17

PCR	PCR well	Sequencer run	Sequencer well	Allele 1 (bp)	Allele 2 (bp)
22.03.06A	6A	03.22.06	4E	143	147
22.03.06A	6B	03.22.06	4F	143	143
22.03.06A	6C	03.22.06	4G	147	147
24.03.06B	1C	03.22.06	5C	143	147
24.03.06B	1D	03.22.06	5D	143	143

2.4.4iii) False Alleles

When genotyping samples using sources of poor quality and low quantity DNA, such as faecal material, it is common to encounter false alleles (Hung *et al.*, 2004). These are occurrences of alleles which do not actually belong to the sample being genotyped. False alleles can occur for two main reasons; firstly false alleles can occur due to contamination events (see section *2.4.4i, Contamination* for further information). Secondly, in a sample run of results from a spraint extract it is not uncommon to find peaks which share the same profile as a microsatellite allele, but that appear to be of the wrong size for an allelic peak. These artefacts, known as false alleles, are probably caused by slippage in the first few cycles of PCR (Taberlet *et al.*, 1996). These false alleles usually occur outside the allele size range expected for the loci, however they may still have the potential to interfere with the reading of allele sizes from other loci used in a multiplex (see section *2.4.3, Multiplexing Microsatellite Loci*). True alleles can be easily identifiable by considering allele sizes produced during the tissue genotyping study. Often false alleles occur in conjunction with the presentation of true alleles. Therefore in cases where three or more allele sizes are identified it should be those that correspond to the sizes of alleles identified during the process of tissue

genotyping that are called as true alleles of that individual. The detection of false alleles is a further reason for repetitions of PCRs.

Also of note, PCRs carried out at the same time may contain the same false allele, in which case it is advisable to carry out a second round of PCRs to check for the elimination of said false allele.

2.4.5 Spraint Genotyping Methods

A basic set of steps that were followed in order to assess quickly and efficiently which samples produced sufficient concentrations of DNA to make genotyping possible, and to produce genotypes from said samples efficiently. The method, based on the multi-tube approach of Taberlet *et al.* (1996), was designed to be easy to use and follow, with the saving of time and money in mind whilst still addressing the need to obtain the best results possible from a difficult and limiting resource. After DNA extraction the steps followed for a successfully genotyped sample were thus:

1) Four initial PCRs, one each for four different microsatellite loci

   Those samples showing good, clear alleles for three or more PCR reactions were taken on to the next step. Unsuccessful samples were discarded as they were unlikely to contain enough DNA to produce a genotype (Creel *et al.*, 2003).

2) Four PCRs were carried out per microsatellite loci. These were run on the sequencer with the products from a compatible microsatellite (see section 2.4.3, Multiplexing microsatellite Loci) amplified from the same sample.

3) PCR repeats (usually run in sets of two to four per microsatellite locus) were carried out until:
   a) One allele was observed 3 or more times, with no other alleles observed[1]
   b) Two alleles were observed 3 or more times each
   c) The DNA supply ran out

4) For samples which did not produce a full genotype before the DNA elution ran out the spraint samples were re-extracted and the process of PRC repeats (step 3) was continued using this second elution. However, the second elution often results in even lower DNA yields and consequently PCR success rate are often lower. Therefore, for the best chance of success as many PCRs as possible should be carried out using elution from the first extractions from spraint samples.

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[1] Hung *et al.* (2006) calculated that after 3 separate amplifications the probability of obtaining a false homozygote was p = 0.00675

2.4.6 Analysis of Spraint Genotyping Data

The results from the multiple PCRs were entered into a specially designed database which allowed easy access to the data for each sample. The database enabled the data regarding individual microsatellite loci and samples to be drawn out for comparisons. This meant that it was easy to view the microsatellite data of a sample and to calculate roughly how many further repeats were needed to obtain a reliable allele call for each locus, ultimately making the laboratory process smoother and more efficient. The database allowed for efficient handling and viewing of all data, both field and laboratory based, for each individual sample.

2.5 Data Handling for Spraint Genotyping Project

2.5.1 Tables and Relationships

The project produced a large amount of data for each spraint sample collected, both in terms of field data and, in particular for those samples that extracted well, in terms of laboratory data. The use of an Access database allowed all relevant data to be linked back to individual samples and allowed quick and easy comparisons of the data of interest.

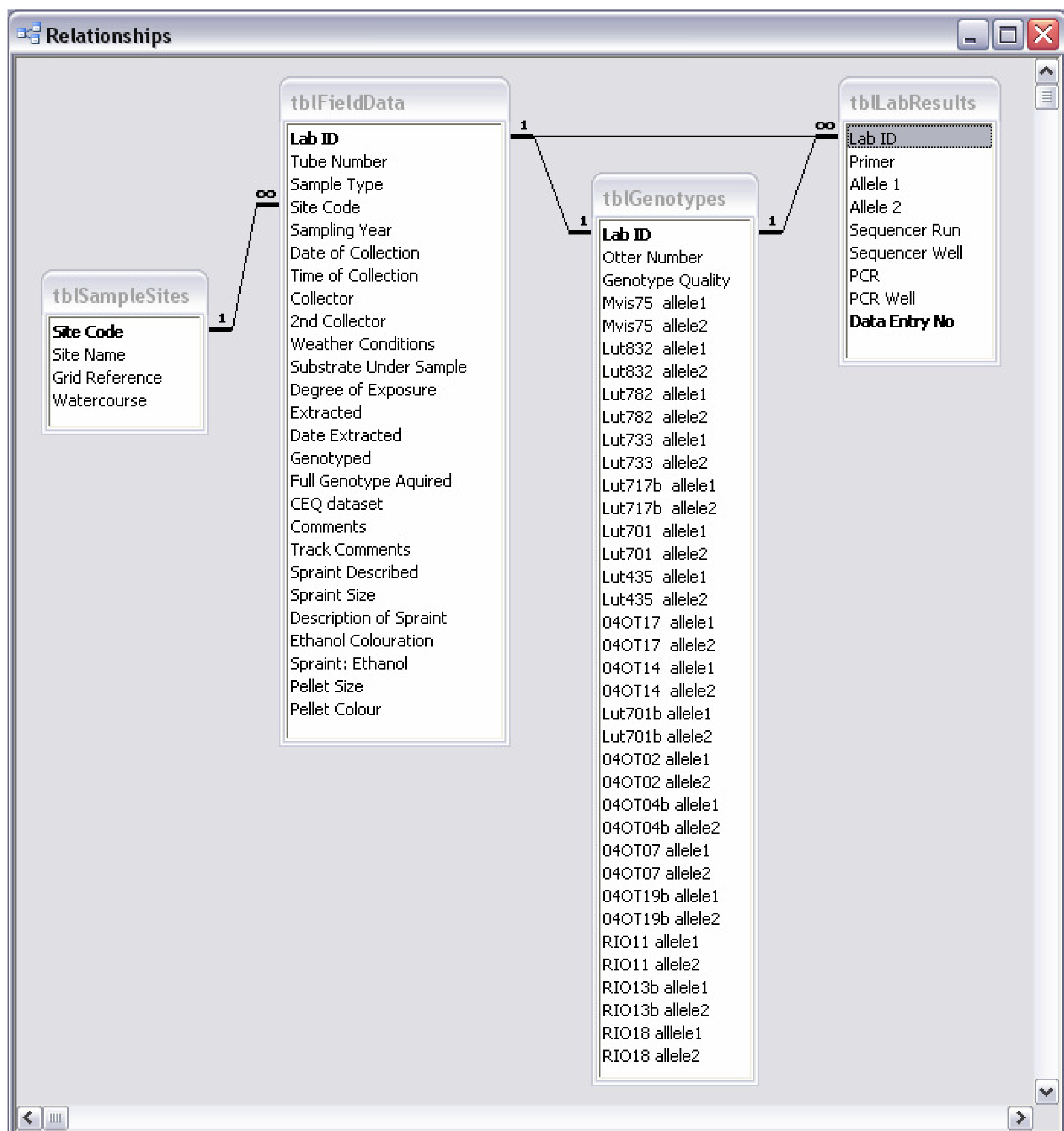


Figure 2.15 The tables and relationships used in the database designed specifically to deal with the data produced during the collection and geneotyping of spraint samples

Figure 2.15 shows the layout of the database, showing the information entered into each of the tables and the relationships between them. Each table has a primary key, highlighted in bold, which is used to identify individual records within the table. For example, in the field data table, tblFieldData, lab ID is identified as the primary key, therefore there can only be one sample with a particular ID number, ensuring there are no duplicate sample numbers within the sample set.

The relationships between tables are indicated by the lines, with the factor in the first table where the line starts being linked to the factor in the second table where the link ends. Relationships usually involve primary keys, but not always as can be seen from the tblLabResults table in Figure 2.15. The type of relationship is denoted by the symbols at either end of the relationship line. In a 1 to 1 relationship, as the relationship would suggest, one record in the first table relates to one, and only one record in the second table for the specific factors the relationship relates to. In this database the field data table, tblFieldData, and genotypes table, tblGenotypes, are linked via lab IDs in a 1 to 1 relationship. In theory data held in tables linked in a 1 to 1 relationship could in fact be entered into one single table, however, as with this data set it is sometimes beneficial to separate the data into two tables to facilitate ease of understanding and use.

A more commonly used relationship is a 1 to α (1 to many) relationship. As the name suggests in this type of relationship one record in the first table, marked with a 1, can relate to several records in the second table, marked with a α. An example would be the relationship between the sample sites table, tblSampleSites, and the field data table, tblFieldData, where it is possible for several spraint samples, identified by individual lab IDs, to be collected from the same sample site, identified by site code (see Figure 2.15).

2.5.2 Forms

Forms are constructed as a method of quickly and efficiently entering data into tables, with a form being constructed using fields from one or more tables. Data entered into a field in a form will automatically appear in the corresponding field in the relevant table and vice versa from table to form because the two are linked and the data is effectively the same data point just in two different locations. Forms are used to view records individually, and the design of forms is such that data relevant to a particular subject can easily be entered at the same time. For example, the form frmFieldData (Figure 2.16) has been set up so that all the data from the data sheets filled out by the collection volunteers at the site of collection (see Figure 2.1, *section 2.1.2, Spraint Collection*) can be entered straight into the one computer based form. The form frmSpraintDescription (Figure 2.17) is set up in a similar manor so that data regarding spraint consistency and IMS colouration can be entered straight into the relevant form and there is no need to jump between forms or across confusing tables when entering data about a particular sample.

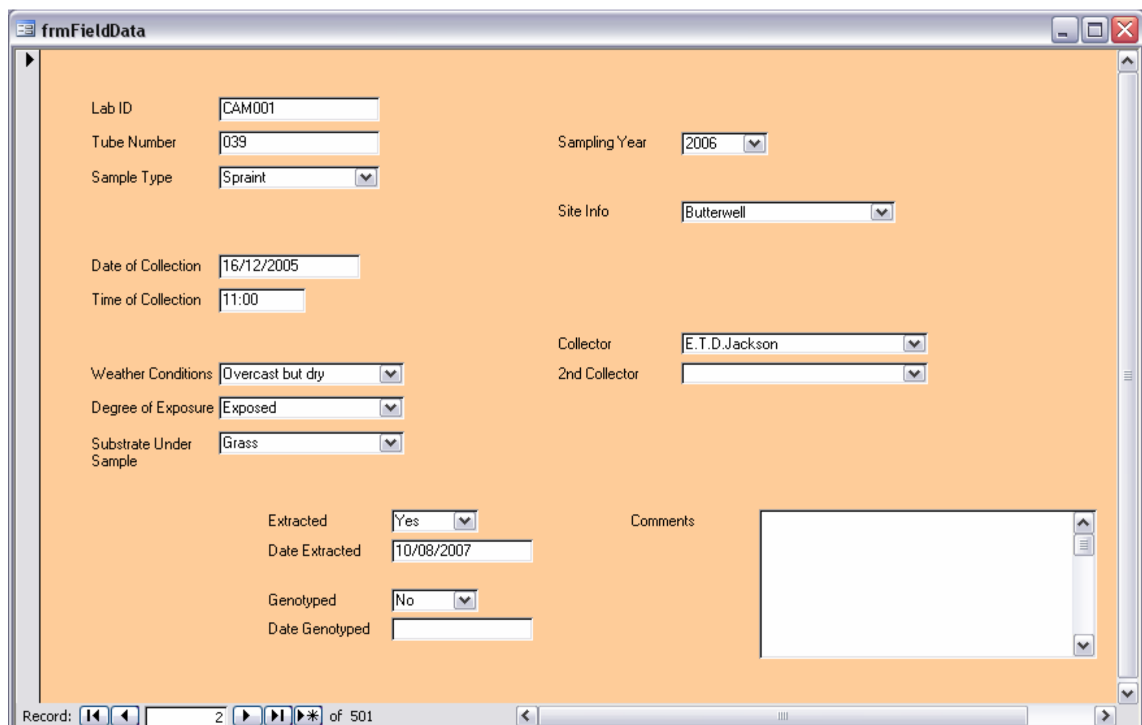


Figure 2.16 Latout of the form frmFieldData used to collate information collected by the volunteers about the field conditions in which the spraint were collected, including dates and locations

Figure 2.17 Layout of the form frmSpraintDescriptions, used to enter information about the appearance of the spraint and the medium it was collected in once it has arrived in the lab. The figure also shows a typical combo box with its dropdown list showing the various options for entering into the 'Pellet Colour' box

Converting data entry boxes into combo boxes makes data entry easier and more efficient. A combo box creates a drop-down list with pre-defined values/statements for entry into the data box. For example, in Figure 2.17, depicting the form frmSpraintDescriptions, the data box 'Pellet Colour' shows a drop-down menu of colour options for the user to choose from. This method of data entry saves time and reduces possible data analysis problems due to miss-spelling or accidental renaming of categories.

The form frmLabResults (Figure 2.18) has been designed so that all the data relating to an individual PCR well and consequent sequence run and sample result can be entered into the form together. The form relates to the table tblLabResults which is in a α (many) relationship (see Figure 2.15) and therefore there can be many data entries in the form frmLabResults for each sample, as well as multiple entries for PCR reaction and sequencer runs/wells.

frmLabResults

Lab ID	CAM002	Sequencer Run	02.22.06
Primer	Lut717b	Sequencer Well	22F
Allele 1	98	PCR	28.02.06
Allele 2	98	PCR Well	6B
		Data Entry No	195

Record: 92 of 3137

Figure 2.18 The form frmLabResults was used to record allele products, tracing htem back to the PCR and sequencer well in which they were run

After all PCRs were carried out and the correct allele sizes were known, the sizes of both alleles for individual spraint samples were entered into the form frmGenotypes (Figure 2.19). Data was entered into the form by viewing the results of all the PCRs and sequencer runs carried out for the sample for each particular microsatellite loci in turn. This was done using a query.

frmGenotypesExtended

Mvis75 allele1		Lut733 allele1		Lut435 allele1	125
Mvis75 allele2		Lut733 allele2		Lut435 allele2	137
Lut832 allele1	193	Lut717b allele1	98	04OT17 allele1	143
Lut832 allele2	197	Lut717b allele2	114	04OT17 allele2	147
Lut782 allele1		Lut701 allele1		04OT14 allele1	138
Lut782 allele2		Lut701 allele2		04OT14 allele2	138
Lab ID	CAM004	Genotype Quality	Good		
Lut701b allele1	155	04OT07 allele1	206	RIO13b allele1	162
Lut701b allele2	159	04OT07 allele2	210	RIO13b allele2	162
04OT02 allele1		04OT19b allele1	184	RIO18 alllele1	185
04OT02 allele2		04OT19b allele2	184	RIO18 allele2	185
04OT04b allele1	164	RIO11 allele1	154		
04OT04b allele2	188	RIO11 allele2	156		

Record: 3 of 53

Figure 2.19 Once all the results were collected the allelic information was entered into the form frmGenotypes using laboratory data viewed through queries

2.5.3 Queries

Queries were used to view the data more effectively. As data is linked through a network of primary keys and relationships between tables then queries can be designed to draw out the relevant sample data and information. Queries can also be used to order data, to help to answer questions about the data, and to exclude any data not required for viewing at that particular time. All the information is linked so filtering or ordering one column of data will automatically change the order of data in other columns in accordance with the order in the filtered column.

Queries about field data were used in the first instance to help select the samples deemed most likely to produce DNA during extraction. For example, we know from previous studies that anal jelly samples are more likely to produce DNA than spraint samples, and so a query was designed so that by entering the phrase "like "Jelly"" into the ***Criteria*** box in design view we could view only those samples recorded in the field as being anal jelly samples (Figure 2.20). By adding and sorting further columns relating to extraction attempts and genotyping success (see Figure 2.20) it was not only possible to sort out which samples have already been extracted, but it was also possible to use the information provided to calculate success rates for extractions from different categories of samples.

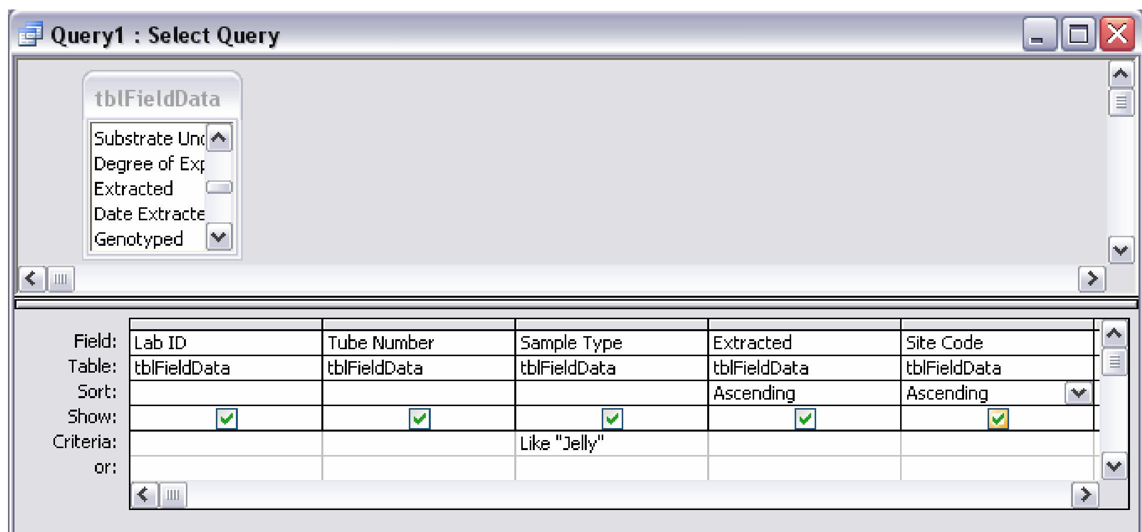


Figure 2.20 An example of a query set-up. Spraint type data has been sorted so that only anal jelly samples will be shown and samples have been sorted so that the extracted samples are separated from those still to be extracted

By sorting laboratory data by sample Lab ID and by microsatellite loci it was possible to use a query to view the allele sizes gained from each of the PCR reactions carried out per sample, per loci (Figure 2.21). Viewing all this information together allowed easy extrapolation of genotype data. As explained in the methods section (section 2.4.5, Methods), if an allele is seen more that three times then it can be considered part of the individuals genotype and therefore can be entered into the awaiting genotypes form (frmGenotypes). Viewing all available information also allows for the best and most informed judgment call when allele sizes are not intuitive, such as in cases where contamination is likely to have occurred in one of the PCR reactions. In cases where there is some uncertainty the allele size was entered into the genotypes table with an asterix or question mark to denote a low level of confidence in the allele as a true allele of the samples genotype. The entering of these alleles into the genotypes form was still important as the issue may be one of a lack of PCR success rather that the allele not being a true allele and by not calling it this may result in an identity being wrongly assigned. It is important that all relevant information is available in the final analysis and assignment of identity.

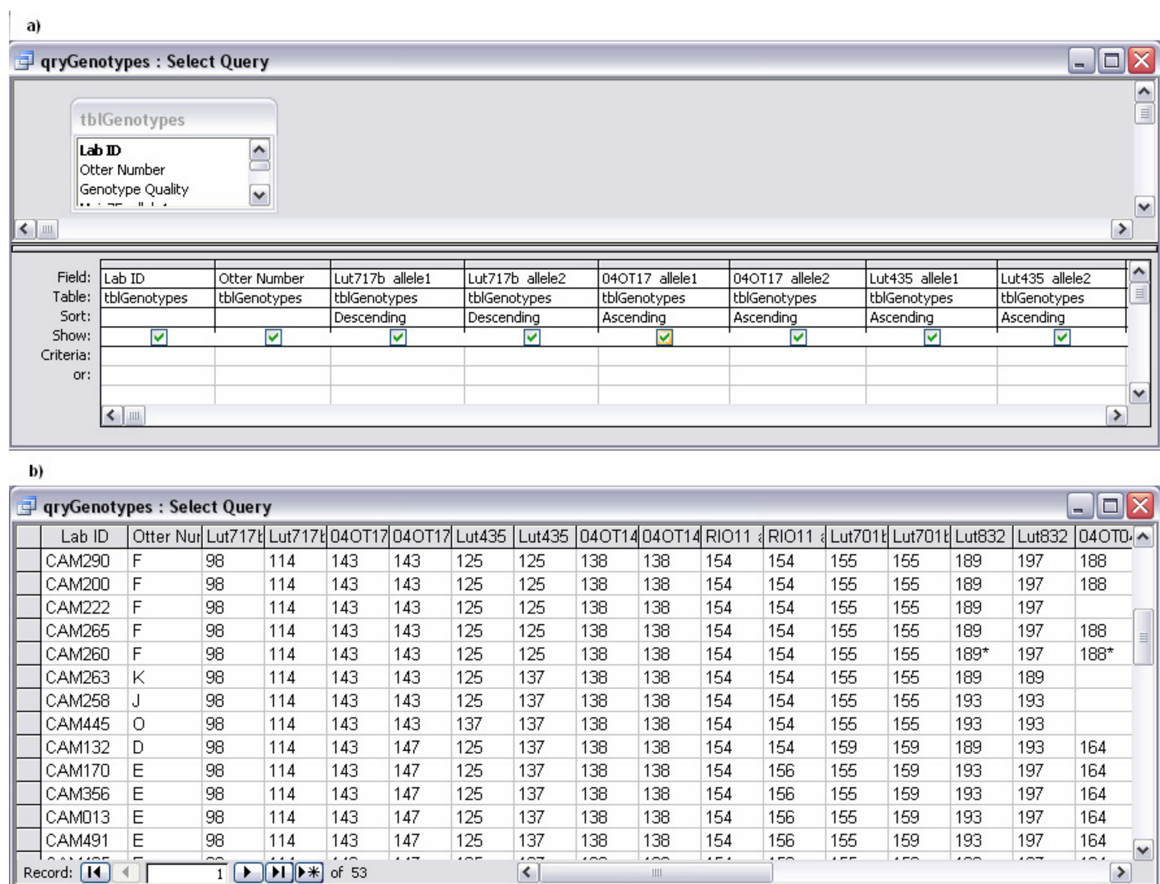
qryLabResults2006 : Select Query

Lab ID	Primer	Allele 1	Allele 2	Sequencer Run	Sequencer	PCR	PCR Well
CAM004	Lut832	193	197	08.11.07	F6	11/08/07A	10B
CAM004	Lut832	193	197	08.11.07	F7	11/08/07A	10C
CAM004	Lut832	193	197	08.11.07	F8	11/08/07A	10D
CAM004	Lut832	193	197	08.11.07	B2	10/08/07A	4B
CAM004	Lut832	193	197	08.11.07	F5	11/08/07A	10A
CAM004	Lut717b	98	114	08.11.07	C7	11/08/07A	2C
CAM004	Lut717b	98	114	08.11.07	C8	11/08/07A	2D
CAM004	Lut717b	98	114	08.11.07	C6	11/08/07A	2B
CAM004	Lut717b	98	114	08.11.07	C5	11/08/07A	2A
CAM004	Lut717b	98	114	08.11.07	A2	10/08/07A	1B
CAM004	Lut701b	155	159	08.11.07	C5	11/08/07B	2A
CAM004	Lut701b	155	159	08.11.07	C6	11/08/07B	2B
CAM004	Lut701b	155	159	08.11.07	C7	11/08/07B	2C
CAM004	Lut701b	155	159	08.11.07	C8	11/08/07B	2D
CAM004	Lut435	125	125	08.11.07	A2	10/08/07A	2B
CAM004	Lut435	125	137	08.11.07	D5	11/08/07A	4A
CAM004	Lut435	125	137	08.11.07	D6	11/08/07A	4B

Record: 1 of 1619

Figure 2.21 A view of a section of results from the query qryLabResults2006, showing the sorted allelic data for a range of microsatellite loci for the spraint sample CAM004. The above query provides an easy format for viewing and assessing allele sizes whilst entering final allele sizes into the form frmGenotypes

The queries labelled qryGenotypes were designed to group samples with identical genotypes and to enable the user to assign an identity to the various genotypes (Figure 2.22). The queries contain columns for the various microsatellites, alleles 1 and 2, which are then sorted into ascending order so that the allele column furthest left is ascended first and the next column along is then ascended within that first ordering and so on across columns of the query. The result is that matching genotypes end up listed together (although note the effects of asterix and question marks) and can then be assigned together to a specific otter identity.



Lab ID	Otter Nur	Lut717b	Lut717b	04OT17	04OT17	Lut435	Lut435	04OT14	04OT14	RIO11	RIO11	Lut701b	Lut701b	Lut832	Lut832	04OT0
CAM290	F	98	114	143	143	125	125	138	138	154	154	155	155	189	197	188
CAM200	F	98	114	143	143	125	125	138	138	154	154	155	155	189	197	188
CAM222	F	98	114	143	143	125	125	138	138	154	154	155	155	189	197	
CAM265	F	98	114	143	143	125	125	138	138	154	154	155	155	189	197	188
CAM260	F	98	114	143	143	125	125	138	138	154	154	155	155	189*	197	188*
CAM263	K	98	114	143	143	125	137	138	138	154	154	155	155	189	189	
CAM258	J	98	114	143	143	125	137	138	138	154	154	155	155	193	193	
CAM445	O	98	114	143	143	137	137	138	138	154	154	155	155	193	193	
CAM132	D	98	114	143	147	125	137	138	138	154	154	159	159	189	193	164
CAM170	E	98	114	143	147	125	137	138	138	154	156	155	159	193	197	164
CAM356	E	98	114	143	147	125	137	138	138	154	156	155	159	193	197	164
CAM013	E	98	114	143	147	125	137	138	138	154	156	155	159	193	197	164
CAM491	E	98	114	143	147	125	137	138	138	154	156	155	159	193	197	164

Figure 2.22 Showing a) the design view and b) the results of a query designed to sort genotypes in order for identities (otter numbers) to be assigned to specific genotypes

Queries were used to extrapolate data relating to a genotyped sample, such as the date and location of sample collection, allowing us to collate information regarding the location and movement of individual otter identities. Queries of field data, such as weather conditions and time of collection, and laboratory information, such as the colour of the IMS the ethanol was stored in, were used to amalgamate the information that was used to calculate which factors significantly affected the success rate of extractions.

2.5.4 Using Access to Handle the Spraint Project Data

In summary, tables and the relationships between them allowed the efficient collection and amalgamation of data in a way that also enabled its efficient use during various methods of analysing the data collected. Forms helped with quick and efficient entry of data into the relevant tables. Finally, queries were used to view and sort the data into formats that aided the various analysis to be carried out. The alternative method would have consisted of the use of a number of excel spreadsheets that would require a large amount of cutting and pasting and sorting by eye. This process would have been time consuming and frustrating and would be likely to lead to errors that may alter the quality and integrity of the final set of analysis. The use of an specifically designed Access database allowed for a quick and efficient data entry process that linked sample data in a way that greatly reduced the possibility for human error and ensured the efficient sorting and analysis of data during the entirety of the project.

2.6 Statistical Analysis

2.6.1 Checking Suitability of Microsatellite Loci

Linkage disequilibrium values were calculated using GENEPOP (version 4.0.7) (Raymond & Rousset, 1995b). For each pair of loci the computer program constructs a contingency table which is used to compute a log likelihood ratio statistic (G-test) using the Markov chain algorithm of Raymond and Rousset (1995a). This provides a P-value for the likelihood that the two loci are linked.

HWE probabilities were calculated using GENEPOP (version 4.0.7) (Raymond & Rousset, 1995b). There are three possible tests for the analysis of Hardy-Weinberg probabilities, an exact test and tests for heterozygosity excess and deficit. The exact test (Guo & Thomas, 1992) provides a probability that a deviation from HWE has occurred by chance, whilst the other two tests for deviations in a particular direction only. The exact test for Hardy-Weinberg proportions is perhaps the most appropriate test for use here as it looks at the probability that there is deviation form HWE given the whole. However, it has been suggested that the tests for heterozygosity deficit and excess are more appropriate in studies with small sample numbers (Rousset & Raymond, 1995). Therefore, it is suggested that all three tests should be used in conjunction in order to assess the extent and direction of deviations from HWE

2.6.2 Population statistics

2.6.2i) Allele Frequencies

Allele frequency was calculated using the number of alleles of that length divided by the total number of alleles genotyped for that locus. In essence it is the proportion of alleles of a particular allele length, worked out per microsatellite locus.

For example, if in a population of five individuals genotypes for microsatellite X are as follows:

Individual	Microsatellite X	
	Allele 1	Allele 2
01	100	104
02	104	104
03	108	108
04	100	104
05	100	108

Then the allele frequency for the allele of 100bp would be:

$$3 \div 10 = 0.3$$

As the calculations for allele frequency are not difficult spreadsheets of tables and equations were set up in Microsoft Excel in order to carry out the calculations. Several of the computer programs used to calculate latter statistics calculated allele frequencies as a side calculation. These could be used to check initial calculations.

2.6.2ii) Allelic Richness

Allelic richness of each microsatellite loci was calculated per population using the computer program FSTAT.

2.6.2iii) Observed and Expected Heterozygosities

Observed heterozygosity (Ho) is a measure of the proportion of individuals that express heterozygosity at a locus. The calculation carried out is simply the number of heterozygotes divided by the total number of genotypes obtained for that locus (Carr, 2002). To obtain an average value of Ho for the population a mean of observed heterozygosities is calculated from across loci.

Expected heterozygosity (He) is a calculation of the level of heterozygosity expected given the allele frequencies found in the population. Expected heterozygosity is calculated as one minus the sum of squared allele frequencies. The calculations were carried out in Excel on the same spreadsheet as calculations for allele frequency. Allele frequencies were squared and then a formula was used to calculate the sum of these values deleted from one for each of the loci per population.

2.6.2iv) Hardy-Weinberg Equilibrium

When considering population level effects it is necessary to consider deviations from HWE across all loci genotyped as a population level effect would be likely to chow itself at several loci combined. One way to view this is to consider the H-W results for each locus in the population and to see if several deviate from HWE. Another is to carry out a χ^2 goodness-of-fit test, based on Fishers exact test contingency tables (Rousset & Raymond, 1995), to combine the probabilities for all loci into one measurement. Therefore, providing a probability that the population is in HWE. All HWE calculations were carried out using GENEPOP (version 4.0.7) (Raymond & Rousset, 1995b)

2.6.2v) *F*-statistics

Measures of *F*-statistics work on the basis that populations have been maintained under similar conditions. Therefore, for *F*-statistics to be accurately measured it is necessary to sample from similar sized populations (Weir & Cockerham, 1984). Sample availability made it difficult to sample from similar populations, however, samples from the Cornish population were taken from the area of eastern Cornwall alone, in order to reduce the size of sampling range to one closer to that seen for the Itchen and Dorset collections.

Measures of between population F_{ST} were obtained using GENEPOP (version 4.0.7) (Raymond & Rousset, 1995b). Pairwise F_{ST} values were calculated using allele identity, avoiding the use of a mutation model which would not have taken into consideration the fact that many of the new alleles have been introduced through the release of captive bred otters not through natural mutation. Even with the use of allele identity the introduction of new alleles through the released otters will still need to be considered when the results of pairwise F_{ST} estimates are interpreted.

Variation in the number of samples analysed per population can influence the outcome of some calculations of F_{ST}. Weir and Cockerham (1984) address this by weighting their formulae according to the sample numbers collected per population. In GENEPOP (version 4.0.7) (Raymond & Rousset, 1995b) calculation of F_{ST} are carried out on individual loci using the weighted analysis of variance methods suggested by Weir and Cockerham (1984) (Rousset, 2007). To obtain an overall value of pairwise F_{ST} between populations the F_{ST} estimates of all loci are amalgamated using an exact nonparametric procedure. The method, known as an exact probability test, uses a Marcov Chain method to produce an unbiased estimate and was designed to replace the long and laborious calculations needed to carry out contingency tables and χ^2 calculations (Raymond & Rousset, 1995a). The probability test has been found to perform as well, if not better than the χ^2 test, and both methods are accurate and unbiased, even for very small samples or low frequency alleles (Raymond & Rousset, 1995a). The exact test used by GENEPOP has recently been modified so that the unbiased estimate is now based on the P-value of a log-likelihood ratio (G) and it was this "G-test" which was used in the calculations presented in this study (Rousset, 2007).

Calculations of the inbreeding coefficient, F_{IS}, were also carried out using GENEPOP (version 4.0.7) (Raymond & Rousset, 1995b). Here calculations are carried out using formulae and parameters designed by Robertson and Hill (1984), as well as by Weir and Cockerham (1984). Both sets of formulae use weighting to adjust for variation in samples size and are worked out per loci, as for F_{ST} , and amalgamated using an exact probability test to provide an F_{IS} value per population sampled. Weir and Cockerham (1984) state that their formulae are suitable for use on small datasets, whilst Robertson and Hill (1984) state that the required sample size for their estimates to be accurate are 4205, 1402, 841 and 601 using 2, 4, 6 and 8 microsatellite loci respectively. They also

state that their formulae were originally designed for use with much larger datasets (Robertson & Hill, 1984), therefore the datasets produced in this study are likely to be too small for Robertson and Hill estimates to be accurate, even given the eleven microsatellite loci used. Weir and Cockerham estimates are taken in preference, although here to sample sizes are probably too small for accurate analysis. All calculations of the inbreeding coefficient (F_{IS}) therefore should be interpreted with caution.

2.6.3 Allele Sharing Neighbour-Joining

An allele sharing distance matrix was constructed using the computer program MICROSAT 1.5 (Minch *et al.,* 1996). This matrix was then used to construct a neighbour-joining tree using the program PHYLIP 3.6 (Felsenstein, 1995) and the file Neighbor within it

2.6.4 Relatedness and Parentage Statistics

Values of genetic relatedness were calculated using the program Relatedness 5.0 (Queller & Goodnight, 1989). Parentage assignment was calculated using Cervus, with an inclusion methodology which assigned a log likelihood value (LOD) to potential parents showing which individuals were likely to be in a parent-offspring relationship given the alleles expressed. All individuals were entered into the program as both offspring and potential parents.

2.7 Producing Mitochondrial Control Region Haplotypes

2.7.1 Obtaining Mitochondrial Haplotype Sequences

Primers L-Pro and 363rev (Ketmaier & Bernardini 2005) were used to sequence >300bp from the 5′ end of the mitochondrial control region. This marker has been used in a number of previous studies of Eurasian otter populations (Mucci *et al.* 1999; Cassens *et al.* 2000; Ferrando *et al*. 2004; Pérez-Haro *et al* 2005) and has been shown to be highly informative in discriminating otter haplotypes. Polymerase chain reaction (PCR) amplifications were carried out in a 25µl reaction volume containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.2mM of each dNTP, 0.75µM (0.75pmol.μl^{-1}) of each primer, 1U of REDTaq DNA polymerase (Sigma-Aldrich) and 2µl of DNA extract. The PCR program consisted of a denaturing stage of 94ºC for 2min, 30 amplification cycles of 94ºC for 15secs, 50ºC for 15secs and 72ºC for 45secs, followed by a final extension period of 72ºC for 5min.

Six PCR reactions were performed on template DNA from each sample to eliminate possible sequencing artefacts; products were then separated on 1% agarose gels. Reactions produced single bands of approximately 365bp which were subsequently excised and extracted using a QIAGEN gel extraction kit. The resulting DNA products were then pooled and sequenced in both directions (Lark Technologies) using the L-Pro and 363rev primers.

A section of the samples, nine samples from East Anglia and six samples from South Korea, were analysed by Klaus-Peter Koepfli, at the Department of Ecology and Evolution, University of California, Los Angeles. These samples were analysed using an identical pair of primers in order ensure that the sequences matched the haplotypes obtained in Exeter as well as possible.

2.7.2 Haplotype Identification

The forward and reverse sequences for each sample were aligned using the program AutoAssembler v.2.0 (Applied Biosystems) to obtain a sequence of >300bp. Sequences were then aligned with each other and with previously defined haplotypes (Lut1-5 from Cassens *et al* 2000, GenBank accession no. AJ006174-78; Lut 6 from Pérez-Haro *et al* 2005); alignments were performed using the program ClustalX (Thomson *et al* 1997); all alignments were then checked by eye to eliminate spurious alignment artefacts.

2.8 Analysing Haplotype Information

2.8.1 Comparisons of Haplotypes

Once haplotype identity had been assigned it was easy to use haplotypes identified in this study as well as haplotypes identified in other studies to compare otter haplotype profiles, i.e. the proportions of individuals containing each haplotype, from different countries around Europe, as well as different locations in the United Kingdom and South Korea.

2.8.2 Construction of a Minimum Spanning Network

A minimum spanning network was constructed, giving a visual representation of the minimum number of mutations needed to produce the haplotypes observed. To do this the number of mutational differences between haplotypes was calculated using the computer program Arlequin (version 3.1). The program outputted a list of pairs of haplotypes which were directly linked (i.e. unlikely to be linked through another haplotype), providing information about the number of mutation needed to get from one haplotype to the other. This was then used to construct a diagram showing the mutational links between each pair of haplotypes and ultimately showing how each haplotype is linked to all others, through central haplotypes or not.

Chapter 3. Microsatellite Genotyping

3.1 The Microsatellite Genotyping Project

The aim of this part of the project was to improve upon techniques used to review the number of otters using a water system using microsatellite genotyping techniques with DNA extractions from spraint samples. Further to this was the aim to use these genotypes to investigate the populations of otters using the River Itchen in Hampshire and the River Camel in Cornwall using these microsatellite genotyping techniques. Tissue samples were also available for this task, however it was hoped that a large amount of the information obtained would have been provided by the spraint genotyping project.

3.2 Genotyping Spraint

3.2.1 Sampling and Genotyping Success

3.2.1i) River Camel Success Rates

The collection volunteers on the River Camel collected between them four hundred and ninety-nine otter spraint samples. Of these two hundred and twenty-seven were extracted and of this number forty-one produced working genotypes. A further three samples produced what could be considered partial genotypes, however these were not considered as successful samples. Of the samples collected from the River Camel catchment 18.1% of extracted samples were successfully genotyped.

3.2.1ii) River Itchen Success Rates

Across the two collection seasons a total of one hundred and four samples were collected from the Itchen river catchment. Sampling was lower on the River Itchen in part because the lower success rates, particularly at the beginning of the project, provided less incentive for the volunteers to collect. Added to this the River Itchen suffered from long periods of high water, during which time it was too dangerous for volunteers to collect.

Of the one hundred and four samples collected extractions were attempted from fifty-nine. Of these four produced usable genotypes and three further samples showed signs of containing DNA but the yield was so low that the samples only produced partial genotypes. If only those samples which gave a full genotype are considered this gives a success rate of 6.8% - far below that seen for the Camel spraint samples. Including the partial genotypes increases the success rate to 11.9%. However, this is still far below what would be expected and implies that there is an underlying problem with the samples collected from the Itchen river catchment.

3.2.2 Assessment of Variation in Genotyping Success

One of the overall aims of the project was to improve and standardize the spraint genotyping technique, and as part of this to improve DNA extraction and genotyping success rates. To this end, factors which may have influenced the likelihood of obtaining a genotype from a sample were investigated. Information collected in the field was used to try to identify any factors which may inhibit or enhance the chances of obtaining a successful genotype from a spraint sample. Factors pertaining to the sample itself, as well as the conditions it was stored in were also investigated.

The DNA source targeted in this study is that contained in the cells shed from the lining of the otter's gut which are consequently excised from the body along with the faecal matter. Once shed these cells are no longer part of the living tissue of the otter and as such loose many of the mechanisms of protection inherent in a living cell (Lindahl, 1993; Handt *et al.*, 1994). The cells become susceptible to the degrative actions of enzymes and acids remaining in the latter stages of the digestive system. Once excised from the body the cells become even more vulnerable, being exposed to a number of possible breakdown mechanisms including bacterial metabolism, hydrolytic and photolytic breakdown (Seutin *et al.*, 1991; Lindahl, 1993; Handt *et al.*, 1994; Hájková *et al.*, 2006). Once the cell wall and nucleus have been breached the DNA itself becomes exposed to the effects of these degradation processes.

Information was collected regarding collection conditions (see section *2.1.2, Spraint Collection*) in order to help assess the effects that certain environmental factors have in facilitating cellular and DNA breakdown. The information collected included factors such as weather conditions, the time of day samples were collected, and levels of exposure to the elements; factors which are likely to reflect the amount of exposure a sample has experienced in relation to various breakdown mechanisms, and in particular to bacterial action.

The way in which a sample is stored and treated in the laboratory also has the potential to affect the chances of obtaining a successful DNA extraction and genotype. The potential effects of the IMS medium used for sample storage in this study has previously been discussed in relation to the River Itchen samples (see section *3.2.1i, River Itchen Success Rates*), however the length of time for which samples were stored,

the amount of spraint material collected and the volume of IMS storage medium used may have an effect on the process of DNA preservation and ultimately on the success rates for obtaining genotypes. The composition of the spraint itself may have a bearing on the amount of otter DNA present in the sample, with the potential for some dietary materials, such as bone fragments, to encourage the sloughing of cells from the gut lining more readily than others. In addition, factors such as the biochemical composition of the spraint may vary according to the dietary material ingested, with different conditions encouraging different rates of sample degradation.

The information presented in this section has been collated using data from the River Camel spraint samples. Data from the River Itchen spraint samples is not considered due to the low success rates obtained from these samples and the unknown cause of this reduced rate of success (see section *3.2.1i, River Itchen Success Rates*). However, it is hoped that by better understanding the causes of variation in success rates on the River Camel, this will shed light on the possible causes of the low success rates obtained from the River Itchen samples.

3.2.2i) Field Based Factors

Collection volunteers were provided with data sheets, not only to record the date and location of sample collections, but also to record information regarding the conditions under which samples were collected (see section *2.1 Sample collection*). This information was then used to investigate the effect that various factors had upon the likelihood of obtaining a successful genotype. The six field data factors included in the analysis were sample type (anal jelly or spraint), weather condition (dry, wet or frost/snow), time of sample collection, level of sample exposure (exposed or protected), substrate under sample (stone, grass or other) and season (autumn = Oct – Dec, winter = Jan – Mar, and spring = Apr – Jun). It should be noted that samples recorded as spraint containing traces of anal jelly were recorded as spraint for the purposes of this analysis. The success rates obtained from "spraint and jelly" samples (16.0%, n = 25) are closer to those of spraint samples (15.2%, n = 145) than anal jelly samples (31.3%, n = 48), suggesting that spraint samples containing evidence of anal jelly content are likely to behave similarly to spraint samples in terms of rates of successful extractions of DNA.

A logistic regression model was carried out, using the Forward:LR method, to extract from the list of six field data factors those which have a significant effect on the likelihood of obtaining a successful genotype. The model extracted spraint type (X^2 = 7.967, df = 1, p = 0.005) and weather (X^2 = 6.854, df = 2, p = 0.032) as having significant effects upon genotyping success rates. However, even after the inclusion of these two factors into the model the -2 log likelihood remained high (-2LL = 156.7), suggesting that there is still a large amount of variation unaccounted for by the model.

The predicted probabilities produced by the model (Table 3.1) indicate that anal jelly samples are more likely to produce a genotype than spraint samples. Of the anal jelly samples analysed 31.25% (±4.89%; n = 48) produced a successful genotype, while only 15.29% (±2.60%; n = 170) of spraint samples produced a genotype. The significant relationship between spraint type and genotype success rate, indicated by the model, shows that obtaining of a successful genotyping is significantly more likely from anal jelly samples than from spraint samples.

Table 3.1 The predicted probabilities produced by the logistic regression model of field collection conditions, predicting the likelihood that a sample possessing the stated characteristics will produce a genotype.

Sample type	Weather conditions	Predicted probabilities
Anal jelly	Dry	0.335
Anal jelly	Wet	0.331
Anal jelly	Snow / frost	0.652
Spraint	Dry	0.092
Spraint	Wet	0.091
Spraint	Snow / frost	0.275

With regards to the weather conditions samples were collected in, the predicted probabilities of the model (Table 3.1) indicate that samples collected in snow/frosty conditions are significantly more likely to produce a genotype that those collected under other weather conditions - classified as dry or wet for the purpose of this analysis. This would concur with the observed success rates for the three weather condition groups (Figure 3.1), where a greater proportion of the samples collected in snow/frost produced a successful genotype.

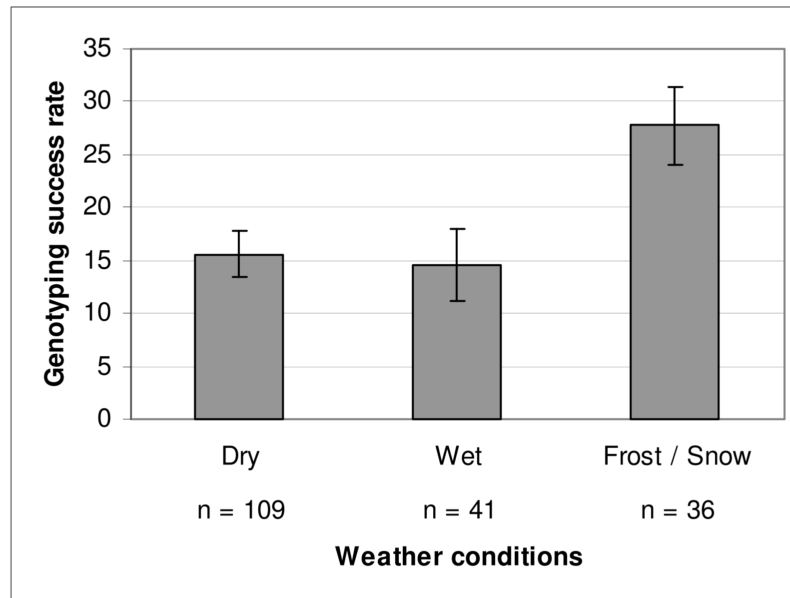


Figure 3.1 Genotyping success rates for samples of spraint collected under different weather conditions.

Of note is the fact that for samples of spraint collected under dry or wet conditions, the successful acquisition of a genotype resulted in high standardized (ZRE) and studentized (SRE) residuals (ZRE = 3.136 and SRE = 2.195 for samples of spraint collected under dry conditions and ZRE = 3.164 and SRE = 2.216 for spraint collected under wet conditions). The high residuals are an indication that the successful genotyping of these samples does not fit the model well and is further evidence of the poor overall fit of the model in terms of predicting which samples are likely to produce a successful genotype.

In summary, of the six factors assessed, only sample type and weather conditions were shown to significantly affect the chances of obtaining a successful genotype. However, these factors alone are not sufficient predictors of whether a particular sample will produce a genotype or not.

3.2.2ii) Sample Observations

Samples varied in terms of the amount and appearance of spraint material collected, as well as in terms of the volume and colouration of the IMS collection medium the sample was stored in. Observations were made in the laboratory in order to try to assess whether any of these factors can be used to predict the likelihood of obtaining a genotype from a sample, potentially providing information which may be useful for the

selection of the more productive samples for analysis in future studies. Only samples of spraint (i.e. not anal jelly) were included in the analysis of sample variation in an effort to negate any possible interactive effects between spraint type and the sample parameters analysed. As the number of anal jelly samples analysed was already small these samples were not subjected to further analysis.

The Forward:LR method was used to carry out a logistic regression analysis of genotype success rates relative to five sample condition factors. The factors entered into the model being storage time (≤2 months, 2-6 months or ≥6 months), IMS colour (clear/pale or amber/dark), IMS:spraint ratio (poor, reasonable or good), spraint description (loose, silty or other) and spraint size (small, medium or large). The model extracted IMS colour ($X^2 = 10.633$, df = 1, p = 0.001) and IMS:spraint ratio ($X^2 = 15.976$, df = 2, $p < 0.001$) as having significant effects upon genotyping success rates. Together these factors account for 68.4% of the variation in the spraint sample data (remaining -2LL = 12.291).

Table 3.2 The predicted probabilities produced by the logistic regression model of spraint sample observations, predicting the likelihood that a sample possessing the stated characteristics will produce a genotype.

IMS colour	IMS:Sprаint ratio [a]	Predicted probabilities
Clear / pale	Poor	1.000
Clear / pale	Reasonable	0.083
Clear / pale	Good	0.167
Amber / dark	Poor	$2.5x10^{-9}$
Amber / dark	Reasonable	$8.2x10^{-19}$
Amber / dark	Good	$1.8x10^{-18}$

[a] ratios based on observational assessment of the volume of IMS in relation to the amount of spraint material collected

The predicted probabilities produced by the model (Table 3.2) indicate that, based on the results of this study, samples that turned the IMS amber or darker are extremely unlikely to produce a genotype. Of those spraint samples analysed that did not colour the IMS to a great extent (i.e. IMS colour remained clear or pale) 20.83% (±9.02%; n = 24) produced genotypes, whereas samples which coloured the IMS amber or darker showed a 0.00% (±8.84%; n = 25) success rate. This is the opposite of what would be expected if, as has been suggested (Graham Roberts, pers. comm.), spraint which are fresher, and would therefore be thought more likely to produce a genotype, colour the collection medium to a greater extent.

In terms of the effects of the IMS:spraint ratios observed, those samples held in relatively low volumes of IMS compared to the amount of spraint material collected (classified as a poor ratio of IMS:spraint) were significantly more likely to produce a genotype than those contained in a more favourable ratio of IMS:spraint. This is signified, not only by the higher success rates observed for this category of spraint (Figure 3.2), but also by the higher predicted probabilities produced by the model for samples showing poor ratios of IMS:spraint (Table 3.2). This result again is counter-intuitive, as we would expect DNA to be preserved more effectively in larger volumes of collection medium compared to spraint material amount.

The standardized (ZRE) and studentized (SRE) residuals are high for the few samples producing genotypes after being held in reasonable (3.7%; n = 27) or good (10%; n = 10) ratios of IMS:spraint (Reasonable ratio, ZRE = 3.317, SRE = 2.328; good ratio, ZRE = 2.236, SRE = 2.074). The poor fit of the residuals suggesting that these samples are not a good fit for the model, backing up the finding that samples with reasonable or good volumes of IMS compared to spraint amount are far less likely to produce a genotype that those held in a poor ratio of IMS:spraint.

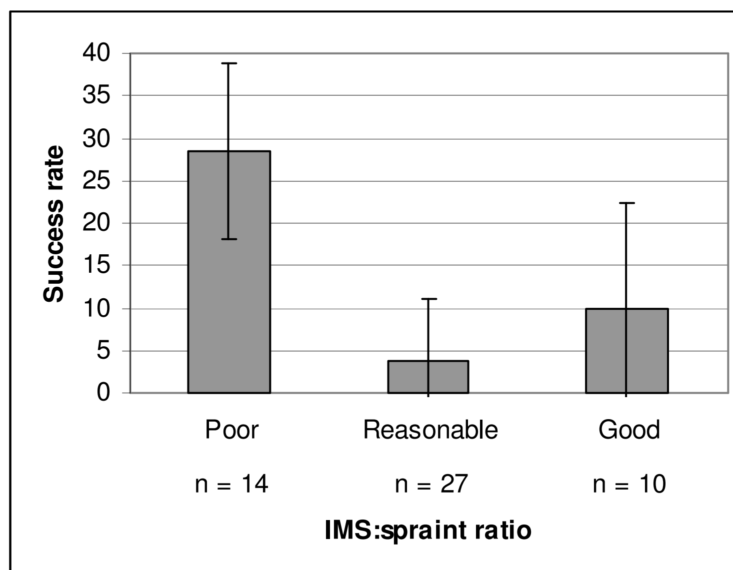


Figure 3.2 Genotyping success rates for spraint samples held in varying ratios of IMS:spraint volume.

3.3 River Itchen Results

For the River Itchen spraint genotyping study only four samples, all collected in May 2006, were fully genotyped. The four samples each produced a different genotype representing a separate individual otter (Table 3.3). From this we can deduce that we had at least four otters using the River Itchen catchment in May 2006

Table 3.3 Genotypes obtained from spraint samples collected on the River Itchen catchment.

Lab ID	Lut717b		Lut435		04OT14		04OT17		Lut832		Lut733		Lut782		Mvis75	
ITC013	118	118	125	125	118	118	159	159	193	193	157	171	188	188	128	128
ITC016	118	118	125	125	118	118	159	159	181	185	171	171	164	188	125	128
ITC023	114	118	125	139	118	122	147	159	193	193	157	157	188	188	125	128
ITC020	114	118	125	139	118	122	147	159			157	177	164	188	125	128

The genotypes obtained from the River Itchen spraint samples are composed using different microsatellite loci to those used to analyse tissue samples and Camel spraint samples. This is because the River Itchen samples were genotyped during the stages of primer development, and the samples were not of a good enough quality to yield DNA from a second extraction. The use of different loci for the study of the different river systems makes comparisons between the two populations difficult, particularly as the number of otters genotyped on the river Itchen is far too low to enable any analysis that would be of any statistical significance.

Comparisons of allele frequencies for the five microsatellite loci which have been analysed for both the River Itchen tissue samples and the River Itchen spraint samples reveal that the addition of the spraint genotypes makes little difference to the allele frequency patterns (Table 3.4). The allele frequencies for all of the loci remain similar between the tissue samples and the total - where tissue and spraint samples genotype are combined in one dataset to produce allele frequencies.

Table 3.4 Allele frequencies for the Itchen spraint samples, the Itchen tissue samples and for the total when both sets of genotypes are combined.

Loci	Allele	Itchen spraint (n = 4) *	Itchen tissue (n = 9) *	Total (n = 13) *
Lut717b	98	0	0.056	0.038
	114	0.25	0.389	0.346
	118	0.75	0.556	0.615
04OT14	118	0.75	0.333	0.462
	122	0.25	0.111	0.154
	138	0	0.444	0.308
	142	0	0.111	0.080
04OT17	143	0	0.278	0.192
	147	0.25	0.444	0.385
	159	0.75	0.278	0.423
Lut435	125	0.75	0.556	0.615
	137	0	0.278	0.192
	139	0.25	0.167	0.192
Lut832	181	0.167	0.222	0.208
	185	0.167	0.278	0.25
	189	0	0.111	0.083
	193	0.667	0.389	0.458

* n = number of individuals genotyped, i.e. number of alleles is twice this value

3.4 River Camel Results

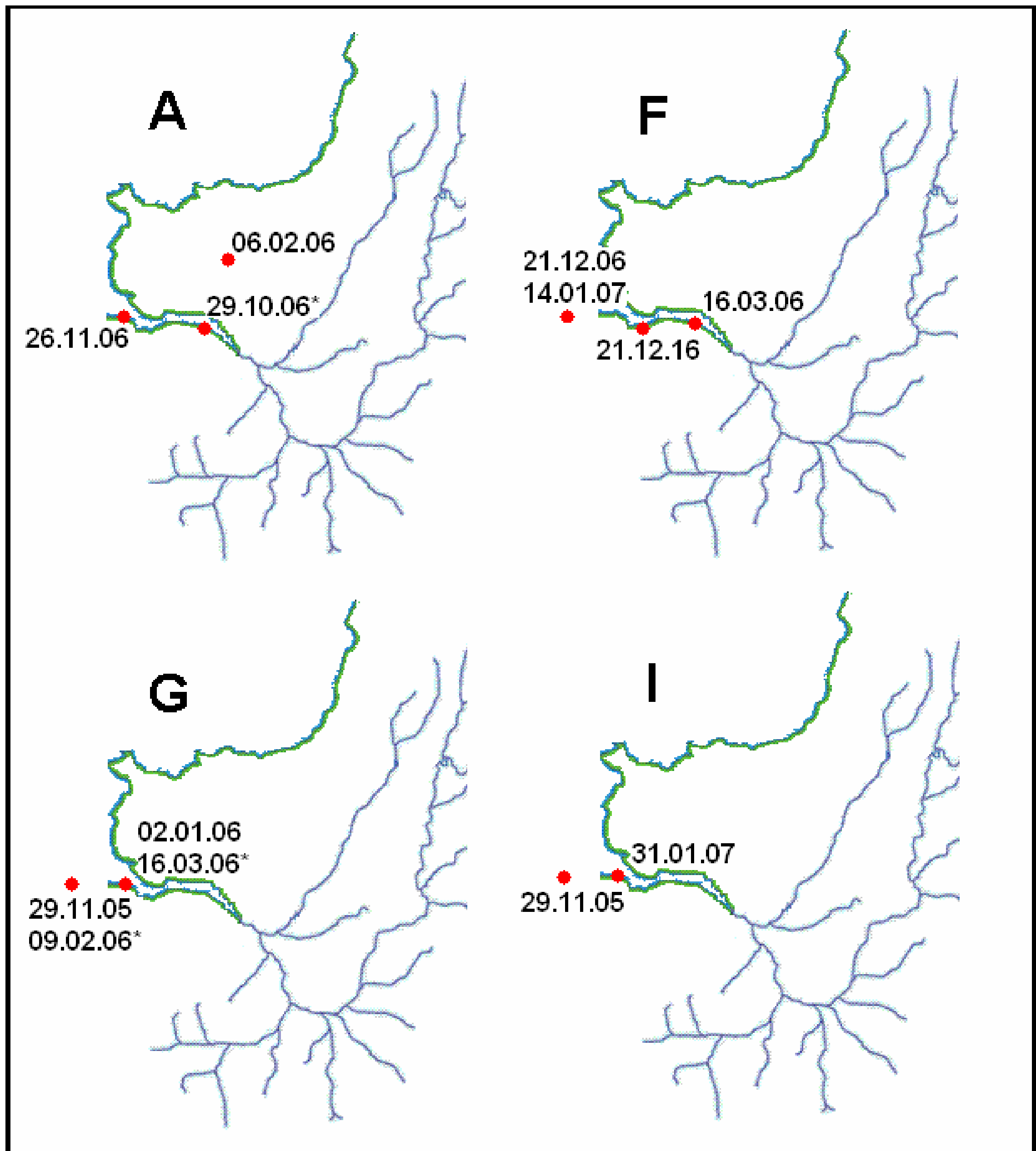
3.4.1 The Number of Otters

Genotypes were obtained from forty-one spraint samples collected from the River Camel across the two collection seasons. From these, sixteen different genotypes were identified, representing at least sixteen different individual otters between them. Nine different genotypes were found in the first collection season, and twelve in the second. This means that a minimum of nine otters used the River Camel at some point during the period from October 2005 to May 2006, and a minimum of twelve otters used the catchment at some point during the period of October 2006 to June 2007. It also means that the River Camel is capable of providing, at least in part, for the needs of at least twelve different otters during a nine month, over-winter period.

Of the sixteen, five genotypes were observed at least once in both collection periods, suggesting that the otters that these genotypes represent were likely to be residents of the river during the period of study. Of the remaining eleven genotypes only three were observed more than once. In the following sections we will examine further the information gained from date and location data in relation to genotype identification, and explore the information that can be gained from the genotypes in terms of relatedness of pairs of individuals and in terms of the population as a whole.

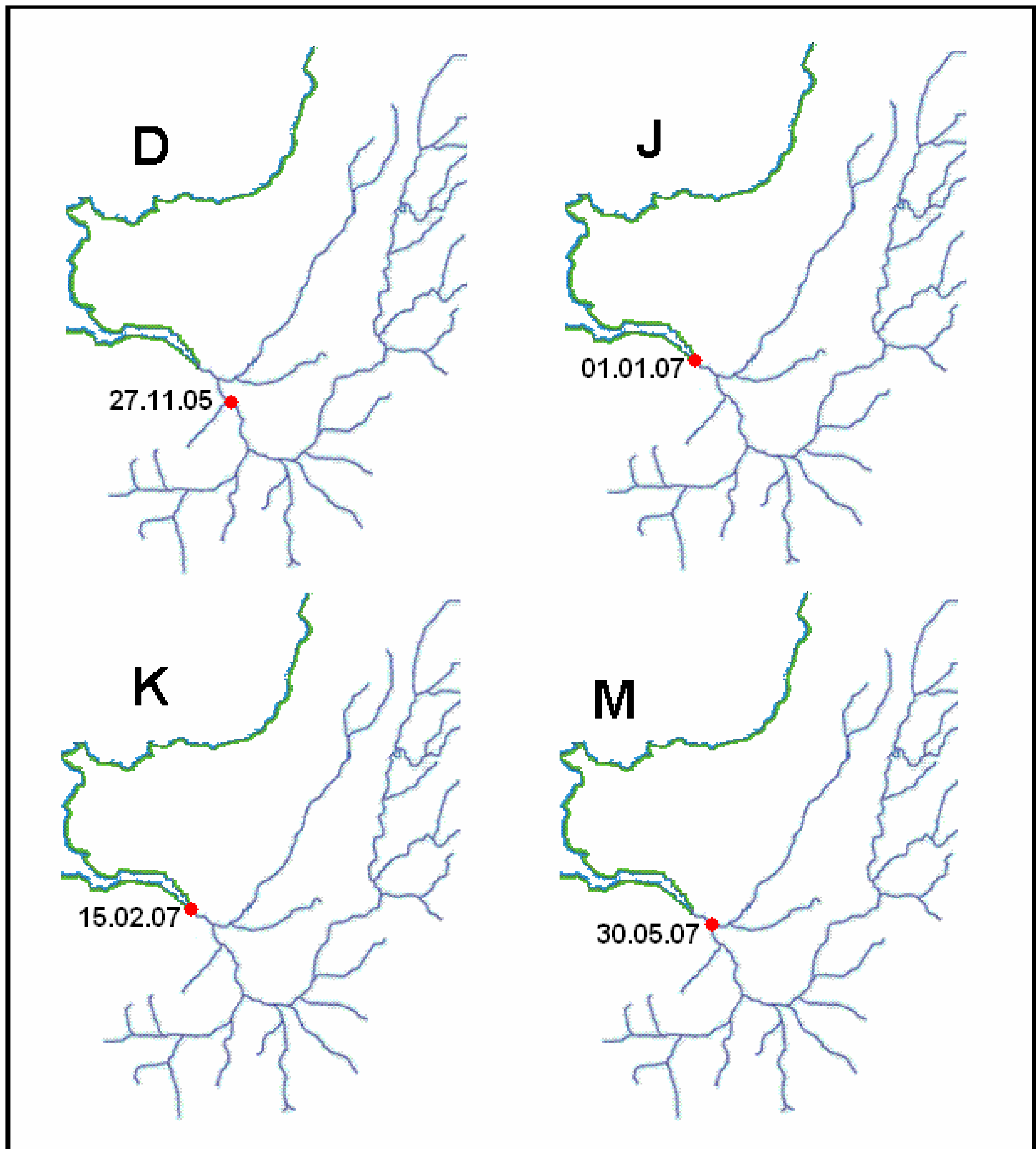
3.4.2 Dates and Locations

The amount of information collected in relation to samples includes information about the dates and locations that samples were collected from (see section 2.1.2, *Spraint Collection* for more information. This has enabled maps to be drawn providing information about the location and movement of individuals within the population as a whole. Using this information three or four distinct areas of otter activity have been identified; with one group of otters using the estuary, a second using an area just above the estuary, a third the lower to mid reaches and a fourth using the upper reaches. It should be noted that none of the otters were observed using more than one of these areas indicating that they were very site specific in their use of habitat/home ranges.



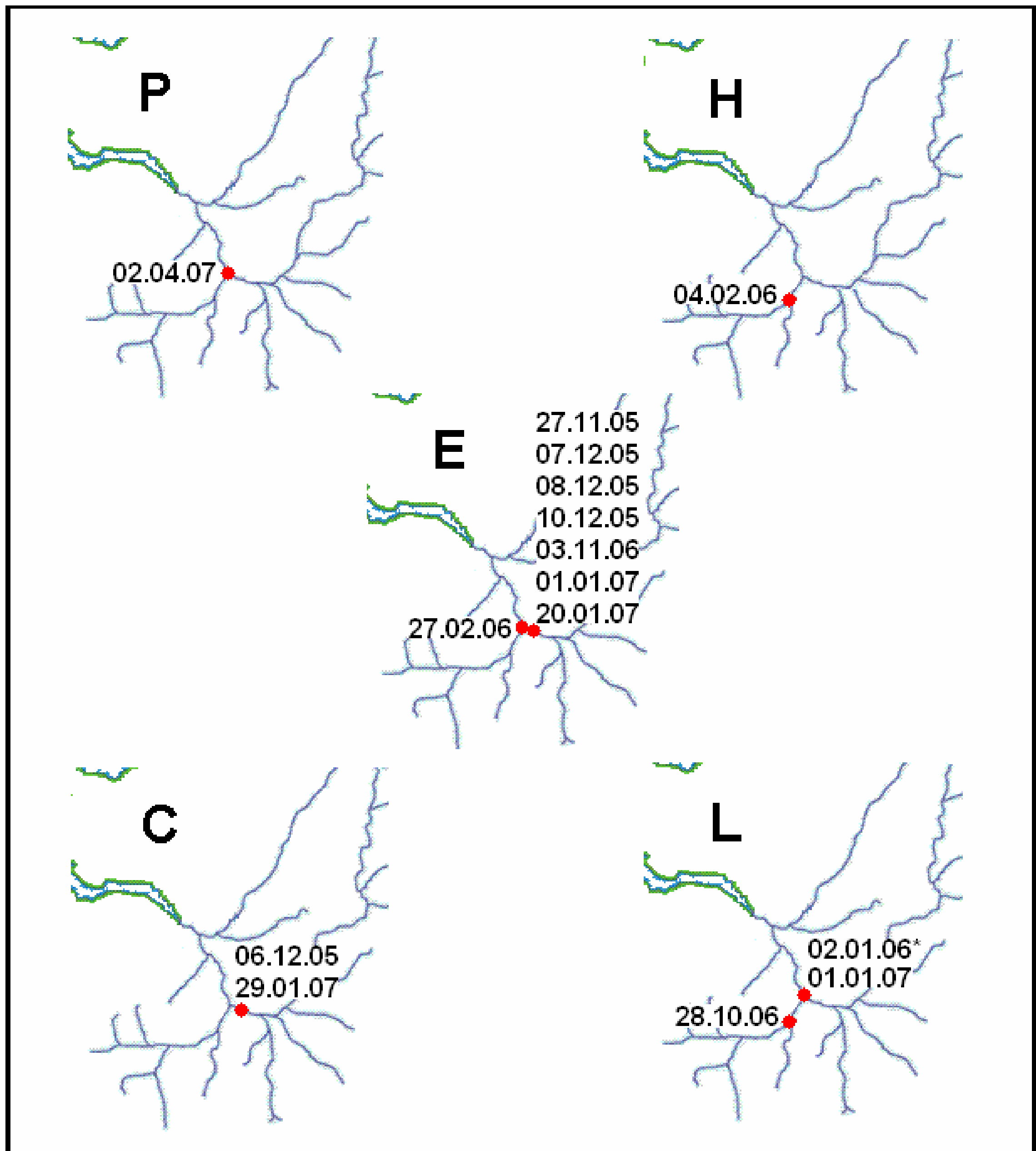
Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
A					Y				Y	Y							
F						Y					Y	Y					
G		Y		Y	Y	Y											
I		Y										Y					

Figure 3.3 Dates and locations for the collection of spraint samples identified via microsatellite genotyping as the individuals labelled A, F, G and I, all of which were found to be using the estuary of the River Camel. Stars denote samples for which there are questions over the assignment. Underneath is a map of the months in which these otters were found, where positives are highlighted in purple. Blue squares represent genotypes which may belong to a separate individual, and green poorly genotyped samples which fit only this individual for the few loci which have been typed.



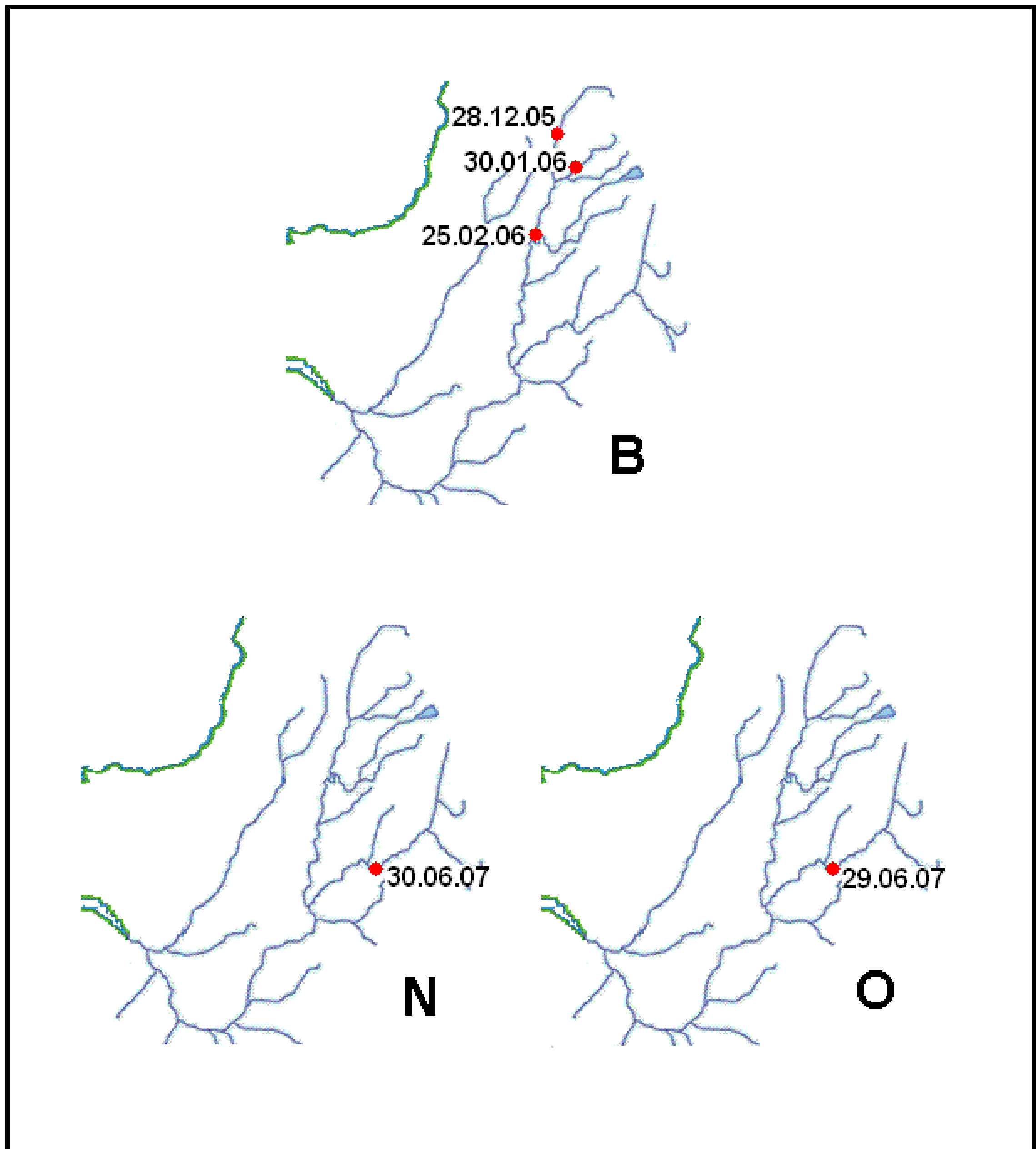
Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
D		Y															
J												Y					
K													Y				
M																Y	

Figure 3.4 Dates and locations for the collection of spraint samples identified via microsatellite genotyping as the individuals labelled D, J, K and M, all of which were found to be using the lower section of the River Camel. Underneath is a map of the months in which these otters were found, where positives are highlighted in purple.



Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
P															Y		
C			Y									Y					
E		Y	Y		Y					Y		Y					
H					Y												
L				Y					Y			Y					

Figure 3.5 Dates and locations for the collection of spraint samples identified via microsatellite genotyping as the individuals labelled P, H, E, C and L, all of which were found to be using the lower to mid section of the River Camel. Underneath is a map of the months in which these otters were found, where positives are highlighted in purple. The green square represents a find for which the genotypic assignment is poorly supported.



Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
B			Y	Y	Y												
N																	Y
O																	Y

Figure 3.6 Dates and locations for the collection of spraint samples identified via microsatellite genotyping as the individuals labelled B, N and O, all of which were found to be using the upper reaches of the River Camel. Underneath is a map of the months in which these otters were found, where positives are highlighted in purple

3.4.3 Relatedness of Individuals

3.4.3i) Values of Pairwise Relatedness

Measures of pairwise relatedness can be used to gain information about how closely related two individuals are to each other. The measure compares the variation and similarities in allele frequencies of the two individuals in question to that of the population, thereby gaining a measure of how much of the similarity between the genotypes is due to chance given the population and how much is likely to be due to a true genetic relationship between the two individuals. The results of pairwise relatedness analysis for the River Camel spraint genotypes are presented in Table 3.5. Relatedness values of 0.5 or above, which represent pairs of individuals that are likely to be related as parent-offspring or as full siblings (see section *1.2.7i, Relatedness*) are highlighted as well as relatedness values of between 0.4-0.5 which are also likely to represent a close relationship between two individuals.

Table 3.5 Table of relatedness values for the pairs of otters genotyped from the River Camel catchment. Pairwise relatedness values above 0.50, indicating a pairing of highly related individuals, are highlighted in purple, whilst values that lie between 0.40 and 0.50 are highlighted in green.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
A	*														
B	-0.4761	*													
C	-0.5884	0.3827	*												
D	-0.3537	0.1205	-0.0279	*											
E	0.262	-0.496	-0.1197	-0.1468	*										
F	0.5097	-0.5096	-0.9256	-0.2383	-0.0125	*									
G	-0.5454	-0.0938	0.1337	-0.0729	0.1426	-0.3887	*								
H	-0.2916	0.0663	-0.0675	0.3402	-0.1219	-0.2389	0.2382	*							
I	-0.2051	-0.3699	-0.4618	-0.2561	-0.3487	0.5893	-0.4915	-0.2455	*						
J	0.1329	-0.1504	0.0969	-0.0136	0.1237	0.2476	-0.3275	-0.0499	0.4616	*					
K	0.3617	-0.2712	-0.5789	-0.1921	-0.5929	0.7248	-0.6418	-0.194	0.6137	0.3548	*				
L	-0.4389	0.2934	0.0688	0.4325	-0.0754	-0.1972	0.2316	0.825	-0.0924	0.2854	-0.2712	*			
M	0.4529	-0.2013	-0.7642	0.2208	-0.1765	0.6741	-0.6291	-0.0587	0.3302	0.2005	0.6371	-0.132	*		
N	0.0559	0.1489	0.1788	0.4338	0.2033	-0.3754	-0.0828	-0.0819	-0.6794	-0.1422	-0.5175	0.1002	-0.0422	*	
O	-0.3095	0.5725	0.443	-0.5207	-0.4917	-0.1786	-0.3284	-0.2268	0.2719	0.4141	0.2857	0.1118	-0.3725	-0.2923	*
P	-0.3599	-0.2975	0.0861	-0.0802	0.3171	-0.0437	0.3978	0.3813	0.2376	0.4687	-0.25	0.4554	-0.2961	-0.4289	-0.0169

The pairwise relatedness values, as can be seen from Figure 3.7, follow a roughly normal distribution. In total, eight pairs of individuals express a value of relatedness greater than 0.5 and a further eight express a value between 0.4 and 0.5 (Table 3.5), so that there is reason to believe that at the time of sampling there were a number of related individuals present within the population(see section *1.2.7i, Relatedness*). The pairwise relatedness data suggest that there are three significant 'families' presented by the data, where pairwise relatedness links several individuals together in a fashion that suggests that there is a family link between them all.

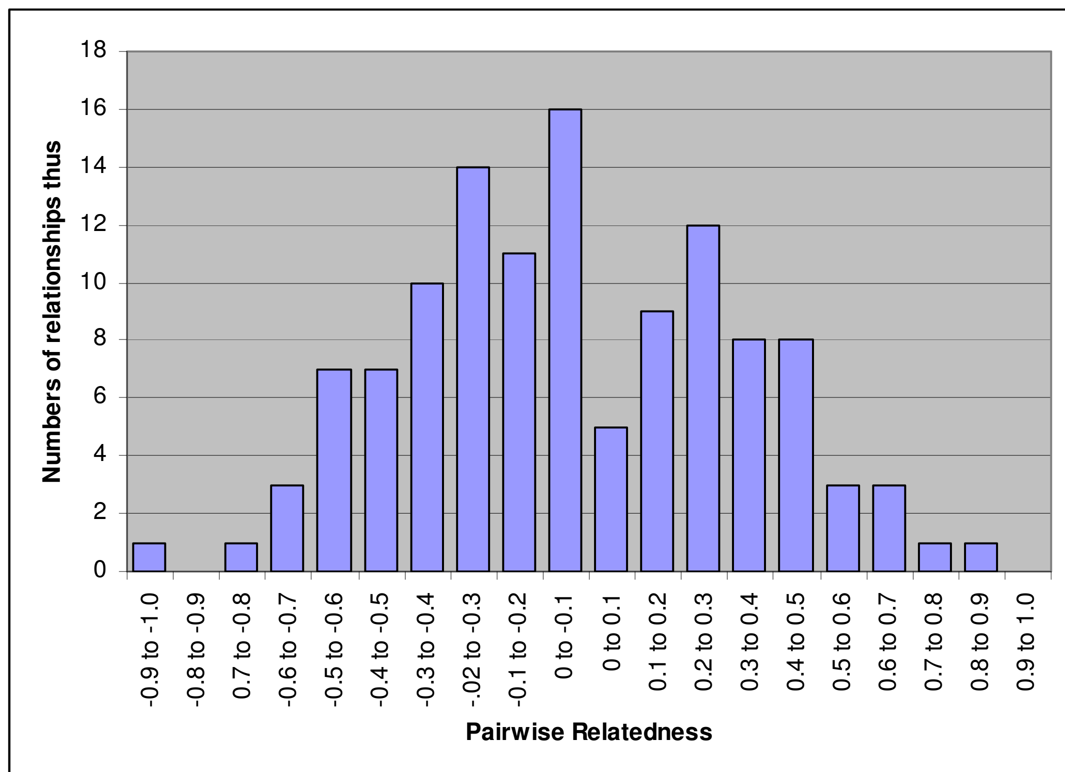


Figure 3.7 The distribution curve for values of pairwise relatedness within the River Camel population, as deduced from the genotypes of the spraint samples analysed.

3.4.3ii) Inferred Family No 1

Family No. 1 involves otters A, F, I, K, and M. The family structure seems to centre around otter F; with all of the other four individuals sharing significant values of relatedness (r >0.5) with this one otter (Figure 3.8). In terms of relationships not involving otter F, I and M both show significant values of relatedness with K (r >0.6), but show a much lower relationship with each other (r = 0.33). In addition, otter A shows values of relatedness with M (r = 0.45) and K (r = 0.36) which could be indicative of anything from a full-sibling relationship to a slightly more removed genetic relationship, such as half sibling, grandparent/child or cousin.

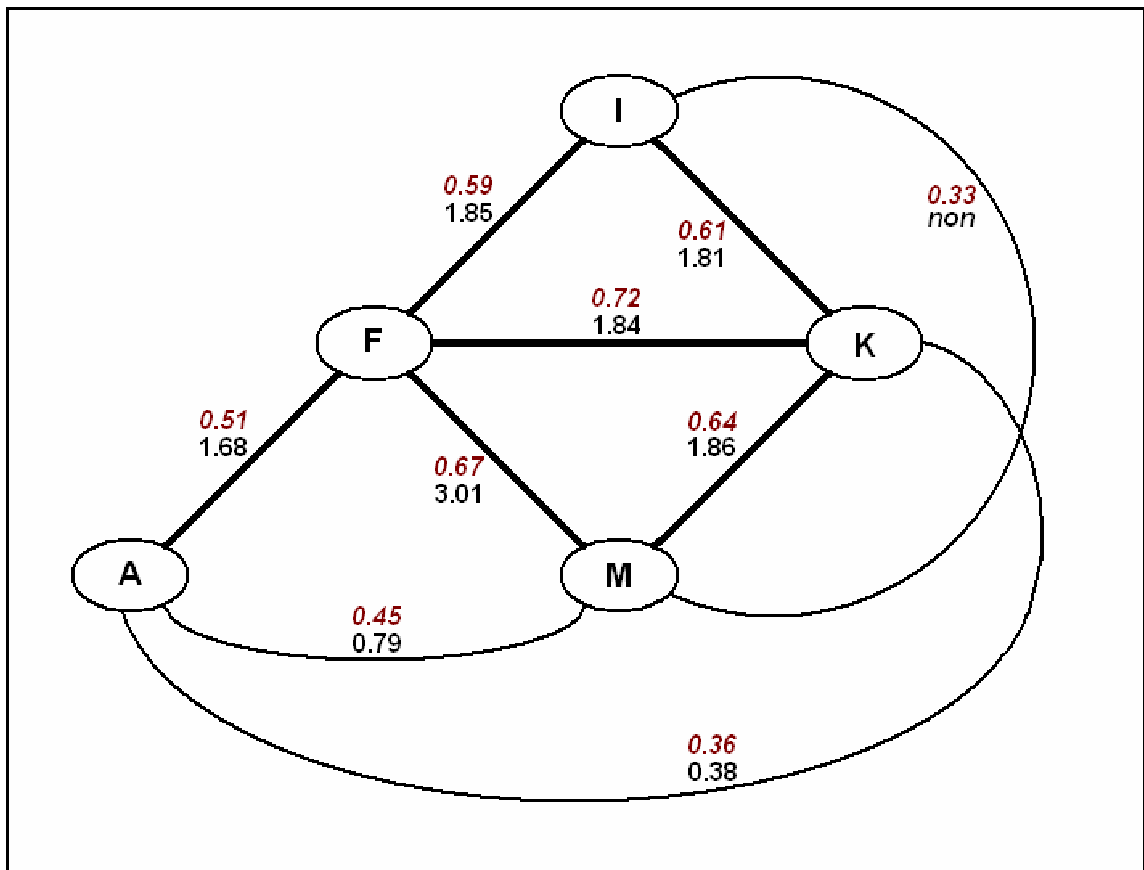


Figure 3.8 Relatedness between otters marked A, F, I, K and M, showing the relationships between various pairs of individuals along with pairwise relatedness values shown in red, and LOD (log likelihood) scores for parentage analysis shown in black. For both measurements, the higher the value the more likely it is that there is a genuine genetic relationship between the two individuals.

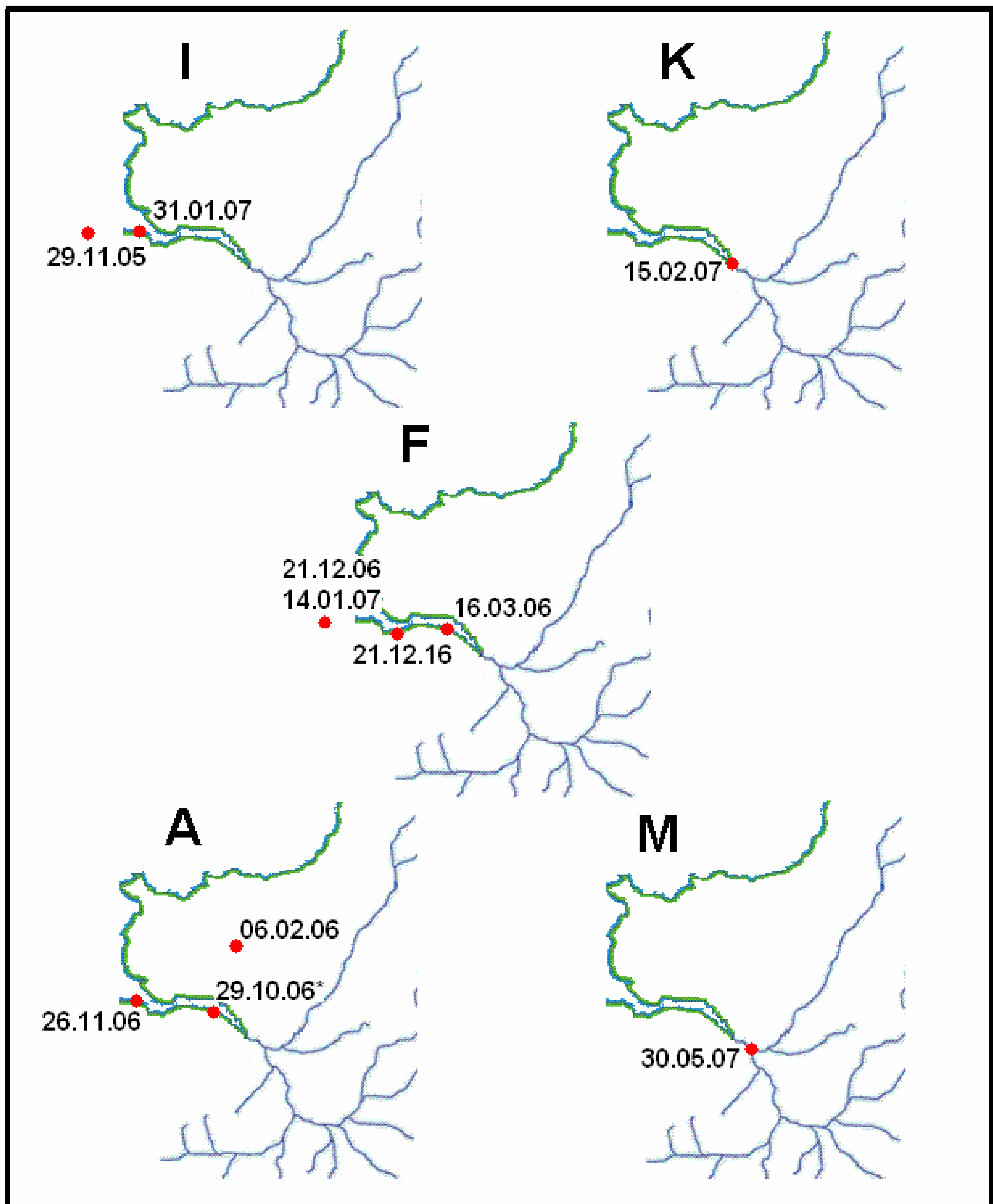
The log likelihood scores obtained from the parentage analysis (Table 3.6) support the findings of the relatedness values for family No. 1. Otter F shows the highest likelihood of being in a parent-offspring relationship with otter M (LOD = 3.01), and also shows relatively high likelihood of parent-offspring relationships with otters I, K, and A (LOD = 1.85, 1.84 and 1.68 respectively). Both I and M show high likelihood scores with K (LOD = 1.81 and 1.86 respectively), backing up the findings of high relatedness values between these individuals. Although the results of the parentage analysis do not clearly indicate which otters are the parent/offspring of which, it does lend support to relationships inferred by the high values of relatedness observed between these individual (Figure 3.8).

Of note is the fact that otter A shows a higher parentage likelihood score with otter E than otter F (Table 3.6), despite the relatively low value of relatedness observed between otter A and E (r = 0.26).

Table 3.6 Parentage analysis results for individuals in inferred family No 1. LOD = log likelihood.

Offspring	Candidate Parent	LOD	Delta	Confidence
A	E	2.026	0.345	-
A	F	1.681	0	
A	M	0.790	0	
A	K	0.376	0	
F	M	3.009	1.158	-
F	I	1.851	0	
F	K	1.841	0	
F	A	1.681	0	
I	F	1.851	0.041	-
I	K	1.810	0	
I	J	0.881	0	
K	M	1.863	0.023	-
K	F	1.841	0	
K	I	1.810	0	
K	A	0.376	0	
M	F	3.009	0.145	-
M	K	1.863	0	
M	A	0.790	0	
M	D	0.166	0	

The otters assigned to family No. 1 were all observed using the lower reaches and/or estuary of the River Camel, between the coastal edge of the collection zone and the confluence of the River Allen just above Wadebridge (Figure 3.9). Three of the otters A , I and F appear to be resident, all being observed at least once in each collection period. The otters K and M are only observed once each, however the positions of these observations, close to the confluence with the River Allen suggests that these otters may have been using the relatively un-surveyed River Allen catchment.



Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
I		Y										Y					
K													Y				
F						Y					Y	Y					
A					Y				Y	Y							
M																Y	

Figure 3.9 Dates and locations for five otters that show significant ties of pairwise relatedness. Underneath is a chart showing the months in which these otters were sampled. Positives are highlighted in purple. The blue square represents a genotype which may belong to a separate individual.

3.4.3iii) Inferred Family No 2

Inferred family No. 2 centres around the relationship between otters H and L (Figure 3.10). These otters show the highest level of relatedness seen between any pair of individuals (Table 3.7). They also express a high parentage likelihood score (LOD = 2.56) suggesting that the relationship between otters H and L is likely to be that of a parent and offspring. In addition to this, both otters have been observed using the same site (Figure 3.11), all be it in different months/ collection periods.

Two further otters, D and P, show reasonably high levels of relatedness (r = between 0.34 and 0.45) to otters H and L (Figure 3.10), suggesting that these otters are likely to be related, although the relationships are unlikely to be close parent-offspring or full-sibling relationships. The relatively likelihoods obtained from parentage analysis would support the finding that otters D and P are unlikely to be the parent/offspring of otters H or L (Table 3.7). However, in support of there being a genetic relationship between the four otters, both D and P were observed in locations used by, or a short distance away from those used by otters H and L (Figure 3.11).

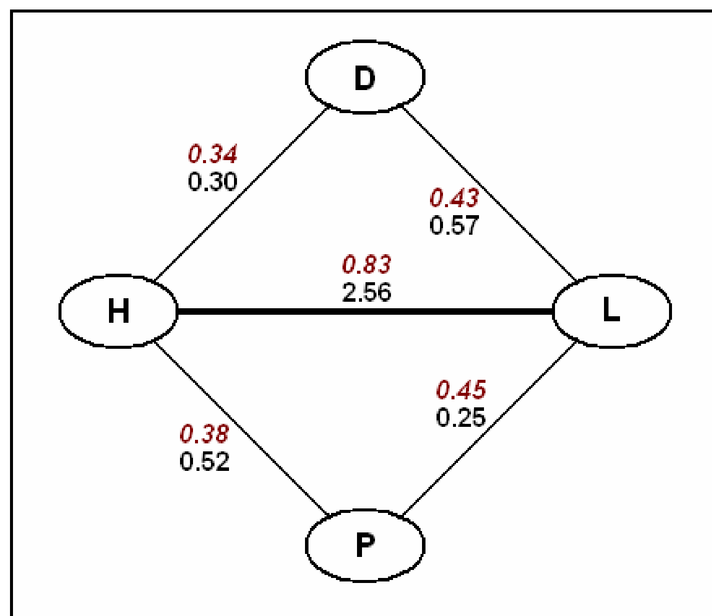
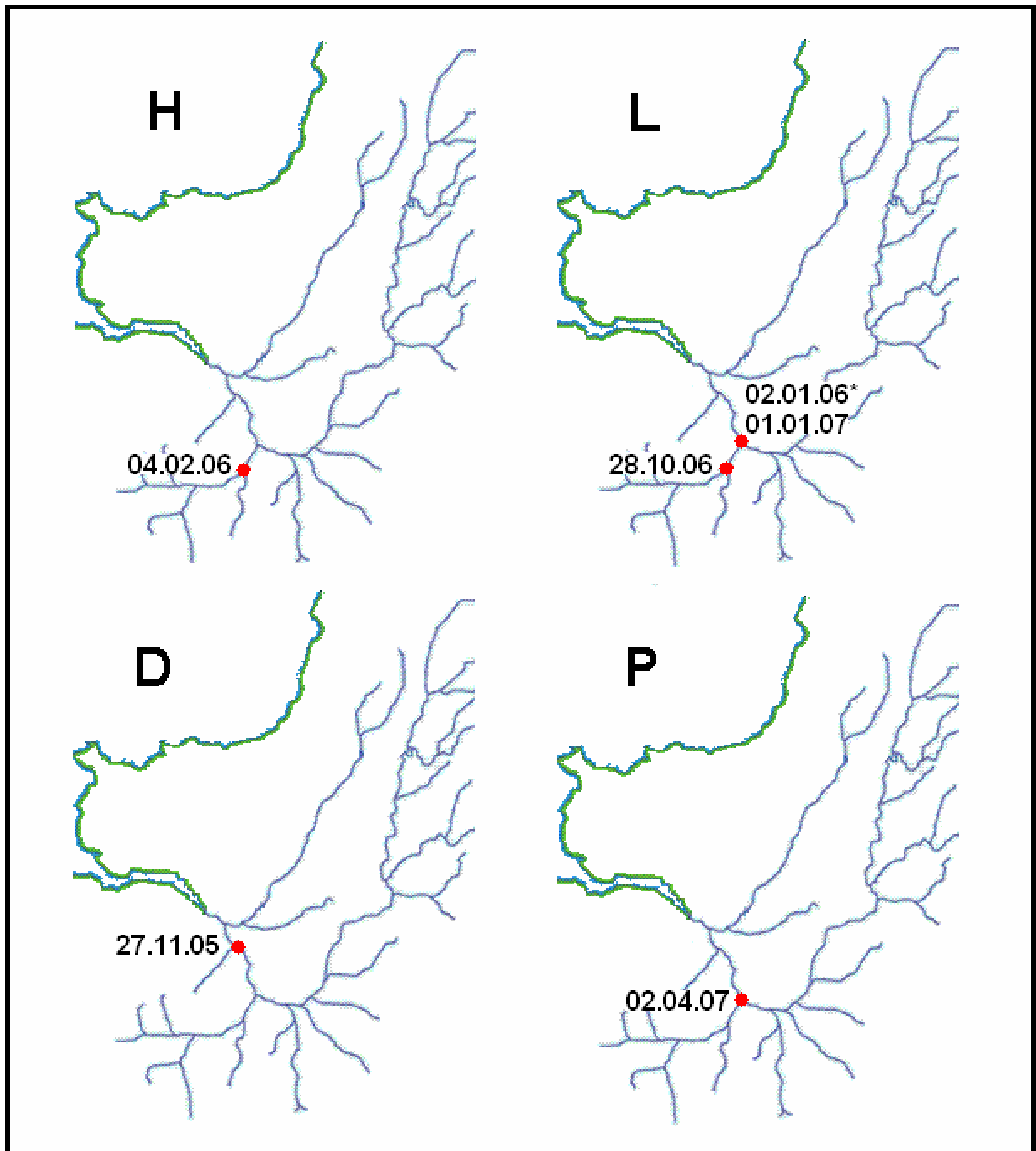


Figure 3.10 Relatedness between otters marked H, L, D and P, showing the relationships between various pairs of individuals along with pairwise relatedness values shown in red, and LOD (log likelihood) scores for parentage analysis shown in black

Table 3.7 Parentage analysis results for the individuals in inferred family No 2. LOD = log likelihood

Offspring	Candidate Parent	LOD	Delta	Confidence
H	L	2.555	2.037	+
H	P	0.518	0	
H	D	0.296	0	
L	H	2.555	1.984	+
L	D	0.572	0	
L	P	0.249	0	
D	N	0.662	0.09	-
D	L	0.572	0	
D	H	0.296	0	
D	M	0.166	0	
D	C	0.064	0	
P	G	1.619	0.883	-
P	E	0.736	0	
P	J	0.686	0	
P	H	0.518	0	
P	L	0.249	0	



Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
H					Y												
L				Y					Y			Y					
D		Y															
P															Y		

Figure 3.11 Dates and locations for four otters that show significant ties of pairwise relatedness. Underneath is a chart showing months in which otters were sampled. Positives are highlighted in purple. The green square represents a find for which the genotypic assignment is poorly supported.

3.4.3iv) Inferred Family No 3

Inferred family No. 3 consists of the otters B, C and O (Figure 3.12). The strongest relationship observed is the relationship between otters B and O (r = 0.57), although the parentage analysis score is very low (LOD = 0.19), suggesting that this is not a parent-offspring relationship. The next highest relationship after parent-offspring would be full siblings. Therefore, the high relatedness value but low parentage score between otter B and O would indicate that these otters are full-siblings. However, they may the results are not conclusive and so they may in fact be slightly more distant relations, such as half-siblings, cousins, etc. The proximity in which these otters were found (Figure 3.13) adds weight to the argument that they are related. It should be noted that the large time gap between the observations of the two individuals is due to variations in sampling effort per location and so it is not possible to conclude whether these otters were or were not present in both sampling periods.

The third otter in this group is otter C (Figure 3.12). This individual shows reasonable levels of relatedness (r = 0.38 and 0.44) and parentage assignment (LOD = 1.93 and 0.89) with both otters B and O, suggesting that there may be a genetic relationship between otter C and both of the other otters. Otter C was observed usinga site significantly further downstream from the other two otters (Figure 3.13), however may still represent a dispersing (or dispersed from) individual.

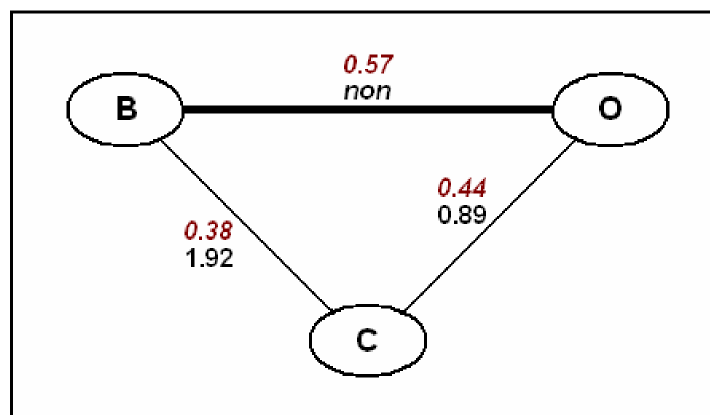
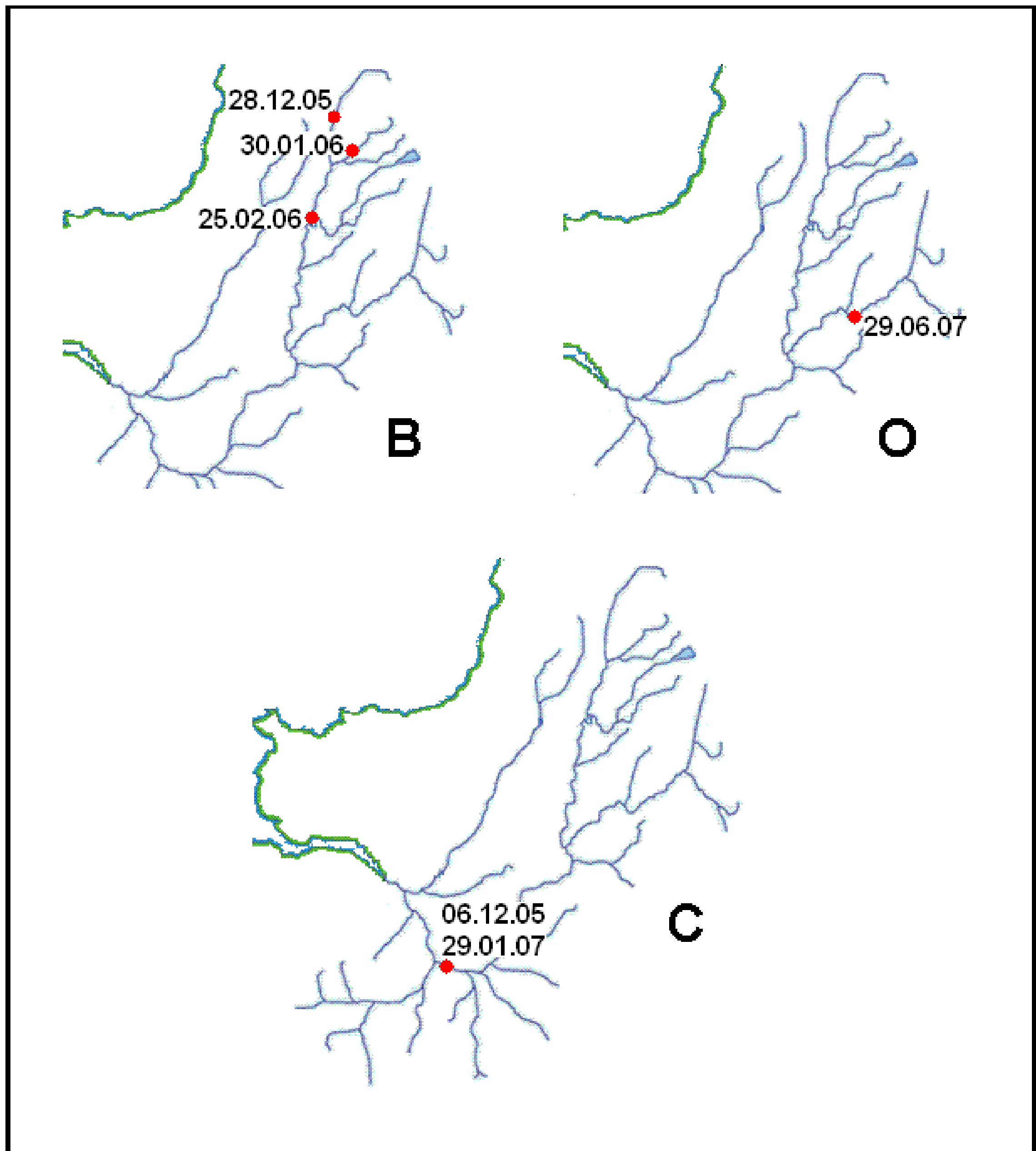


Figure 3.12 Relatedness between otters marked B, O and C, showing the relationships between various pairs of individuals along with pairwise relatedness values shown in red, and LOD (log likelihood) scores for parentage analysis shown in black



Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
B			Y	Y	Y												
O																	Y
C			Y									Y					

Figure 3.13 Dates and locations for three otters that show significant ties of pairwise relatedness. Underneath is a chart showing the months in which these otters were sampled. Positives are highlighted in purple.

Table 3.8 Table of parentage analysis results for individuals in inferred family No 3. LOD = log likelihood

Offspring	Candidate Parent	LOD	Delta	Confidence
B	C	1.925	1.738	+
B	O	0.187	0	
C	B	1.925	1.034	-
C	O	0.891	0	
O	C	0.891	0.704	-
O	B	0.187	0	

3.4.3v) Further Relationships of Possible Consequence

One further set of relationships that are worth mentioning are the relationships observed between I, J and P. I and J express a significant level of genetic relatedness to each other (r = 0.46), as do J and P (r = 0.47). However the level of relatedness between I and P is much lower (r = 0.24). Score obtained from parentage are also relatively low (between I and J LOD = 0.88 and between J and P LOD = 0.69), suggesting that there are no parent-offspring relationships between the trio. Otters I and P are both part of different 'family groups' and therefore are unlikely to be significantly related to J as if they were then J would be likely to also show relatedness to other members of their inferred family groups (see Table 3.5).

3.4.4 Population Genetics of River Camel Otters

3.4.4i) Summary Statistics for the River Camel Samples

The River Camel otters show varying levels of heterozygosity at the ten loci analysed (Table 3.9), and the number of alleles expressed per loci was generally low. Some loci were more informative than others. For example, locus 04OT14 expressed just two alleles, one of which was observed in only one individual and as a result heterozygosity was extremely low (Ho = 0.063), whilst locus 04OT07 expressed four different alleles and showed a relatively high level of heterozygosity (Ho = 0.786).

Three of the loci studied were almost out of Hardy Weinberg Equilibrium (HWE), expressing probabilities just above 0.05, suggesting that the River Camel population may be under some kind of selective pressure.

Table 3.9 Summary statistics for the genotypes obtained from River Camel spraint samples.

	N	A	He	Ho	HWE
Lut701b	16	2	0.500	0.250	0.054
Lut717b	16	2	0.498	0.438	0.638
04OT14	16	2	0.061	0.063	-
04OT17	16	2	0.305	0.250	0.434
Lut435	16	2	0.469	0.375	0.591
RIO11	16	2	0.305	0.375	1.000
04OT04b	12	2	0.469	0.417	1.000
04OT19	15	2	0.320	0.133	0.056
Lut832	16	3	0.580	0.500	0.054
04OT07	14	4	0.704	0.786	0.424

N = no. of individuals
A = no. of alleles
He = expected heterozygosity
Ho = observed heterozygosity
HWE = HWE probabilities

3.4.4ii) Comparisons with the Wider Cornish Population

A low level of genetic differentiation (F_{ST} = 0.008) was observed between the River Camel sample set and the wider Cornish sample set, suggesting that there is very little genetic difference between the two populations. The inbreeding coefficients for the two sample sets suggest that if anything the River Camel population (F_{IS} = 0.186) is less inbred that the Cornish population as a whole (F_{IS} = 0.229).

3.5 River Itchen vs. Cornish and Dorset Populations

3.5.1 Allele Frequency Data

Allele frequency data for the three populations sampled using DNA extracted from tissue samples are presented in Table 3.10. One of the most striking findings to come out of the allele frequency data was the number of private alleles; two from Cornwall, three from Dorset and four from the River Itchen. However, the number of private alleles is likely to be overestimated, particularly in the case of rare alleles, due to the low sample numbers used in the study.

Of perhaps more relevance is the number of alleles which are present in the River Itchen and Dorset samples only (n = 7) and in the Cornish and Dorset samples only (n = 5) versus just one allele (allele 142bp for locus 04OT14) that is present in the River Itchen and Cornish samples but not the Dorset samples, despite the Dorset sample consisting of the smallest number of tissues samples (n = 5).

Given the apparent absence of alleles from some populations, allele frequencies are relatively similar between populations, with the highest frequency allele in one population generally corresponding with the highest frequency allele in another and so on. However there are exceptions, for example locus RIO11 in the Dorset population, where allele 154bp is the lowest frequency allele despite being the highest frequency allele in the other two populations.

Table 3.10 Allele frequencies for the tissue samples from the three different sampling locations.

Loci	Allele	Cornwall (n = 16) *	Dorset (n = 5) *	Itchen (n = 9) *	Total (n = 30) *
Lut701b	147	----	0.200	----	0.034
	151	----	0.300	0.375	0.155
	155	0.563	0.400	0.375	0.483
	159	0.438	0.100	0.250	0.328
Lut717b	98	0.375	0.300	0.056	0.267
	110	0.031	----	----	0.017
	114	0.594	0.300	0.389	0.483
	118	----	0.400	0.556	0.233
04OT14	118	----	----	0.333	0.100
	122	----	----	0.111	0.033
	138	0.938	1.000	0.444	0.800
	142	0.063	----	0.111	0.067
04OT17	143	0.906	0.600	0.278	0.667
	147	0.094	0.400	0.444	0.250
	159	----	----	0.278	0.083
Lut435	125	0.406	0.500	0.556	0.467
	135	0.063	0.200	----	0.067
	137	0.531	0.300	0.278	0.417
	139	----	----	0.167	0.050
RIO11	150	----	0.400	0.111	0.100
	154	0.719	0.200	0.722	0.633
	156	0.281	0.400	0.167	0.267
04OT04b	164	0.406	0.600	0.722	0.533
	188	0.594	0.100	----	0.333
	192	----	0.100	----	0.017
	196	----	0.200	0.278	0.117
04OT19	180	0.313	0.200	----	0.200
	184	0.688	0.200	0.444	0.533
	188	----	0.600	0.556	0.267
RIO18	181	0.063	0.100	----	0.050
	185	0.938	0.900	1.000	0.950
Lut832	181	----	0.100	0.222	0.086
	185	0.167	0.200	0.278	0.207
	189	0.133	0.100	0.111	0.121
	193	0.400	0.500	0.389	0.414
	197	0.300	0.100	----	0.172
04OT07	206	0.250	0.100	0.222	0.217
	210	0.188	----	----	0.100
	214	0.281	0.200	0.222	0.250
	218	----	0.100	----	0.017
	222	----	0.100	0.333	0.117
	226	0.281	0.500	0.222	0.300

* n = number of individuals genotyped, i.e. number of alleles is twice this value

3.5.2 Summary Statistics

The results show that allelic richness (a measure of allele number, standardised for variation in sample size) was, for many loci, highest in the Dorset population, and if anything was slightly higher in the River Itchen population than in the Cornish population (Table 3.11). Measures of allelic richness averaged across the eleven loci were 3.18 for the Dorset population, 2.78 for the River Itchen population and 2.31 for the Cornish population.

Measures of observed heterozygosity suggest that the Dorset population was the most genetically diverse population surveyed (Ho = 0.509), whilst the Cornish population was the least (Ho = 0.344). The heterozygosity of the river Itchen lies in between that of the other two populations (Ho = 0.415) and shows the River Itchen to be relatively diverse.

The only significant departure from HWE detected was at locus 04OT19 in the Cornish sample set. This was due to a significant heterozygosity deficit (p = 0.0075), as can be seen by viewing the disparity between observed (Ho = 0.125) and expected (He = 0.430) values of heterozygosity for this locus in this sample set. The locus 04OT07 also shows a significant heterozygosity deficit (p = 0.0025) in the Cornish population, however the deviation from HWE is not significant (p = 0.062). Overall the deviation from HWE in the Cornish population is not significant (χ^2 = 24.399, df = 22, p = 0.327).

The microsatellite loci 04OT14 is almost out of HWE (p = 0.052) in the River Itchen population due to significant heterozygosity deficit (p = 0.013). Overall the deviation from HWE is not significant in either the River Itchen otter populations (χ^2 = 23.323, df = 20, p = 0.273), or the Dorset otter population (χ^2 = 13.5736, df = 18, p = 0.756).

Three pairs of loci show significant linkage disequilibrium; loci 04OT04 and 04OT07 in the Cornish population (p = 0.043), loci Lut717 and Lut435 in the River Itchen population (p = 0.005), and loci 04OT17 and Lut832 in the River Itchen population (p = 0.049). In each case the linkage disequilibrium is only significant in one population, suggesting the loci are not significantly linked to each other.

Table 3.11 Summary statistics per microsatellite loci, per population for the three populations analysed using DNA extracted from tissue samples. N = no. of individuals, A = allelic richness standardised for the minimum number of individuals in a population (n = 5), He = expected heterozygosity, Ho = observed heterozygosity, HWE = HWE probabilities.

	Lut701b	Lut717b	04OT14	04OT17	Lut435	RIO11	04OT04b	04OT19	Lut832	04OT07	RIO18
Cornwall											
N	16	16	16	16	16	16	16	16	15	16	16
A	2.00	2.31	1.53	1.69	2.53	1.98	2.00	1.99	3.70	4.12	1.53
He	0.492	0.506	0.117	0.170	0.549	0.404	0.482	0.430	0.704	0.744	0.117
Ho	0.500	0.313	0.125	0.188	0.438	0.438	0.563	0.125	0.533	0.438	0.125
HWE	1.000	0.099	1.000	1.000	0.290	1.000	1.000	0.008**	0.379	0.062	1.000
Itchen											
N	8	9	9	9	9	9	9	9	9	9	9
A	2.99	2.56	3.63	2.99	2.93	2.75	1.99	2.00	3.79	3.93	1.00
He	0.656	0.537	0.667	0.648	0.586	0.438	0.401	0.494	0.710	0.741	0.000
Ho	0.625	0.556	0.333	0.444	0.444	0.444	0.333	0.222	0.667	0.889	0.000
HWE	0.511	0.723	0.052	0.182	0.134	0.530	1.000	0.172	0.408	0.496	---
Dorset											
N	5	5	5	5	5	5	5	5	5	5	5
A	4.00	3.00	1.00	2.00	3.00	3.00	4.00	3.00	5.00	5.00	2.00
He	0.700	0.660	0.000	0.480	0.620	0.640	0.580	0.560	0.680	0.680	0.180
Ho	0.800	0.600	0.000	0.400	0.800	0.400	0.400	0.600	0.800	0.600	0.200
HWE	0.696	0.544	---	1.000	1.000	0.113	0.240	1.000	0.483	0.228	---

** = significantly deviated from HWE, $p < 0.01$

3.5.3 Genetic Differentiation and Inbreeding Coefficients

F_{ST} estimates indicate that the most genetically differentiated populations were Cornwall and the River Itchen, whilst the most genetically similar populations were the River Itchen and Dorset populations (Table 3.12).

Table 3.12 Genetic differentiation (F_{ST}) between pairs of populations.

	Cornwall	Itchen
Itchen	0.189	
Dorset	0.128	0.034

Calculations of the inbreeding coefficient F_{IS} indicate that the least inbred population is the Dorset otter population (F_{IS} = 0.142) whilst the River Itchen (F_{IS} = 0.215) and Cornish (F_{IS} = 0.229) populations exhibit similar levels of inbreeding.

3.5.4 Allele-Sharing Neighbour-Joining Tree

The allele-sharing neighbour-joining tree (Figure 3.14) shows a definite grouping of genotypes derived from the Cornish otter population, with all the Cornish samples found within one main branch of the tree. With a couple of exceptions (namely samples M1053 and M1064) which fell in with the Cornish grouping, otters collected from the River Itchen and Dorset lie on a separate branching system. This system is less compact than the Cornish allele-sharing portion of the tree, and the longer branch lengths indicate greater genetic differentiation between the individuals. Otters from Dorset are admixed with otters from the River Itchen suggesting that the two populations are genetically similar to each other.

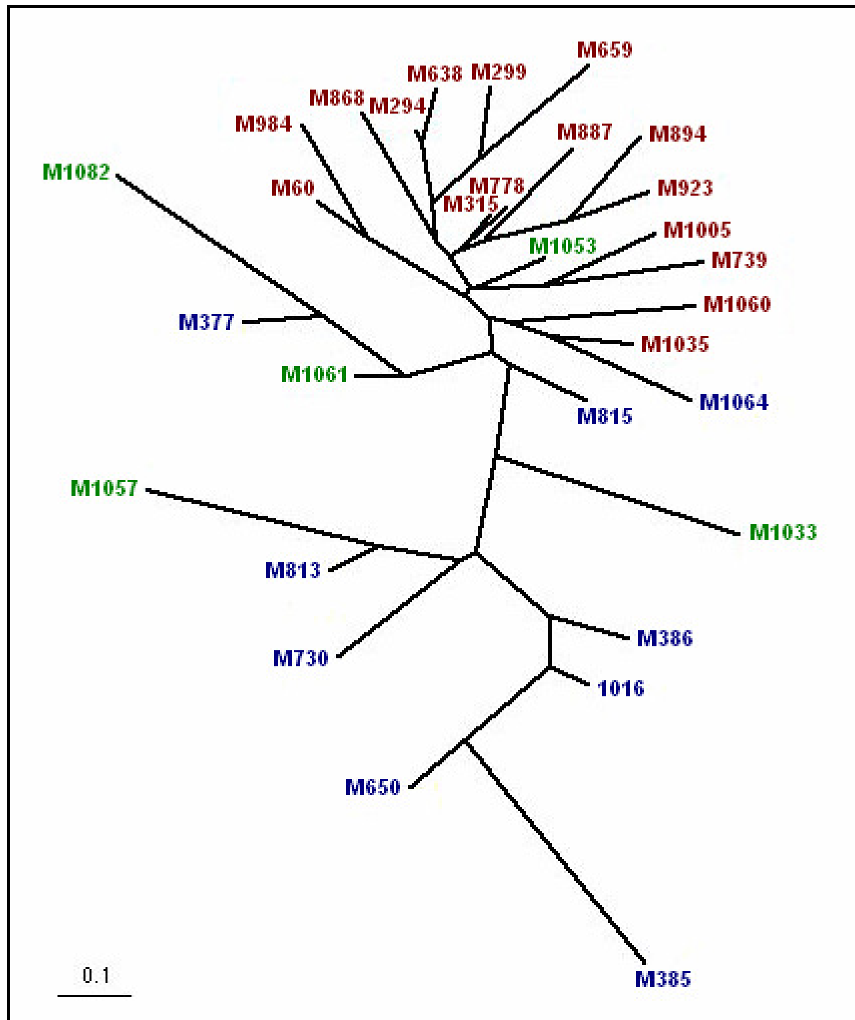


Figure 3.14 An allele sharing neighbour joining tree connecting individuals from the three populations; the Cornish population in red, the River Itchen population in blue, and the Dorset population in green.

3.6 Genotyping Project Discussion

3.6.1 Successful Genotyping of Spraint Samples

3.6.1i) Improvements in the Genotyping of Spraint

The main improvement made in this project to the spraint genotyping technique has been the increase n the number of microsatellite loci successfully genotyped. In addition to a set of otter primers, originally designed by Dallas *et al.* (1998) and commonly used in the genotyping of otter spraint (Hung *et al.*, 2004; Hájková *et al.*, 2006; Arendal *et al.*, 2007), a suit of new European otter primers (Huang *et al.* 2005) and North American river otter (*Lontra canadensis*) primers (Beheler *et al.*, 2005) were also used to genotype spraint samples for this study.

This increase in the number of microsatellite loci genotyped helped to increase confidence that the correct separation of genotypes had been identified. The increase in the level of genetic information acquired also enabled analysis of genetic measures such as relatedness of individuals (Hung *et al.*, 2004).

3.6.1ii) Success Rates

Of the samples collected from the River Camel catchment which were extracted for this study 18.1% were successfully genotyped. This is similar to the 20% success rate obtained in a previous study of otters in South West England (Coxon *et al.*, 1999; Dallas *et al.*, 2003). Other studies have obtained higher rates of success, but in general success rates are low.

In a study of otters from Sweden Arrendal *et al.* (2007) found that 63% of samples successfully amplified products for the Lut717 microsatellite locus. However, of these only 49% went on to produce a complete genotype, providing an overall success rate of 30.9%. In a study of North American river otters (*Lontra canadensis*) Hansen *et al.* (2008) found that 85% of spraint samples produced a product in at least one PCR reaction, suggesting that the majority of samples produced DNA. However, only 44% of the samples produced a genotype for three of the four microsatellite loci analysed and just 8% amplified products for all four loci, suggesting that many of the samples of

DNA produced were of low concentration and quality (Hansen *et al.*, 2008). One of the best success rates was obtained by Hung *et al.* (2004) in a study of populations on two rivers on the island of Kinmen of the coast of China. They found that 91% of samples amplified products of the mitochondrial cytochrome *b* marker, suggesting that a high proportion of samples contained DNA. Of these, 65% produced complete genotypes, providing an overal success rate of 59.2%.

The results of studies of spraint genotype success rates indicate that a relatively high proportion of samples contain DNA. However, the quantity and quality of the DNA produced is often highly variable and in the majority of samples not sufficient to produce a genotype (Dallas *et al.*, 2003; Hung *et al.*, 2004; Arrendal *et al.*, 2007; Hansen *et al.*, 2008)

The fact that our success rates are closest to those of Coxon *et al.* (1999), who sampled in a similar location as us, i.e. in SW England, may be an indication that the location of a study is important in terms of the success rate. For example, if the diet of otters in different locations is different or the weather conditions or chemical composition/contamination of the water in the rivers sampled, this may affect the success rates for spraint samples collected in that area. The laboratory procedures and locations of laboratory work were different between the Coxon *et al.* (1999) study and this study, which would indicate that the similarities in the success rates are due to something in the field and not the laboratory.

It is not clear what caused the low success rates seen in the River Itchen samples. Several of the collection volunteers had experience collected samples for a previous successful spraint genotyping study (Coxon *et al.*, 1999; Coxon *et al.*,2003; Chanin, 2004) and so their collection techniques and handling of samples in the field would not be expected to be at fault. Similarly the samples were not treated any differently to the more productive River Camel samples and so it would follow that the laboratory treatment would also not be considered to be at fault.

One major difference between the River Itchen and River Camel samples collections was the collection medium used to store the samples after collection. During the first few months of collection samples from the river Itchen were stored in tubes of industrial ethanol. It was considered that impurities in this industrial ethanol may have been

causing the low success rates and therefore the collection medium was switched to biological grade ethanol. When this in turn did not improve success rates it was decided to switch again and this time the collection tubes and storage medium used were chosen to match exactly those used in the more successful River Camel collections. This too failed to improve success rates suggesting that the problems encountered with the River Itchen samples were not related to the collection medium used. Indeed the greatest success rates seen were from samples collected during the first few months of collections whilst the industrial ethanol was still in use. In the future would recommend that collection tubes are prepared in the laboratory with the correct medium, i.e. 95% ethanol (Frantzen *et al.*, 1998) in order to control for collection medium effects and to assure the quality of the collection medium used.

The lack of improvement in success rates with the use of collection and storage techniques standardised to match those used on the River Camel, and the apparent lack of any field or laboratory based problem with the handling of the samples suggests that there is some other reason for the low success rates obtained from the river Itchen samples. It was hoped that by reviewing possible factors that affected success rates of extractions from the River Camel samples it might be possible to pinpoint possible reasons for the low success rates seen for the River Itchen samples and to highlight any possible causes for low success rates for the benefit of any future spraint genotyping studies.

3.6.2 Causes of Variation in Success Rates

The possible causes of variation in success rates were investigated using observational data collected both in the field at the time of sample collection and in the laboratory at the time of sample extraction. Possible correlations between the factors analysed and the genotyping success rates were analysed using logistic regression models. An initial model included six factors relating to the field conditions at the time of sample collection, with a second model, carried out using data relating to spraint samples only (i.e. excluding anal jelly sample information), designed to analyse factors relating more specifically to the sample and its storage.

3.6.2i) Methods of Data Collection

The categorisation of field based factors was based on information recorded by collection volunteers at the time of sample collection. Volunteers were likely to show differences of opinion and this may have biased the results. For example, a sample found under the shade of a small tree may have been recorded by one volunteer as "under trees", by a second as "protected" and may have been considered "exposed" by a third. If a volunteer had a particularly prolific sampling site situated under the protection of trees, then the way in which they interpreted the level of exposure of the site may have biased the results of the whole study in terms of analysis of the effects of level of sample exposure on success rates.

The design of the recording sheet itself may have caused problems, with several of the categories providing volunteers with space to write information as opposed to encouraging them to make a straight decision about how to classify the conditions. This in turn transferred the interpretation of the collection conditions on to the data analyst, who was not present when the samples were collected and so can only make a decision based on the information provided on the data sheet. The presence of greater numbers of categories on the data collection sheet than were used in the analysis causes further flaws in the analysis, with decisions about reclassification of data being made in the laboratory, where in fact they would have been more accurately made in the field. For example, a tick box for "under trees" meant that this data then had to be sorted into either the "exposed" or "protected" category, a distinction which would have been better made in the field where it could have been assessed based on the extent of tree cover.

The time of sample collection and the season of the year were the only two field based factors analysed not open to this bias as they were based on fact rather than on opinion. Conversely, the weather conditions, sample type, level of sample exposure and substrate under the sample were based on the opinion of the volunteer and were therefore open to variation due to differences of opinion and interpretation, etc.

3.6.2ii) Field Based Factors

In the regression model of field based factors the time of sample collection, level of sample exposure, substrate the sample was collected from and the season of the year the sample was collected in, all failed to show a significant relationship with genotyping

success rate (see section *3.2.2i, Field Based Factors*). Either there is no relationship between these factors and genotyping success, or there was not enough power in the data and the model to prove the relationship.

It would seem logical that samples collected later in the day would, on average, have been exposed to the effects of degradation for longer and would therefore be less likely to yield DNA and ultimately a genotype. However, the results of this analysis indicate that the time of day at which a sample was collected did not significantly alter the chances of obtaining a genotype. This finding parallels what has been observed in previous otter spraint genotyping studies (Coxon *et al.*, 1999). Lampa *et al.* (2008) found that the length of time (up to 20 hours) between the defecation by the otter and the collection of a sample had no significant effect upon the DNA amplification success rate (Krustal-Wallis test: $H (2, n = 59) = 2.82$, $p = 0.24$). The results of analysis for the time of collection indicate that, within less than a day of defecation, the length of time a spraint has been left in the field does not significantly affect the chances of obtaining a genotype.

The season of the year during which a sample was collected was not found to significantly alter the chances of obtaining a successful genotype. We would expect that any differences between seasons would be linked to differences in the weather conditions experienced. As weather conditions were considered separately as an independent factor in the model then the differences between seasons will only be linked to differences in genotyping success rates if there is another factor, other than weather, acting upon samples collected in different seasons. This may be the case if temperature is having an effect within the weather categories and if temperature is significantly different between the seasons. The lack of a relationship between seasons and genotype success rates suggests that either season is not related to temperature (in terms of the samples collected for this study), or that genotype success is not related to the temperature at the time of sample collection. It should be noted that volunteers had been informed that cold weather is beneficial for preserving the DNA in samples (Hájková *et al.*, 2006). This is likely to have biased them towards collecting on colder days, potentially negating the effects of temperature variation between seasons. A much more accurate way to test whether temperature has an effect upon spraint genotyping success would be to take measurements of the temperature on the day of collection,

specifically at the site of sample collection under the conditions the sample would have been exposed to.

The weather conditions recorded on the day a sample was collected show a highly significant correlation with genotype success rates. Samples collected on days when snow and frost were recorded were found to be significantly more likely to produce a genotype than those collected under other weather conditions. This suggests that, rather than weather itself being important, it is likely to be the temperature on the day of collection that dictates the chances of genotyping success. The most likely explanation being that the DNA in the spraint is preserved better at lower temperatures because the action of bacterial metabolism is slowed down. Again this suggests that a measurement of temperature on the day of collection in any future study may help to clarify whether sample genotyping success in related to temperature.

Several other studies have found similar effects of weather conditions, with colder weather conditions generally being more favourable for obtaining genotypes from spraint. For example, in a study carried out on two rivers in Slovakia and Czech Republic 31% of samples were collected in such cold conditions that they were considered deep frozen when collected from the field (Hájková *et al.*, 2006). Hájková *et al.* (2006) found that, although for samples containing anal jelly being frozen at the time of collection increased the chances of successful genotyping (82% frozen vs. 47% unfrozen), for spraint samples it made little difference and if anything resulted in a lower success rate (34% frozen vs. 41% unfrozen). However, for unfrozen samples genotyping success rates were significantly reduced by increasing temperature at time of collection (ANOVA; $F = 26.24$, $df = 1, 8947$, $p < 0.001$; Hájková *et al.*, 2006).

The results of the analysis for this study show that the type of spraint material collected influences the chances of obtaining a genotype. Samples of anal jelly were significantly more likely to produce a genotype than samples of spraint. The increased chances of obtaining a genotype from anal jelly is a trend noted in several otter spraint genotyping studies (Hung *et al.*, 2004; Hájková *et al.*, 2006; Arrendal *et al.*, 2007).

Anal jelly is a substance produced by a gland situated close to the anus of the otter. The anal jelly itself does not pass through the gut of the otter at any point and so is effectively a seperate DNA source to that of spraint. The fact that anal jelly does not

pass through the gut may be advantageous as this means that the cellular material contained within the sample is not subjected to the breakdown processes of the lower digestive system and the sample is far less likely to contain bacteria and potential PCR inhibitors which spraint samples may pick up from the gut. The fact that spraint samples recorded as containing anal jelly are no more successful than spraint samples (genotyping success rates of: spraint and jelly = 16.0% (±5.4%); spraint only = 15.2% (±2.3%)) suggests that there are likely to be specific DNA breakdown mechanisms causing the lower success rates with spraint, rather than it being a case of lower starting DNA content, which should be alleviated by the presence of anal jelly in a spraint sample.

In summary, weather conditions and sample type were the only field based factors entered into the model to show a significant relationship with genotype success. Samples collected in frost or snow were significantly more likely to produce a genotype that those collected under other weather conditions, suggesting that cold temperatures are advantageous for DNA preservation in otter spraint samples. The field data model also showed that anal jelly samples were significantly more likely to produce a genotype than spraint. It is likely that lower temperatures lead to slower bacterial metabolic breakdown and that anal jelly samples are subjected to lower levels of metabolic breakdown due to differences in their source and physical make up. In future studies, samples of anal jelly and samples collected under cold weather conditions should be extracted and genotyped in preference to their counterpart samples, but due to sampling constraints samples should not be excluded from analysis purely due to their sample type or the weather conditions under which they were collected.

3.6.2iii) Sample Observations

Many of the physical properties of anal jelly samples are different from those of spraint. In order to avoid any potential bias this may cause, the analysis of laboratory observation data was carried out for spraint samples alone (including those spraint samples recorded as containing evidence of anal jelly). Fewer anal jelly samples were collected and so there were not the sample numbers required to carry out a separate analysis. For the spraint samples genotyping success rates were found to be significantly altered by the level of IMS colouration and by the ratio of IMS: spraint.

There was no significant relationship between genotyping success rate and storage time, spraint description or the amount of spraint material collected.

If the storage medium was not 100% effective at preserving the DNA contained in the sample then we might expect to see a trend whereby samples stored for longer periods of time were less likely to yield a genotype. The samples analysed in this study showed no significant decline in genotyping success rate over time and samples extracted more than 6 months after collection were just as likely to yield a genotype as those extracted within two months of collection.

Lampa *et al.* (2008) found that samples which were extracted within 1 day of collection were significantly more likely to produce DNA than those extracted 1 week after collection (Wilcoxon tests; $Z = 2.366$, $p = 0.018$). However, the difference in extraction success rates between samples extracted 1 week and 2 weeks after collection was not significant (Wilcoxon tests; $Z = 1.400$, $p = 0.161$). Hájková *et al.* (2006) found no effect of storage time when swabs from spraint samples were stored frozen for between 0 and 234 days (ANOVA; $F = 0.10$, $df = 1, 8947$, $p = 0.747$), although if only a few of the samples were extracted within a week of collection then the signal may not have been strong enough to provide statistical significance. The combined results of studies would suggest that DNA degradation occurs during the first few hours of storage (Lampa *et al.*, 2008), after which levels of DNA content in the stored spraint sample are relatively stable.

Only a portion of the spraint material deposited by the otter was collected by the volunteers, with the remainder of the spraint left behind so as not to disturb too greatly the scent marking patterns of the otters (Kruuk, 1992). Studies of faecal genotyping success rates are often concerned with the effects of the dietary material (Arrendal *et al.*, 2007; Broquet *et al.*, 2007). The fact that only part of the spraint was collected, and that which was collected was probably actively chosen by the volunteers on the basis of its appearance and ease of collection, means that a full dietary analysis of the sample would not only have been time consuming, but also inaccurate.

It is feasible that the contents of a spraint could have a bearing on the likelihood of obtaining a genotype. For example, bones and exoskeleton material may act to scrape larger numbers of cells from the inside of the otters gut, or particular diets may create

conditions in the spraint which are more optimal for bacteria, resulting in greater levels of breakdown of the cellular and genetic material. However, this is not the only study to suggest that the contents of a faecal sample does not have a significant bearing on the likelihood of obtaining a genotype. Arrendal *et al.* (2007) found no significant difference in the genotyping success rates of otter spraint samples containing different prey types ($\chi^2 = 4.73$, df = 2, p = 0.094). Comparisons of the faecal genotyping studies of species with differing diets have found that diet has no significant effect on genotyping success rates, herbivores and omnivores being just as likely to produce a result as carnivores (Broquet *et al.*, 2007).

The spraint descriptions made in this study were made as observations of consistency (loose, silty or other) and are therefore not likely to be an accurate reflection of diet. Of note is the fact that the group classified as "other" contained spraint of varying description which were grouped together in this fashion so as to reach the sample numbers required for analysis. The results of the model suggest that there are no significant differences in genotyping success rates between the three description groups of spraint, although the vagueness of description groups suggests that this finding should not be taken as proof that there is no relationship between the contents of a spraint sample and the chances of obtaining a genotype.

It might be expected that the collection of greater volumes of spraint material would lead to larger volumes of DNA in the sample tube and ultimately result in a greater chance of obtaining a genotype. However, the results of this study show that the amount of material collected had no measurable effect upon the likelihood of obtaining a genotype.

One factor which did have a significant effect upon genotyping success rates was the ratio of IMS:spraint. Samples with larger amounts of spraint material in comparison to IMS volume (classified as a poor ratio of IMS:spraint) were more likely to produce a genotype than those where a small amount of spraint was collected in comparison to IMS volume (reasonable or good ratios of IMS:spraint). This is counter-intuitive to what would be predicted whereby greater volumes of collection medium, in this case IMS, would be expected to lead to higher levels of DNA presevation. The result observed here suggests that there may in fact be a negative effect, where the IMS interacts with the spraint material to lead to a reduction in DNA presevation.

Alternativelt poor ratios of IMS:spraint may result in greater concentrations of DNA in the storage medium, ultimately leading to a greater chance of a successful DNA extraction.

The other factor to show a significant relationship with genotype success rate was IMS colouration. Spraint samples which coloured the IMS amber or darker were unlikely to produce a genotype, which is reflected not only in the low success rates but also in the low predicted probabilities produced by the model. On the other hand, spraint which left the IMS clear or only slightly coloured were significantly more likely to produce a genotype. It has been suggested that the extent of colouration of the collection medium is related to the freshness of the spraint, with spraint which look freshest colouring the collection medium to the greatest extent (Graham Roberts, pers. comm.). However, the results observed here suggest the opposite, with samples which coloured the IMS the most being significantly less likely to produce a successful genotype. The reasons for this are unclear, but suggest some kind of chemical reaction between something contained in the spraint and the IMS, which not only resulted in greater colouration of the IMS but also in a lower genotype success rate for the sample.

Given what is known about DNA preservation from non-invasive sources (Frantzen *et al.*, 1998; Murphy *et al.*, 2002), we would expect greater volumed of IMS compared to spraint material to preserve greater concentrations of better quality DNA. We would also expect those samples that colour the IMS to produce greater concentrations of better quality DNA. However what we observe with the outcome of the model is the exact opposite. The results would suggest that the spraint samples may have reacted with the IMS in a negative fashion. Results indicate that excess volumes of IMS were detrimental for the preservation of DNA and that certain samples interacted with the IMS in a negative fashion, resulting in unsuitable conditions for DNA preservation, extraction or PCR, ultimately leading to low genotype success rates. It is not clear whether the same result would be evident should an alternative collection medium be used. IMS is known to be a poor DNA collection medium (Boyle *et al.*, 2004). A more conventional collection and DNA storage medium, such as 95% biological grade ethanol, would be suggested for use in any future spraint genotyping studies. Assuptions about the DNA quantity and quality obtained from samples held under certain conditions should not be made until further study has been carried out to

ascertain whether the resutls obtained in this study are specific to the use of IMS, or whether they will still be evident when using other collection and storage techniques.

3.6.3 The River Itchen Spraint Study

Due to problems obtaining genotypes from spraint samples only four otters were genotyped from the River Itchen in this study. However the allele frequencies obtained from these four samples match well with allele frequencies obtained from tissue samples collected from the same general area. So although the sample numbers are low, making more comprehensive statistics unreliable, the allele frequency data increases faith in the fact that the spraint samples have been genotyped correctly.

3.6.4 The River Camel Spraint Study

3.6.4i) Number of Otters using the River Camel

The results of the River Camel spraint analysis show that the river is capable of supporting at least 12 otters during a single 9 month period. As the River Camel is approximately 40km long this gives a calculated otter density of approximately 0.3 otters per km of river. This otter density is lower than the densities observed in many otter spraint genotyping studies (Table 3.13), but is similar to the density of otters observed for the River Itchen population (Coxon *et al.*, 1999). The River Camel and River Itchen are likely to support similar numbers of otters given that the length of waterway, weather conditions encountered and levels of human activity are similar for both of the river systems. Therefore the otter densities observed using the River Camel in this study could be considered realistic given the similarity to the numbers observed using the River Itchen in a previous study (Table 3.13; Coxon *et al.*, 1999).

Table 3.13 Otter densities calculated using the number of otters detected in various spraint genotyping studies.

Location	Length of river (or stretch of river surveyed)	Number of otters observed	Calculated otter density	Reference
River Torridge, South West England	<320km	10	>0.03	Coxon *et al.* (1999)
Central Sweden	~180km	20	~0.11	Arrendal *et al.* (2007)
River Itchen, Southern England	37km	13	0.35	Coxon *et al.* (1999)
River Tone, South West England	33km	22	0.67	Coxon *et al.* (1999)
River Itchen, Southern England	37km	35	0.95	Chanin, 2004
Hoshui-Chinsha Stream, Kinmen, China	6.2km [a]	27	1.5-1.8 [b] (4.35)	Hung *et al.* (2004)
Chienpu Stream, Kinmen, China	2.3m [a]	11	2.2-3.0 [b] (4.78)	Hung *et al.* (2004)

[a] stated length of survey area - length of waterway used by otters may be considerable larger
[b] density given are those recorded by Hung *et al.* (2004), in parenthesis are densities calculated using river length and otter number data

Despite the apparent similarity to results obtained from previous population surveys, the number of otters genotyped from the River Camel census should be considered a minimum count as opposed to a true count of the numbers of otters using the river catchment over the period of study. The practicalities of spraint sample collection meant that coverage of the catchment was greatly biased towards certain areas. Sample collections were carried out by volunteers and so sampling effort was dependent on the personal situation and enthusiasm of the volunteers assigned to a particular area of the catchment. Variation in the ease of access of sprainting site is also likely to have impacted upon the number of samples collected from a site, with greater planning and effort required for the collection of samples from sites which were more difficult to access. Some of the collection volunteers lived or worked close to their assigned sampling sites, making collections easier for them to carry out compared to those volunteers situated further from the river.

The estuary and lower reaches of the river were the most prolifically covered sections of the river system. Conversely, the River Allen and mid-reaches of the River Camel were poorly represented, with few samples collected and no genotypes obtained despite evidence that otters did use these stretches of river during this period of study. The

upper reaches of the river were sampled reasonably well during the first season of study, but were poorly represented during the second collection season.

The Rutherford tributary, situated close to the lower end of the catchment, and the De Lank tributary situated near the upper reaches of the Camel, were the only two major tributaries (bar the River Allen) to be surveyed. Several streams leading into the estuary were surveyed and were treated as estuary sites in terms of the analysis, although very few samples were collected from sites on smaller streams. On both the Rutherford and De Lank tributaries samples were collected from sites situated relatively close to the main river. It is not clear whether the smaller waterways typical of many of the tributaries, particularly further up stream from the river, are capable of supporting otters, as food sources are likely to be limited in these areas. If these areas are used it is likely to only be as a temporarily feeding ground. Collecting samples from these areas is unlikely to prove beneficial in terms of surveying the population of the river as a whole, particularly as the effort required to find samples in these locations is likely to be much greater.

The fact that there were large areas of the river not covered sufficiently by the census means that there may be otters which were using the river system during the period of study which were not identified during the genotyping census. In any future studies of this type it would be advisable to make attempts to standardize the collection effort made across the entire study area. However, the nature of variation in otter habitation and the need to use collection resources (i.e. volunteer time) effectively means that a fully unbiased and effective collection method is unlikely to be practical to implement.

Being able to know when all the individuals in the population have been accounted for is a problem faced in many such non-invasive genotyping studies. The use of a mark-recapture based technique, whereby the number of times repeat genotypes recovered and the number of new genotypes being found are considered, can be used to help calculate an estimate of the true population size (Frantz *et al.*, 2003). Although this technique is likely to be very effective for certain species it is unlikely to provide accurate results for species, such as the otter, where habitat use is not uniform and territorial behaviour means that individuals are not spread homogeneously throughout the survey area. The method will also not take into account the effects of variation in

sampling effort and therefore the technique will only be useful if levels of sampling effort mirror levels of otter occupancy.

Further problems arose from the fact that the study was carried out over a relatively long period of time. Otters which were present at the beginning of the study may not necessarily have been present at the end and vice versa. For this reason the numbers of otters present in the two different sampling periods were considered separately. However, even this is likely to cause inaccuracy as some areas, for example the upper reaches, were only surveyed during the first season and other areas were only surveyed during the second period, for example the De Lank tributary. Despite the fact that the areas probably supported similar numbers of otters in the different seasons, they were not considered as such due to variation in the final otter count. One issue of sampling over time was that of transient individuals, which were perhaps only present on the river for a short period of time when they were counted as present in the survey. Taking into account the date of sample collection can help to negate some of the effects of time, providing an indication of which otters were likely to be resident and which otters may have been transient individuals. However, otters only observed once could still be resident otters, only observed once due to chance or sampling technique. Alternatively, some transient individuals may not have been sampled at all.

The census of the otter population using the River Camel should only be considered a minimum count of the otters using the site during this time period. The coverage was good for many of the main stretches of river. One of the main reasons for lower coverage in certain areas was the lower numbers of spraint present, suggesting that these areas support lower numbers of otters anyway and so their lower coverage in the survey was not likely to have been of such great consequence. Areas not surveyed as well included many of the tributaries which, due to their size, and consequently lower food resources, are unlikely to support resident otter populations.

3.6.4ii) Dates and Locations

Four otters were identified using the first site of interest, the River Camel estuary (Figure 3.3). Three of the four otters were observed at least once in each of the two collection seasons suggesting that there are at least three resident otters using the area. It should be noted however, that the results suggest that the territories of otters A and I are only observed to overlap at the site known as 'Old Town Point', situated close to the mouth of the estuary. The fourth otter, G, is observed only during the first collection season and is not seen after spring 2006. The disappearance of this otter appears to coincide with the appearance of otter F, although making assumptions based on this fact may be misguided as the location and date are based on chance findings and it is possible that both these otters were present before and after this period and have just not been picked up in the sampling. The location of otter G is in the southern section of estuary close to the town of Padstow. Individual G has been observed using the same two sites as individual I, even to the extent that both were observed using the same site on night before/morning of the 29th November 2005. These two sites are situated at the leading edge of the sampling area and so it is not known if these two individuals were also using sites past Padstow, closer to the coast.

Four otters were observed using the area just above the estuary, around the town of Wadebridge and close to the confluence of the River Allen (Figure 3.4). The first otter, individual D, was found in just one occasion during the first collection season. The other three individuals were also only found on one occasion each. These otters were all found on dates within the second collection season.

Five otters have been found to use the stretch of river between the village of Rutherford, on one of the tributaries of the main river, and the Butterwell farm site situated in the lower to mid reaches of the river (Figure 3.5). Of these, otter E was observed the most often with a total of eight dates and locations recorded; in fact, this otter was observed the most frequently of all otters. From the results we can clearly see that otter E is a resident around the Butterwell farm area, with seven out of the eight findings occurring at this site and observations spread across from November 2005 to January 2007. Individual C appears to be another apparent resident at the Butterwell site, seen once in both December 2005 and January 2007.

The remaining three otters using the lower to mid-Camel have been observed using of stretch of a tributary that runs between the village of Rutherford and the confluence with the River Camel. Otters H and P were observed only once each, otter H using the area near Rutherford in February 2006 and otter P using the area near the confluence with the Camel in April 2007. Otter L used both these sites during the beginning of the 2006-07 sampling season. Otter L may also have been observed in January 2006, although this sample provided a poor genotyped and so there is less support for the genotype obtained accurately representing this particular individual.

Sites in between the lower and upper Camel, including some on the neighbouring River Allen tributary, were surveyed, however none of the spraint samples collected produced usable genotypes. Volunteers found these stretches of river difficult to survey, and this reduced the number of samples available for analysis. The fact that some samples were collected from these regions suggests that these areas were being used by otter and that if it had been possible to genotype some samples from these locations more otters, perhaps not seen in other locations, may have been identified. However, given that no genotypes were obtained it is not possible to ascertain whether the otters producing the spraint are new to the study or are travelling from another location on the river.

In the upper reaches of the River Camel three otters were observed (Figure 3.6). One of these, otter B, was observed three times during the first collection season, at three different sites across the upper Camel. The other two otters were observed during the latter part of the second collection season and were only observed once each; it should be noted however, that these otters were located as part of a short term collection designed to increase the coverage of the survey and therefore the lack of sightings for these otters can be put down to an absence of collection coverage on the De Lank tributary in previous months and not necessarily down to an absence of otters.

A sample found at a site known as ‘Gam Bridge’, situated towards the upper reaches of the River Camel produced a partial genotype. This genotype provided enough information to ascertain that it was not one of the individuals that has been assigned to the upper reaches of the river. However, it is not possible to discern whether the otter is one of the individuals sampled on the lower reaches that has travelled up the river, or if it is a separate individual altogether. Therefore, from this point forward this individual will be excluded from further analysis.

In summary, the sixteen otters found to be using the River Camel are separated into groups using distinct geographical areas. Some of the otters are observed using the same area over several months and across collection seasons, suggesting that they are permanent residents on that stretch of river. Other otters are observed only once or twice and less information can be deduced from these, as it is not possible to distinguish between otters that were only observed once because they were only in the area for a short period of time from those that were only observed once through chance.

3.6.4iii) Relatedness

Values of pairwise relatedness have been used with the River Camel spraint genotypes to identify three groups of otters in which high values of pairwise relatedness, relative to background levels, are observed. The links of high pairwise relatedness within these groups suggests that each one forms what is likely to be a family unit.

Values of parentage, although not reliable by themselves given the limited amount of information known for each individual otter, can be used to back up the relationships inferred by the relatedness values. Parentage analysis results can also be useful in helping to infer relationships in situations where values of relatedness are high. For example, if the value of relatedness is high but the parentage analysis (LOD) score is low then the relationship between the two individuals in question is likely to be one of full-siblings. Whereas if both the value relatedness and the parentage score are high then the relationship is likely (though not certain) to be one of a parent-offspring pair.

Pairwise relatedness values can help build up a picture of which otters are likely to be genetically related and to roughly what extent. Parentage analysis can provide some guidance, either in support of their being a relationship or as a guide to whether that relationship is likely to involve a parent- offspring pair or not. However the lack of information about the otters involved often makes it hard to deduce the true family structure. For example, a high parentage score (LOD) will infer that there is likely to be a parent-offspring relationship between two otters. However without information about the ages of the otters it is not possible to know which is the parent and which is the offspring.

Geographical proximity of otters in an inferred relationship will help to increase the confidence in the relationship as otters living in close proximity are more likely to be related, particularly if they represent a mother and cub. However, as otters are highly mobile and as, males in particular will disperse from their natal home range, then geographical distance between two otters showing high levels of relatedness is by no means indicative of that relatedness value being false. Similarly close geographical proximity is not in itself a sign of relatedness.

The first family inferred from genetic relatedness and parentage values consists, in part, of three otters, A, F and I, which appear to be residents of the River Camel Estuary. All three otters having been detected at least once in each collection season and all three having only ever been observed using the estuary of tributaries leading off of the estuary. Also included in this family group are otters K and M, both of which are only detected once and both in close proximity to the confluence of the River Allen.

The second family to be constructed using values of relatedness centres around a strong genetic bond seen between otters H and L/ Parentage analysis and demographic data suggests that these two individuals are linked in a parent-offspring bond. In addition to this central pair, two other otters appear to be genetically linked to both individuals in this first pair. Although the lower values of relatedness observed for these two otters suggests that the relationships are likely to be once removed, i.e. half-siblings, cousins, etc.

The third family observed again centres around a closely related pair of individuals, only in this family the central pair do not share a high parentage score and so the relationship is most likely to be that of full-siblings. The third otter in this group again shows values of relatedness which are more consistent with a relative once removed. This individual is also geographically situated at a distance from the primary pair of individuals.

In summary, the values of pairwise relatedness indicate that there were two or three strongly supported family groups inhabiting the River Camel during the period of study (Oct05 to June07). A lack of information regarding the age and gender of the otters involved makes it difficult to depict true family bonds and to draw family trees.

However, significant values of relatedness and parentage analysis scores can be used to infer the nature of the genetic bonds observed between two otters.

3.6.4iv) Population Genetics of the River Camel Population

The River Camel spraint genotypes expressed variable levels of heterozygosity for the different loci studied. However, for nine out of the ten loci, observed heterozygosity (Ho) was equal to or below 0.5, indicating low levels of genetic diversity in the River Camel population. Similarly, the low number of alleles observed is also indicative of a low level of genetic diversity.

Three of the ten microsatellite loci analysed for the River Camel spraint samples show departures from HWE that are just above the value of statistical significance ($p = 0.054 – 0.056$). As this departure from HWE affects several loci it would suggest a population level effect indicative of some sort of selective pressure. One of the assumptions of HWE is that there is random mating within the population. Given the relatively small special scale and the high numbers of related individuals observed in the population it is likely that this high level of relatedness caused the departures from HWE seen for the River Camel sample set.

The River Camel spraint genotypes were compared to the wider Cornish population in part in order to test the accuracy of the spraint genotypes (Broquet & Petit, 2004). If the genotypes obtained from the spraint material were inaccurate then this is likely to show as a large genetic differentiation between the spraint genotype set and the Cornish tissue genotype set.

The comparison can also be used to see if the River Camel population was typical of a Cornish otter population. Indeed the level of genetic differentiation between the River Camel and Cornish otter populations was low ($F_{ST} = 0.008$), suggesting that the River Camel otter population is genetically integrated into the wider Cornish population. However, during the interpretation of this analysis it is necessary to be aware that the two data sets are effectively measuring over two different time periods and geographical areas (population sizes), therefore this is likely to bias the genetic analysis output.

3.6.5 River Itchen vs. Cornish and Dorset Populations

3.6.5i) Allele Frequency Data

High numbers of alleles are found in Cornwall and Dorset but not in the River Itchen samples (n = 5). Similarly, high numbers of alleles are found in the River Itchen and Dorset samples but not in the Cornish samples (n = 7). Whereas only one allele is seen in the River Itchen and Cornish populations but not the Dorset population, despite the number of samples genotyped from Dorset being particularly small (n = 5). This would suggest that the Dorset population was acting as a sort of half-way house, containing genetic information in common with populations in the South West, as represented by the Cornish sample set, and also sharing genetic information in common with the River Itchen otter population.

3.6.5ii) Summary Statistics

All microsatellite loci were in HWE for all populations except locus 04OT19 in the Cornish population. The lack of HWE for locus 04OT19 is unlikely to represent a selection pressure in the Cornish population as this is the only loci out of HWE for the population. Similarly the locus 04OT19 is only out of HWE in the Cornish population, therefore there is unlikely to be a selective pressure upon the locus itself, such as linkage to a gene.

The results suggest that out of all three populations the Dorset population was the most genetically diverse, showing high levels of allelic richness as well as the highest level of heterozygosity (Ho = 0.509). The Cornish otter population showed the lowest levels of genetic diversity, with the lowest observed levels of allelic richness and the lowest measure of heterozygosity (Ho = 0.344).

The results of statistical analysis may have been affected by the low sample numbers and the disparity between the sizes of the sample sets used for the analysis. The Dorset population data is constructed using the lowest sample set (n = 5), whilst the Cornish population is represented by the largest sample set (n = 16). If the size of the sample sets used has affected the results of analysis then it is most likely to have resulted in an underestimation of the genetic diversity of the populations with the lowest numbers of

samples analysed, in this case the Dorset population. Therefore, the Dorset population is seen to express the highest levels of genetic diversity, despite the small sample set of genotypes analysed.

3.6.5iii) Genetic Differentiation and Inbreeding Coefficients

The most geographically distant otter populations, the Cornish and the River Itchen populations, are also the most genetically different from each other ($F_{ST} = 0.189$). The two populations which are the closest in terms of genetic differentiation are the River Itchen and Dorset populations ($F_{ST} = 0.034$). The Dorset otter population being relatively close to the River Itchen population in terms of genetic differentiation as well as geographic distance when compared to its distance and genetic differentiation from the Cornish population ($F_{ST} = 0.128$). Therefore, overall the three populations show a pattern of genetic differentiation indicative of isolation by distance.

The F_{IS} values indicate that, of the three populations analysed, the Dorset population shows the lowest likelihood of inbreeding. The Cornish and River Itchen populations show similar values of F_{IS}, suggesting that these populations show similar levels of inbreeding.

3.6.5iv) Allele-Sharing Neighbour-Joining Tree

As with genetic differentiation, the Allele-Sharing Neighbour-Joining tree indicates that the River Itchen and Dorset populations are significantly more similar to each other than to the Cornish otter population. In fact the tree places individuals from the Dorset and River Itchen populations admixed together on the same branch, indicating that the populations are so genetically similar that the allele-sharing method was not powerful enough to separate out individuals from the two populations. In contrast the Cornish otters sit on their own relatively tight and close knit branching system, suggesting that not only is this population genetically differentiated, but that otters in this population are more similar to each other than are otters from the other two populations studied.

3.6.5v) Overall Discussion - River Itchen, Cornish and Dorset Populations

The Dorset and River Itchen otter populations show distinct genetic similarities to each other, with relatively low genetic differentiation between the populations and admixture observed between the two populations in a tree of allele-sharing genetic distances. The genetic similarities between these two otter populations are probably due, in part to the geographic closeness of the populations, but are also likely to have been influenced by distinct similarities in the demographic histories of the two populations. The Dorset and River Itchen otter populations both suffered from severe population declines during the 1960s to 1980s, where small pockets of otters remained in relative isolation (Crawford, 2003). Small but significant numbers of captive bred otters were released into both populations during the late 1980s, early 1990s (Crawford, 2003). In addition the leading edge of expansion of the South West England population stronghold is thought to have now reached both the Dorset and the river Itchen populations (Crawford, 2003).

Genetic differences between the three populations studied show a general pattern of isolation by distance, although other unidentified factors may be involved. The Cornish otter population did not decline to the same drastic extent during the 1960s and 70s (Crawford, 2003). Despite this the present day Cornish otter population is genetically the least diverse of all three of the populations analysed, suggesting that the River Itchen and Dorset populations have recovered sufficiently from the drastic historic declines observed. The relatively high genetic diversities indicate that the release of captive bred otters into the River Itchen and Dorset populations has significantly influences the genetic footprint of the populations, introducing new genetic material into the populations and in terms of the microsatellite loci studied, increasing the number and frequencies of alleles observed.

Chapter 4 Mitochondrial Control Region Haplotypes

Work using haplotypes of otters to assess the impact of captive release programmes on the River Itchen otter population was written up in a journal paper format, presented here in *Section 4.1*. Co-authors on the paper were academic supervisors Jamie Stevens and Charles Tyler, and Geoff Hobbs from the University of Cardiff for his capacity in supplying samples for analysis. The paper was rejected from Molecular Ecology on the grounds of the subject being out of their remit the journal. The paper was then submitted to the journal Conservation Genetics. Conservation Genetics commented that they thought the paper was suitable for publication in the journal, but that more work was needed to enhance the knowledge about the haplotypes of South East Asian otters in order to support some of the claims made by the paper.

4.1 Haplotypes inform on the impact of captive breeding programmes on wild English otter (*Lutra lutra*) populations: A case study of the River Itchen in Hampshire

4.1.1 Haplotype Paper Abstract

Otter numbers in Britain, as in many other areas of Europe, declined drastically between the 1950s and 1970s. Populations in Britain suffered greatest across central and eastern England, and a captive breeding and release programme was set up to try to counteract these declines. The programme appears to have been successful in supporting the subsequent recovery of wild otters in locations across southern England, however, to what extent is uncertain. Information on the origin of otters used in the captive breeding programme is limited and not easily accessible. In this study, we used >300bp from the 5′ end of the mitochondrial control region to haplotype otters from across England and Wales. The information gained, combined with information from previously published work, was used to determine the haplotypes of the otters used in the captive breeding programme, to help elucidate the haplotypes naturally present in England and Wales and to assess the effects that releases have had on the haplotype profiles of the resident otter population of the River Itchen in Hampshire, southern England. The results suggest that eastern Germany is the most likely source for the otters used to found the captive breeding programme. The effects of introducing otters from a captive breeding programme, that are likely to have suffered negatively due to inbreeding and loss of genetic diversity, into depleted English populations and the possible effects of using a geographically distant founder population are discussed.

4.1.2 Haplotype Paper Introduction

During the 1950s to 1970s otter populations across Europe declined drastically (Mason and Macdonald 1986). The causes of these declines were complex and likely included a combination of loss of appropriate habitat, human disturbance and persecution, and, perhaps most significantly, the adverse effects of a range of organochlorine pollutants, known to have toxic and endocrine disrupting properties (Kruuk & Conroy, 1996; Simpson *et al.* 2000; Roos *et al.* 2001).

In Britain the greatest declines were seen across mainland England where declines were so drastic that localised extinctions occurred in several regions (Crawford, 2003). Overall, populations of otters in Scotland, Wales and the southwest of England remained relatively healthy, although numbers did drop significantly in some of these regions, particularly in more industrial areas (Chanin & Jefferies 1978; Mason & Macdonald 1986; Dallas *et al.* 1999). Additionally, in some localised areas of England isolated populations persisted, for example, on the River Itchen in Hampshire. The Itchen is a chalk stream habitat which continued to provide an ideal habitat for otters, and throughout the national declines in the 1950s to 1980s a resident population of otters remained on the river (Crawford, 2003).

In the 1980s a captive breeding programme was set up by The Otter Trust (on behalf of the Nature Conservancy Council) with a view to enhancing wild populations of otters in England through release schemes. Records are sporadic and sometimes contradictory, but it is thought that between 117 (The Otter Trust, 2006) and 168 (Crawford, 2003) otters were released between the start of the programme in 1983 and its end in 1999; unfortunately, reliable records as to the geographical origins of the otters used to initiate the captive breeding programme are not readily available. Releases were concentrated mainly in East Anglia, the Thames Valley and Yorkshire, but small numbers of captive bred otters were also released into many other areas across England, e.g. the River Itchen.

In 1993-94 four otters, three females and one male, were released onto the River Itchen and, prior to this, in 1989 two captive bred otters were released onto the neighbouring catchment of the Hampshire Avon (Crawford, 2003; Graham Roberts, *pers. comm*). In recent years, the recovery of otter populations in the neighbouring Dorset Region and the movement of otters eastwards from southwest England is also likely to have led to natural migration into the Itchen population.

This study was carried out to investigate the extent to which released captive otters have integrated into the River Itchen otter population and to gain a better understanding of the origins of the otters used in the British captive breeding programme. The ultimate aim was to increase our wider understanding of the genetic effects of the release programme upon resident otter populations, such as that of the River Itchen. The 5´ end of the mitochondrial control region was analysed in otters from four locations from

across England and Wales. This section of DNA, roughly 300bp in length, has been used successfully in a number of previous studies (Mucci *et al.* 1999; Cassens *et al.* 2000; Pérez-Haro *et al.* 2005) to characterise otter haplotypes from populations across Europe, but as yet little work (Ferrando *et al.* 2004) has been carried out on otters from the England and Wales.

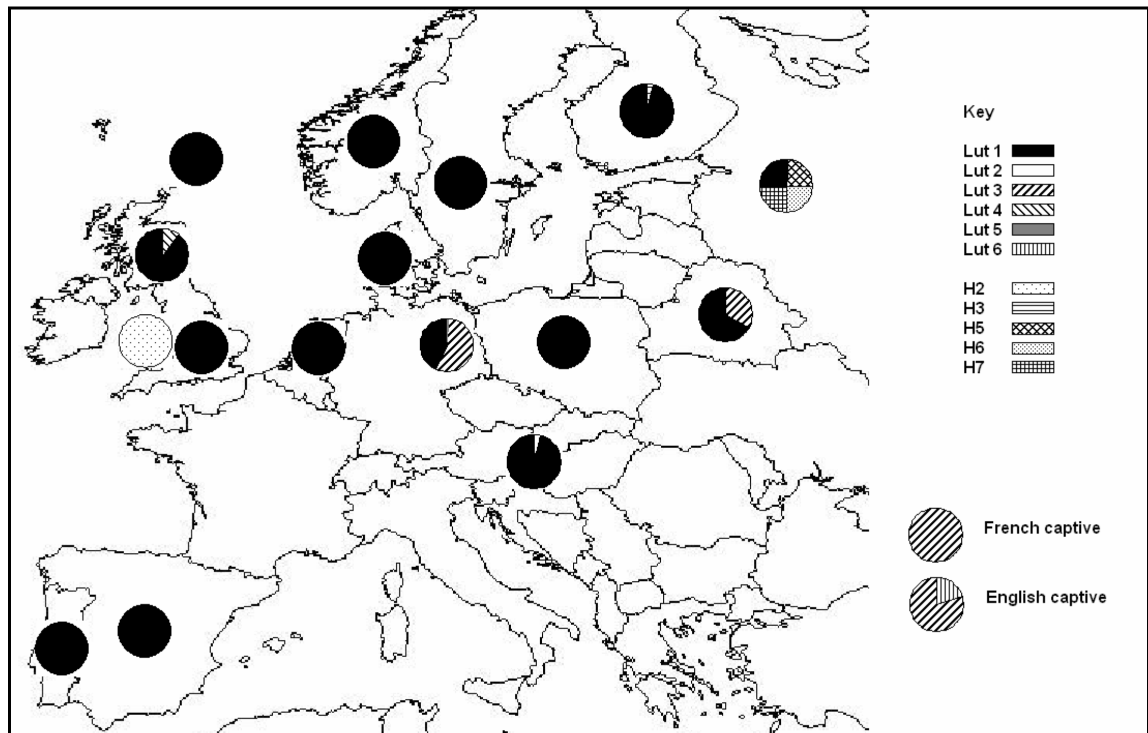


Figure 4.1 Haplotypes of otters from across Europe as the result of amalgamation of data from Mucci et al. (1999), Cassens et al. (2000), Ferrando et al. (2004) and Pérez-Haro et al. (2005). Sample sizes are as follows: Shetland (4); Scotland (19); England (1); Wales (1); Finland (26); Norway (4); Sweden (5); Denmark (30); Holland (5); Eastern Germany (76); Poland (1); Belarus (3); Russia (4); Austria and Hungry (25); Portugal and Spain (82); French captive (2); English captive (5). Note, for the English captive sample, four individuals expressing the haplotype Lut6 have been treated as one because they are known to have originated from a single female.

Across Europe the haplotype Lut1 dominates, with isolated incidents of haplotype variation (Figure 4.1). Other sections of the control region and cytochrome b region also show a lack of genetic variation (Effenberger & Suchentrunk, 1999; Mucci *et al.* 1999; Ketmaier & Bernardini 2005) and microsatellite analysis suggests historic bottlenecking of European otter populations (Pertoldi *et al* 2001; Randi *et al* 2003). As with many other European plant and animal species (Taberlet *et al.* 1998; Hewitt 1999; Hewitt 2000) it is likely that during glaciation in the Pleistocene period otter populations were pushed south into refugia, candidate locations being the Iberian

Peninsula, Italy and the Balkans (Sommer & Benecke 2004; Sommer & Nadachowski 2006). The effects of population isolation in refugia combined with founder effects at the leading edge of subsequent species range expansion would have led to a loss of genetic diversity (Taberlet *et al.* 1998; Hewitt 2000), and Lut1 likely became fixed in the population during this period; then, as otters spread back across Europe, mutational events produced the isolated cases of low haplotype diversity seen in several of the countries studied (Cassens *et al.* 2000; Randi *et al.* 2003). Greater genetic diversity in Russia and Eastern Europe (Ferrando *et al.* 2004) suggests that otter populations in these areas were less affected by the glaciation periods and that Russia and Eastern Europe may also have acted as a refuge from which otters subsequently spread. As in many countries the German otter population declined drastically in the mid 1900s (Schwenk 1986; Mason & Macdonald 1986). In central and western Germany otters are still largely absent, however, otters have recovered well in eastern Germany and the high prevalence of haplotype Lut3 in this population is probably an artefact of severe bottlenecking during the recent period of population declines (Cassens *et al.* 2000).

Haplotyping otters derived from the English captive breeding programme may allow us to speculate about where the otters used in the programme originated from. Several studies have looked at the haplotypes of captive otters. To date the majority of otters originating from English captive stock have been found to express haplotype Lut3 (n=4) (Mucci *et al* 1999; Pérez-Haro *et al* 2005), and re-alignment of sequences derived from direct descendants of the Norfolk otter breeding programme (n=8) suggests that they too express the haplotype Lut3 (Ketamier & Bernardini 2005, Genbank accession no. AY860321-AY860328). A further four otters originating from the English captive stock expressed the haplotype Lut6 (Pérez-Haro *et al* 2005), although all four individuals were produced by the same female and so effectively represent just one individual. The high prevalence of Lut3 in captive otters would suggest that the otters used in the breeding programmes originated from a wild population also containing a high prevalence of the Lut3 haplotype.

In this study we used mitochondrial haplotype analysis to investigate possible sources of otters used in the English captive release programme and to gauge the effect such releases have had on genetic diversity within the populations receiving these otters, using the River Itchen in Hampshire as a case study population. The low numbers of otters inhabiting East Anglia in the 1980s, and the sharp increase in numbers following

releases in the area, would suggest that a large proportion of the otters in the present day East Anglian population are direct descendants of captive bred otters. In the absence of any usable archived otter tissues from the captive breeding programme, otters from East Anglia were included in the study as possible representatives of the captive bred otters used in the original release programme. Populations from Cornwall and Wales were included as representatives of populations known to have been largely unaffected by the releases of captive bred otters as comparisons to the River Itchen population which is known to have received at least four captive bred otters.

4.1.3 Haplotype Paper Materials and methods

4.1.3i) Sample collection and preparation

Kidney and liver tissue samples were obtained during *post mortem* examinations of otter carcasses as part of a UK-wide Environment Agency otter monitoring project; *post mortem* examinations were carried out at the Wildlife Veterinary Investigation Centre (VWIC), Cornwall and by the Cardiff University Otter Project (CUOP). A total of 40 otters were analysed: 13 from Wales, 7 from East Anglia, 11 from Cornwall and 9 from the River Itchen in Hampshire. DNA was extracted from tissue samples stored frozen at –20°C using a standard phenyl-chloroform extraction (Sambrook *et al.* 1989).

4.1.3ii) Sample Analysis

Primers L-Pro and 363rev (Ketmaier & Bernardini 2005) were used to sequence >300bp from the 5´ end of the mitochondrial control region. This marker has been used in a number of previous studies of Eurasian otter populations (Mucci *et al.* 1999; Cassens *et al.* 2000; Ferrando *et al*. 2004; Pérez-Haro *et al* 2005) and has been shown to be highly informative in discriminating otter haplotypes. Polymerase chain reaction (PCR) amplifications were carried out in a 25µl reaction volume containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.2mM of each dNTP, 0.75µM (0.75pmol.μl^{-1}) of each primer, 1U of REDTaq DNA polymerase (Sigma-Aldrich) and 2µl of DNA extract. The PCR program consisted of a denaturing stage of 94ºC for 2min, 30 amplification cycles of 94ºC for 15secs, 50ºC for 15secs and 72ºC for 45secs, followed by a final extension period of 72ºC for 5min.

Six PCR reactions were performed on template DNA from each sample to eliminate possible sequencing artefacts; products were then separated on 1% agarose gels. Reactions produced single bands of approximately 365bp which were subsequently excised and extracted using a QIAGEN gel extraction kit. The resulting DNA products were then pooled and sequenced in both directions (Lark Technologies) using the L-Pro and 363rev primers. The forward and reverse sequences for each sample were aligned using the program AutoAssembler v.2.0 (Applied Biosystems) to obtain a sequence of >300bp. Sequences were then aligned with each other and with previously defined haplotypes (Lut1-5 from Cassens *et al* 2000, GenBank accession no. AJ006174-78; Lut 6 from Pérez-Haro *et al* 2005); alignments were performed using the program ClustalX (Thomson *et al* 1997); all alignments were then checked by eye to eliminate spurious alignment artefacts.

4.1.4 Haplotype Paper Results

The results of our analysis are presented in Figure 4.2, with sample numbers given in Table 4.1. Haplotype Lut1 was expressed by all otters sampled in Cornwall and also in a number of Welsh otters. In Wales the majority of individuals expressed the haplotype Lut6, a haplotype previously only seen in otters of English captive origin (Pérez-Haro *et al* 2005). Another Welsh otter expressed what appears to be a new haplotype, referred to here as Lut7. This haplotype, Lut7, arises due to a C-T transversion at position 153, as seen in Lut6, and a T-C transversion at position 237, as seen in Lut3 (Table 4.2). It should be noted that the one otter previously sampled from Wales expressed a haplotype labelled H2 (Ferrando *et al*. 2004), which they identified as being different from Lut1. However, Ferrando *et al*. (2004) did not compare their haplotype H2 to Lut6 (Pérez-Haro *et al* 2005) and with the sequence currently unavailable in database it is not possible to compare haplotype H2 to either Lut6 or Lut7.

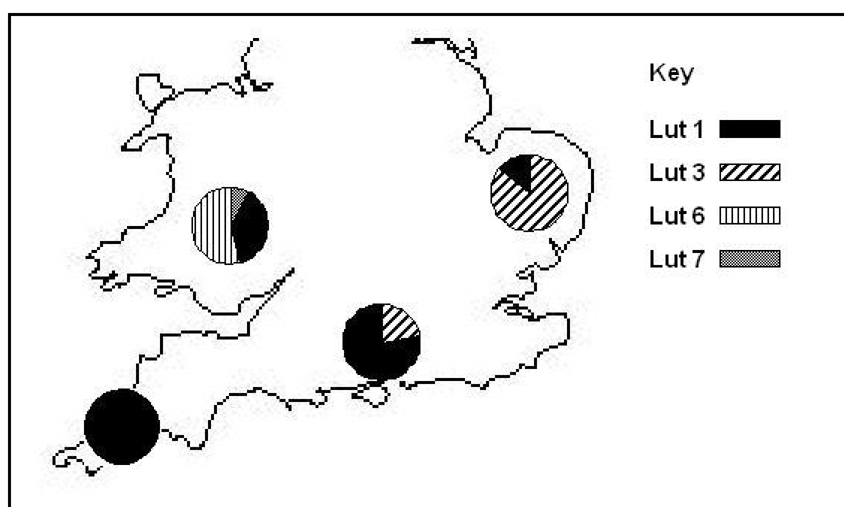


Figure 4.2 Haplotypes from the four British locations analysed in this study: Wales (13), East Anglia (7), Cornwall (11) and the River Itchen in Hampshire (9).

Table 4.1 Numbers of otter haplotypes found for each of the four locations, plus a total for the United Kingdom including haplotypes collected previously for England, Wales and Scotland (Ferrando *et al* 2004).

Country	Lut1	Lut3	Lut4	Lut6	Lut7	H2	n
Wales	5			7	1		(13)
Cornwall	11						(11)
Itchen	7	2					(9)
East Anglia	1	6					(7)
Total	24	8		7	1		(40)
UK Total	**47**	**8**	**2**	**7**	**1**	**1**	**65**

Table 4.2 Haplotypes from around Europe. Lut1 to Lut5 described in Cassens (2000), Lut6 in Pérez-Haro (2005). Lut7 is a new haplotype described in this paper. Dots indicate identity to reference sequence, and dashes indicate a gap.

	72	95	101	153	237
Lut1	G	G	-	C	T
Lut2	·	A	·	·	·
Lut3	·	·	·	·	C
Lut4	·	·	C	·	·
Lut5	A	·	·	·	·
Lut6	·	·	·	T	·
Lut7	·	·	·	T	C

In the East Anglian population, which we suggest may be representative of captive bred otters, a significant majority of animals expressed the haplotype Lut3, with only one otter expressing the haplotype Lut1. On the Itchen the majority of otters expressed Lut1, the haplotype which may be considered from the Cornish and Welsh results to be representative of the native English otter population. However, the haplotype Lut3 was also present in the Itchen population in a significant proportion of individuals (22%), suggesting that the release of captive bred animals onto the river system has impacted upon the genetic make-up of the resident river population.

4.1.5 Haplotype Paper Discussion

Haplotyping can provide a powerful tool to quickly and cheaply trace the genetic origins of animal populations and, as such, has been used in the analysis of a number of animal populations resulting from, or augmented by purposeful or accidental introductions (Tsutsui *et al.* 2001; Couceiro *et al.* 2008). In the current study, we used this approach to facilitate comparison with a number of previous studies on otters (Ferrando *et al.* 2004; Ketamier & Bernardini 2005; Pérez-Haro *et al* 2005), selecting a molecular marker comprising >300bp from the 5' end of the mitochondrial control region; a region which has previously been shown to provide a level of resolution appropriate for elucidating phylogeographic patterns in European otter populations (Ferrando *et al.* 2004; Pérez-Haro *et al* 2005).

As in previous studies of haplotype variation across Europe (see Figure 4.1), our data indicate that Lut1 is also common in English and Welsh otters, being found in all four populations studied, however, the proportion of the population expressing Lut1 varied considerably. In Cornwall, an area which has received few if any captive bred otters, all 11 individuals sampled expressed haplotype Lut1, while in the Itchen, Hampshire, Lut1 was found in 7 out of 9 individuals typed.

In Wales, we detected the presence of a unique haplotype, Lut6. The absence of haplotype Lut6 from the other (English) populations analysed in this study suggests that Lut6 has not yet spread to southern England, and suggests that Lut6 may be the result of a mutation in this particular component of the British population. Outside of Britain, haplotype Lut6 does not appear to exist in wild populations, having been reported only

from Spain, in a group of captive otters presumed to be of 'English origin' (Pérez-Haro *et al* 2005). Using microsatellite analysis, Dallas *et al*. (2002) found a lower than expected level of genetic exchange between populations in Wales and southwest England. In mammals, such as the otter, genetic drift is often lower for mitochondrial markers than for nuclear markers such as microsatellites, due to the combined effects of maternal inheritance and male biased dispersal (Moore 1995; Nyakaana & Arctander 1999; Blundell *et al.* 2002; Trujillo *et al.* 2004). Our findings concerning the existence of haplotype differences between these two areas (i.e. the presence of Lut6 in otters from Wales and an absence of this haplotype in southern England), are thus consistent with the findings of Dallas *et al*. (2002).

One otter from the Welsh sample set expressed the novel haplotype Lut7. Given the nucleotide sequence of Lut7, the most parsimonious explanation is that this haplotype is derived from either Lut3 or Lut6, each representing a single nucleotide change within the 300bp sequence (see Table 4.2). However, Lut3 is absent from Wales, appears to originate in Eastern Europe (Cassens *et al.* 2000; Ferrando *et al.* 2004) and is only found in British otter populations assumed to be associated with the captive release programme (Mucci *et al.* 1999; Pérez-Haro *et al* 2005), while Lut6 is present in, and appears unique to, Wales. Accordingly, we suggest that Lut7 is most likely derived from Lut6.

In East Anglia, where the majority of otters are likely to have descended from animals released from the captive breeding programme, all but one of the otters (i.e. 86%) expressed the Lut3 haplotype, suggesting that Lut3 may have been the major haplotype of otters used in the captive breeding programme. This trend was also found by Mucci *et al.* (1999) in four otters presumed to be from the same captive breeding programme (Pérez-Haro *et al* 2005). Again, this raises the question of where the otters used in the captive breeding programme originated.

With a high proportion of individuals in East Anglia expressing haplotype Lut3, German otters appear to be a likely source population. At the time of initiating the English breeding programme in the early 1980s the population of otters in eastern Germany was reported to be recovering well (Cassens *et al.* 2000; Effenberger & Suchentrunk 1999) and would therefore have been a favourable source population. Moreover, if the founder population was small, the effects of bottlenecking and/or

inbreeding in the resulting captive population would explain the higher prevalence of the Lut3 haplotype in populations supplemented by captive bred animals (Lacy, 1987), e.g. the present day River Itchen and East Anglian populations.

Outside of Britain, to date the Lut3 haplotype has been found only sporadically. The main stronghold for haplotype Lut3 appears to be eastern Germany (Figure 4.1), where 55% of the otter population express it (Cassens *et al.* 2000; Ferrando *et al.* 2004), however further sampling from eastern Europe is required before any conclusions over the extent of the European distribution of Lut3 can be drawn. One of three otters sampled from Belarus by Ferrando *et al.* (2004) expressed haplotype Lut3. Further east, in Russia, haplotypes show a much greater degree of diversity, implying that otters from eastern Europe may be more genetically diverse than those from central and western Europe.

Unfortunately, without historic samples for analysis, it is not possible to conclude unequivocally that the haplotype Lut3 is not a native haplotype of Britain. However, it would seem highly unlikely that the otters used in the programme were taken from the dwindling populations of mainland England and none of the more numerous populations of Wales, Scotland or southwest England were found to contain any individuals that expressed the haplotype Lut3. Thus, our findings suggest that it is unlikely that otters from Britain were used as the main source for the captive breeding programmes.

The presence of the Lut3 haplotype in two of the nine of the otters sampled from the River Itchen, where at least four captive-bred otters were released, indicates that captive-bred animals have successfully integrated and bred with the local population, and in turn this has changed the genetic composition of the resident population. The use of maternally inherited mitochondrial DNA as a marker is likely to have resulted in an underestimation of the effects as it will only detect the influence of captive bred females. The genetic descendents of captive bred males are not detected using this method. To do so, other methods such as microsatellite analysis would be required, but this would also require a greater number of animals to be sampled for the resolution required.

If not well managed, captive breeding often leads to a lowering of genetic diversity (Lacy, 1987) and can, even within a few generations, lead to the selection of traits mal-adaptive to life in the wild where survival pressures are far greater than those experienced in captivity (Nicholls & Pullin 2000; Frantzen 2001; Salonen & Peuhkuri 2004). The population of otters on the River Itchen became small and isolated (Crawford 2003) and as a result is likely to have suffered from a lack of genetic diversity and the effects of inbreeding. The introduction of animals from a second population low in genetic diversity and possibly suffering from the effects of inbreeding - in this case otters from the captive breeding programme - is likely to initially increase the genetic diversity of the resulting population (Lynch, 1991). However, the extent of negative effects resulting from possible outbreeding depression is unknown (Michaux *et al.* 2004) and the use of animals in the captive breeding programme from locations geographically distant from the recipient population increases the risk still further (Lynch, 1991).

4.2 Additional Haplotypes

Additional samples were analysed in order to increase our understanding of the origins of the otters used in the captive breeding program. These additional samples were obtained and analysed by Klaus Koepfli, at the department of Ecology and Evolution, University of California, Los Angeles, using the same primer pair used for the British samples. The samples themselves have been used in analysis for work published elsewhere, however this particular haplotype sequence was obtained specifically for use in this project. Nine additional East Anglian samples were analysed, as well as six Eurasian otter samples collected from South Korea.

4.2.1 Extra East Anglian Haplotypes

The additional East Anglian samples were samples originally collected by Don Jefferies and so are assumed to be different animals to those collected and analysed by the Environment Agency. Of these nine samples, two expressed the haplotype Lut1, five expressed the haplotype Lut3 and two expressed the haplotype Lut6. Combining this with the samples previously analysed for East Anglia brings the total number of otters analysed to sixteen, with three expressing Lut1, eleven expressing Lut3 and two expressing the haplotype Lut6. As identified previously, the Lut1 haplotype expressed by East Anglian otters is shared by a large proportion of British otters, and the Lut6 haplotype found to be expressed in two of the new samples is also present in Welsh otters (figure 4.3), suggesting that this too is a native British haplotype. However, Lut3 remains the most dominant haplotype in East Anglia, with 69% of otters expressing it.

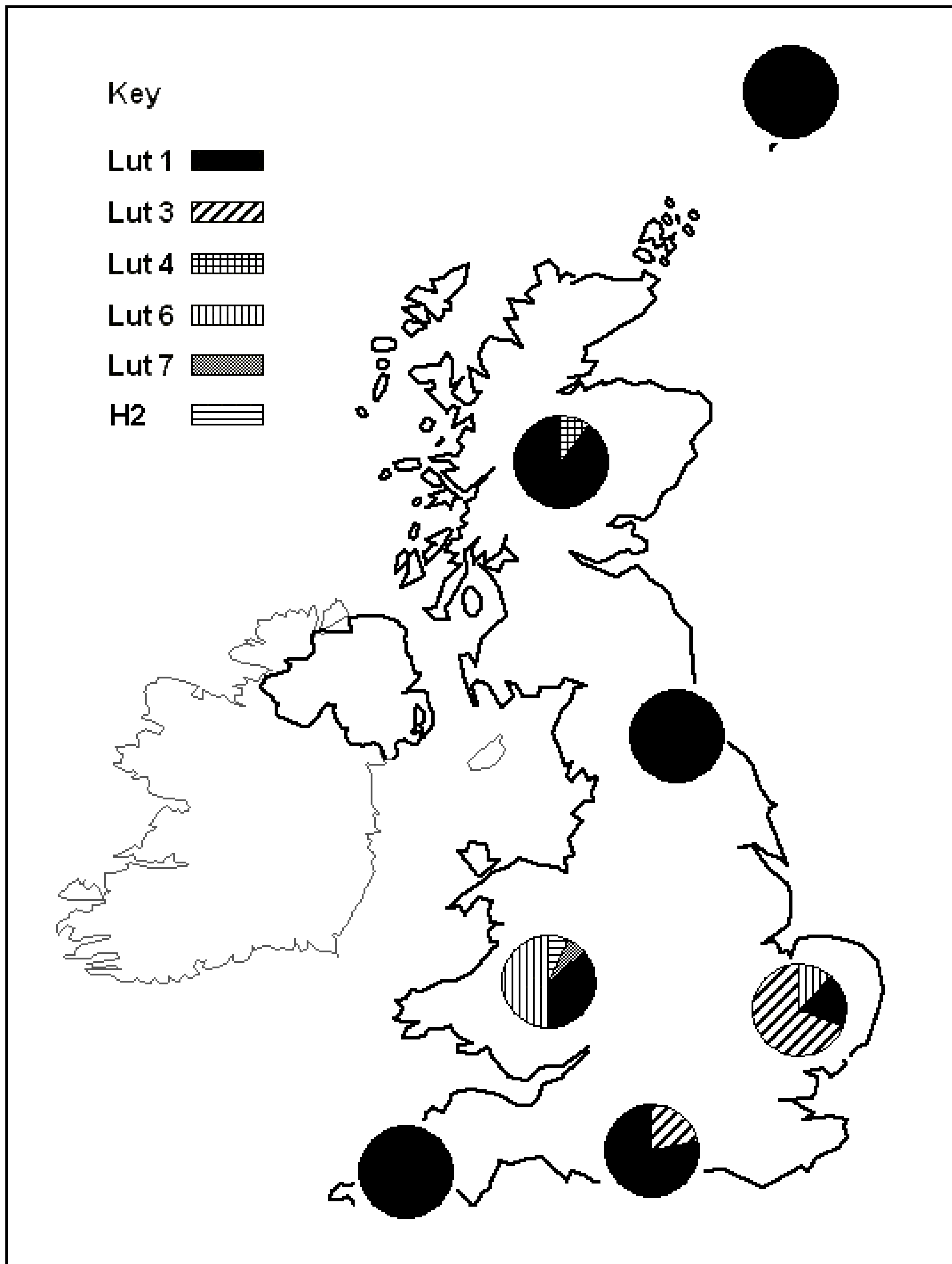


Figure 4.3 Map of the haplotypes found across Britain; included is data from this study as well as data from Cassens (2000) and Ferrando *et al.* (2004). Sample numbers are as follows; Shetland Isles (4), Scotland Mainland (19), Northern England (1), Wales (14), East Anglia (16), Cornwall (11) and Southern England (9).

4.2.2 South East Asian Haplotypes

The six South Korean otter samples expressed two new haplotypes, as yet not identified in otters, labelled here as LutSK1 and LutSK2. The first haplotype, LutSK1 was found in five of the six otters, suggesting that this is the most dominant haplotype in South Korean otters.

The haplotypes LutSK1 and LutSK2 differ from European haplotype Lut1 in three or four main locations (Table 4.3). In two of these instances the South Korean otters express a single base pair difference to that seen in European haplotypes; at base pair position 56 G is expressed in the South Korean otters where A is expressed in the European haplotypes, similarly at position 296 T is expressed instead of C. At base pair position 237 the South Korean haplotypes express the base C. This is in common with the European haplotypes Lut3 and Lut7, but different to all other European haplotypes, including Lut1, which express the base T at this position. In addition, in the haplotype LutSK2 an insertion of a C at the 101bp location mirrors an identical substitution event seen in the haplotype Lut4. The result is that the haplotypes expressed by otters from South Korea are two (LutSK1) and three (LutSK2) base pairs different from the haplotype Lut3 and fit into the minimum spanning network at this position accordingly (Figure 4.4).

Table 4.3 Shows the base pair differences between European haplotypes and the two new South Korean haplotypes, LutSK1 and LutSK2.

	56	72	95	101	153	237	296
Lut1	**A**	**G**	**G**	–	**C**	**T**	**C**
Lut2	•	•	**A**	•	•	•	•
Lut3	•	•	•	•	•	**C**	•
Lut4	•	•	•	**C**	•	•	•
Lut5	•	**A**	•	•	•	•	•
Lut6	•	•	•	•	**T**	•	•
Lut7	•	•	•	•	**T**	**C**	•
LutSK1	**G**	•	•	•	•	**C**	**T**
LutSK2	**G**	•	•	**C**	•	**C**	**T**

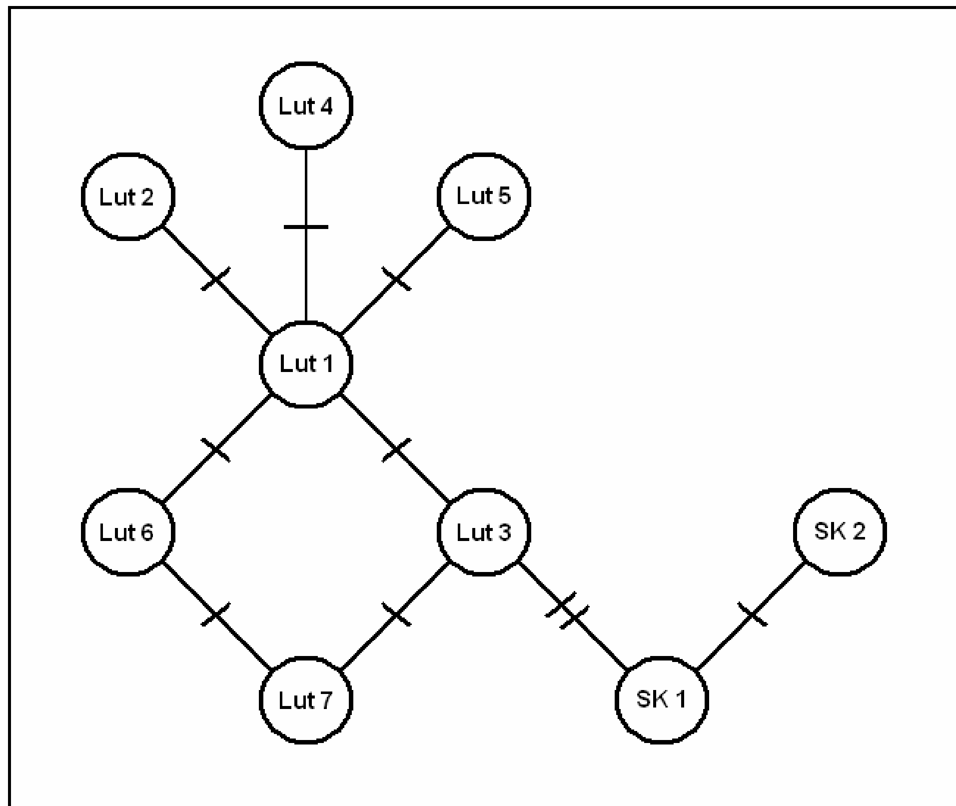


Figure 4.4 Minimum-spanning network for the European and South Korean haplotypes combined

4.3 Haplotype Project Discussion

As discussed in section *4.1.5 Haplotype Paper Discussion*, it appears that the most dominant haplotype expressed by otters in the East Anglian region, a population influences greatly by the release of large numbers of captive bred otters (Crawford, 2003), is the haplotype Lut3. The haplotype Lut1 is present in the East Anglian populations, but to a much lesser extent (19% of total) than the haplotype Lut3 (69%). With the new set of samples analysed the haplotype Lut6 was also found to be present at low concentrations (12.5%) in the East Anglian population (Figure 4.3). As the most prominent haplotype in many European samples (Figure 4.1), it is perhaps not surprising that the haplotype Lut1 is present in the east Anglian population. However, the haplotype Lut6 has only been seen before in samples from Wales and samples of captive otters of presumed 'English origins' (Pérez-Haro *et al* 2005). The presence of haplotype Lut6 in the East Anglian populations suggests that either the haplotype was present in the area before the declines and persisted in the dwindling wild population that remained, or that at least some otters of British origin were successfully used in the captive breeding programme.

Perhaps the most interesting finding is the high prevalence of the haplotype Lut3 in the East Anglian population, and its presence also in the River Itchen population. The implications of which have already been discussed in detail in section *4.1.5 Haplotype Paper Discussion*.

It is known that the Norfolk otter breeding facility, the Otter Trust, did successfully breed from otters originating from South East Asia (Paul Chanin, personal comm.). It is also known that the Otter Trust released a substantial number of otters into British ecosystems (Crawford, 2003). Whether the otters originating from Southeast Asia were used to breed the otters that were released into the wild in Britain is not known. However, if otters from South East Asia are found to genetically match otters in areas of Britain where high numbers of releases were carried out then this would prove to be considerable evidence in support of Asian otters having been used in the breeding programmes.

Although not particularly close to Indonesia, where the otters rumoured to have been used in the otter breeding programme supposedly came from (Paul Chanin, personal comm.), South Korea is in South East Asian area and therefore is more likely to genetically resemble the otters of Indonesia than are European otters.

The minimum spanning network (Figure 4.4) places LutSK1 and LutSK2 two and three mutations away from Lut3 respectively, the haplotype Lut3 being, in terms of this section of control region, the closest European haplotype to the two South Korean haplotypes observed. If anything this complicated the situation further as it implies that the haplotype Lut3 is only half as likely to have originated from the LutSK1 haplotype two mutations away, as it is from the Lut1 haplotype one mutation away. Given that South Korea is a considerable distance from Indonesia making an exact match unlikely, this similarity makes making a firm conclusion about the true origins of otters used in the captive breeding programme impossible.

Greater analysis if other regions, such as the 5′ end of the mitochondrial region to the cytochrome b region may help to shed more light on this. However, previous studies which have tried to produce phylogenetic trees using these regions have failed to obtain the definition and clarity needed to understand phylogenetic relationships.

Chapter 5 PBDEs in Otters

5.1 The Measurement of PBDEs in Otter Liver Tissue

Polybrominated diphenyl ethers (PBDEs) have been used over the last few decades to flame retard a wide range of materials (Vethaak *et al.*, 2002; Hooper *et al.*, 2004). PBDEs are used as additives rather than as a component of the structure of the material itself, and therefore over time they leach out into the surrounding environment (Darnerud *et al.*, 2001; Alcock *et al.*, 2003). As a result PBDEs are found in workplace environments (Sjodin *et al.*, 1999; Harrad *et al.*, 2006; Harrad *et al.*, 2008), and are building up to high concentrations in the wider environment (Strandberg *et al.*, 2001; Darnerud *et al.*, 2001; de Boer *et al.*, 2003). Despite recent bans on the manufacture, sale and use of PBDEs in the European Union (The European Parliament and the Council of the European Union, 2003; BSEF, 2008), their widespread previous use, high mobility and environmentally persistent nature, means that their presence in the environment is likely to remain a concern for many years to come (Martin *et al.*, 2004; de Wit *et al.*, 2006; Li *et al.*, 2006).

PBDEs have a tendancy to biomagnify up aquatic food chains (Boon *et al.*, 2002; Wolkers *et al.*, 2002b; Jenssen *et al.*, 2004). Congeners BDE-47, -99 and -100 tend to be found at particularly high concentrations in invertebrate and fish species (Ikonomou *et al.*, 2002; Covaci *et al.*, 2004; Schlabach *et al.*, 2004), and these congeners also tend to dominate the congener profiles of many marine mammal and fish-eating bird species (Boon *et al.*, 2002; She *et al.*, 2002; Jaspers *et al.*, 2006; Braune *et al.*, 2007). As a top predator, with fish making up a large proportion of their diet (Clavero *et al.*, 2003; Sales-Luís *et al.*, 2007), otters are likely to bioaccumulate PBDEs, in particular the congeners BDE-47, -99 and -100.

The decline in otter numbers observed in countries across Europe in the 1950s to 1980s has been linked in some studies to the release of dieldrin into the environment (Chanin & Jefferies, 1978) and in other studies to the release of PCBs (Roos *et al.*, 2001; Ruiz Olmo *et al.*, 2002; Barbosa *et al.*, 2003), suggesting that otters as a species are likely to be particularly sensitive to halogenated organic pollutants. PBDEs are similar in both structure and mode of toxicity to PCBs (see section *1.3.4 Toxicity*), although PBDEs are generally less potent in the responses they elicit (Hallgren, 2001; Hallgren and

Darnerud, 2002). Despite recent reductions in their use (The European Parliament and the Council of the European Union, 2003; BSEF, 2008), PBDEs are still increasing in concentration in many environments and species of biota (de Wit *et al.*, 2006; Braune *et al.*, 2007). As a top predator, otters are a species particularly susceptible to exposure to PBDEs, and their suspected susceptibility to the toxicological effects of organic pollutants highlights a particular need to monitor concentrations of PBDEs in these animals. In addition possible mixtures effects, i.e the combined toxicological effect of several pollutants acting together, are not fully understood, supporting the need to carry out the analysis of PBDE concentrations alongside the measurement of other problematic organic pollutants, such as PCBs and DDTs.

In this study the liver tissue of 129 otters collected, mainly as road kill, from locations across England and Wales (see section *2.1.3 Tissue Collection for Chemical Analysis*) was analysed for PBDE content, as well as for a range of PCB congeners, DDT breakdown products and HCB. The aim was to identify the sources of PBDE contamination in otter and the potential detriment effects upon the health of otter populations. The main focus of the project was on PBDE contamination, with measures of PCB and DDT concentrations carried out for comparative purposes. Potentail causes of variation in PBDE concentrations, such as geographical locations and the age and gender of the animal, were assessed in order to identify groups of otters more vulnerable to the effects of contamination. In summary, the aim was to assess PBDE exposure in otters and whether PBDEs are building up to concentrations which are likely to be detrimental to the health of the otter population, in particular in light of combined exposure with other organic pollutants.

5.2 Methods of Extraction and Measurement

5.2.1 Extraction and Clean-up

Samples were extracted in batches of six, each batch including a blank for the detection of contamination levels. Samples of seal blubber (always from the same individual) were used as an internal standard and as such were extracted alongside the liver samples approximately once ever other batch. These could then be used at the end to check for any major variations in concentrations obtained throughout the study. In total 192 sampes were analysed: 33 blanks, 15 internal standards and 129 liver samples - 15 of which were analysed in full or partial duplicate.

As deca-BDE is light sensitive it was important to keep UV radiation to a minimum throughout the extraction and clean-up process. Therefore amber glassware was used whenever possible as well as methods of covering and/or storing the samples that kept exposure to a minimum. The majority of soxhlet extractions were carried out in a room with UV filters on the windows. However, due to the electrical, water supply and fume hood requirements of the equipment used, it was not possible to carry out many of the sample clean-up and transfer processes in this same room, and therefore the samples were subjected to greater levels of UV radiation during these later stages of the extraction process.

To reduce contamination all glassware used for samples was cleaned using a cleaning solution (Decon) and warm water before being rinsed in millicon purified water and oven dried. Glassware was then placed in a furnace at 400ºC overnight. Before use all glassware was rinsed using acetone, then hexane, then DCM. Glassware which was not suitable for cleaning in this manor, such as glass pipettes and GCMS vials were baked overnight at 400ºC to remove as much contamination as possible.

5.2.1i) Soxhlet Extraction

Care was taken to ensure that liver tissue analysed did not include material form the outer 5mm of the main sample as these areas are the most likely to be contaminated and the most likely to have defrosted during any sample transfer operations. Samples of approximately 5g of liver (1g of seal blubber) were weighed out on tin foil before being chopped up as fine as possible and entered into a blender to be blended with a measure of anhydrous sodium sulphate. The sodium sulphate must previously have been baked at 400ºC overnight in order to make it anhydrous and capable of absorbing the water from the liver sample. The mixture of liver and sodium sulphate was blended until roughly homogenised before being stored in a clean and covered glass beaker ready for extraction. In between samples any equipment that came into contact with the sample, including the metal bell of the blender, was washed using cleaning solution and warm water before being rinsed using acetone, hexane then DCM. Blank samples were treated as similarly to samples of liver as possible. To this end, for blank samples a small amount of anhydrous sodium sulphate was be exposed to the cleaned sampling equipment before being added to a larger amount of sodium sulphate and blended briefly before storage and extraction.

Soxhlet thimbles were pre-extracted in the soxhlets for 2-4 hours before being used for the extraction of samples. Large thimbles were required to hold the sample material including the relatively large amount of sodium sulphate needed. Samples were spiked with 25µl of internal standards (at 20 $ng.\mu l^{-1}$ $^{13}C_{12}$-PCB-153, -138, -180 and -209, along with $^{13}C_{12}$-BDE-209 and BDE-49 and -51, also at 20 $ng.\mu l^{-1}$) before being soxhlet extracted overnight using DCM as a solvent. Extractions were carried out in a room with UV filters on the windows and foil was wrapped around the flasks and soxhlet bodies to reduce the amount of light exposure to the sample.

5.2.1ii) Lipid Determination

Approximately 10% of the extract was removed and transferred to a pre-weighed vial. The exact percentage of sample removed for lipid determination was assessed by working out by weight how much of the sample is removed from the flask (using volumes before and after the removal minus the flask weight). The amount removed was also weighed directly as a back up, although sample evaporation made this method

more unreliable. The vials with extracts for lipid determination were left in an undisturbed and relatively dust free environment for evaporation to occur. The vials were weighed on days two and three after extraction, and again on every day after this until no difference between measurements were observed, indicating that the sample was completely dry. The difference between the weight of the clean vial and the weight of the vial plus the dried sample extract was taken as the lipid weight and was then adjusted to account for the whole sample.

5.2.1iii) Sulphuric Acid Clean-up

The remaining sample (not used for lipid determination) was reduced and transferred to a clean test tube in hexane before being put through a 15% fuming sulphuric acid clean-up. During the clean-up the sample was mixed thoroughly with an equal amount of 15% fuming sulphuric acid before centrifugation to separate the two phases. The lower sulphuric acid phase was then removed and the process was repeated until the acid remained clear (approximately 7 cycles). Approximately 1ml of purified water was then added and mixed with the sample before centrifugation and removal of the water layer. A small amount of anhydrous sodium sulphate was then used to absorb any water remaining in the sample before the sample was transferred with hexane washes to an amber vial.

5.2.1iv) GPC Column Clean-up

The sample, held in an amber vial in hexane, was reduced down to a few drops before being eluted through a GPC column (35mm d, 70cm l, filled with 70g Biobeads) with 1:1 DCM:hexane at approximately 5ml/min. After rejection of the first 16ml fraction, a 30ml fraction was collected. This was then reduced down and transferred to pure hexane before transfer into a GCMS vial containing 25µl of dodecane containing the internal standards, BDE-69, BDE-181, PCB-30, $^{13}C_{12}$PCB-141 and $^{13}C_{12}$PCB-208.

5.2.2 Chemical Analysis

5.2.2i) GCMS Analysis

Analysis of the samples for PBDE congener content was carried out using gas chromatography/mass spectrometry (GCMS). Analysis of the lower congeners (BDE-17, -28, -32, -35, -37, -47, -66, -71, -75, -77, -85, -99, -100, -119, -128, -138, -153, -154, -166, -183, -190, -196 and-197) was carried out using a Fisons MD800 GC-MS used in SIM mode. The GC column used was a standard DB-5 column, 30m long with a 0.18mm id. The mass spectrometer was used in ECNI (electron capture negative ion) mode with ammonia used as the reagent gas. 1µl of analyte solution was injected insplitless mode into a split/splitless injector that was maintained at 300ºC. The temperature program, used under constant pressure, was: hold at 100ºC for 2 min, 4ºC/min to 300ºC and 10ºC/min up to 320ºC. The GCMS interface temperature was 270ºC and the ion source temperature 300ºC. Concentrations of nona and deca-BDEs (BDE-206, -207, -208 and -209) were determined using a HP6890 GC attached to a VG Autospec Ultima used in EI+ mode, with a resolution of at least 10,000. Separation was achieved on a 15m long 0.18mm id, DB5 MS column. PCBs and OCs were analysed on a Thermo Trace GC-MS in SIM mode using an EI+ source. Separation was achieved on a 50m 0.25mm id CP-Sil 8 column.

5.2.2ii) Conversion of Congener Concentrations

The concentrations obtained from the analysis of GCMS runs were measured in $pg.\mu l^{-1}$ of sample extract injected into the GCMS machine. This needed to be converted to $ng.g^{-1}$ of sample, either as grams of liver or as grams of lipid., as well as being corrected for sample removal for lipid determination and sample dilutions and for background laboratory BDE concentrations. The steps for sample conversion were as follows:

Step 1. The concentrations were adjusted from the 1 µl loaded onto the GCMS to the 25 µl sample volume.

$$\beta_1 = \beta_0 \times 25$$

Step 2. Concentrations were adjusted for the removal of a propotion of the sample for lipid determination.

$$\beta_2 = (\beta_1 \div r) \times 100$$

Where: r = percentage of sample remaining after removal for lipid determination

Step 3. Concentrations were adjusted for sample removal for sample dilutions.

$$\beta_3 = (\beta_s \div s) \times 100$$

Where:

For concentrated samples

s = percentage of sample remaining after removal for the dilute sample run

For dilute samples

s = percentage of sample removed for the dilute sample run

Step 4. Adjusting to account for background laboratory concentrations and conversion from pg to ng.

$$\beta_4 = (\beta_3 - t) \div 1000$$

Where: t = average of the blanks (adjusted as above for sample volume, removal for lipid determinationa nd for sample dilution, etc)

Step 5. Adjusting to the sample weight.

$$\beta_5 = \beta_4 \div L$$

Where: L = liver or lipid weight

Sample concentrations were the compared to limits of detection, with those samples showing concentrations which were below the limit subsequently being classed as such. The limits of detection were calculated as three times the standard deviation of the blanks adjusted for the liver or lipid weight of the sample being analysed.

$$LOD_i = (3 \times \delta) \div L_i$$

Where: δ = the standard deviation of the blanks

5.2.3 The use of Sample Dilutions

During preliminary study it was found that concentrations of some BDE congeners were too high to be read accurately on the GCMS readouts. This can be seen by considering the calibration curve constructed using seven standards of varying concentrations run at the beginning and end of every batch of approximately twenty-four samples, a typical example of which can be seen in figure 5.1.

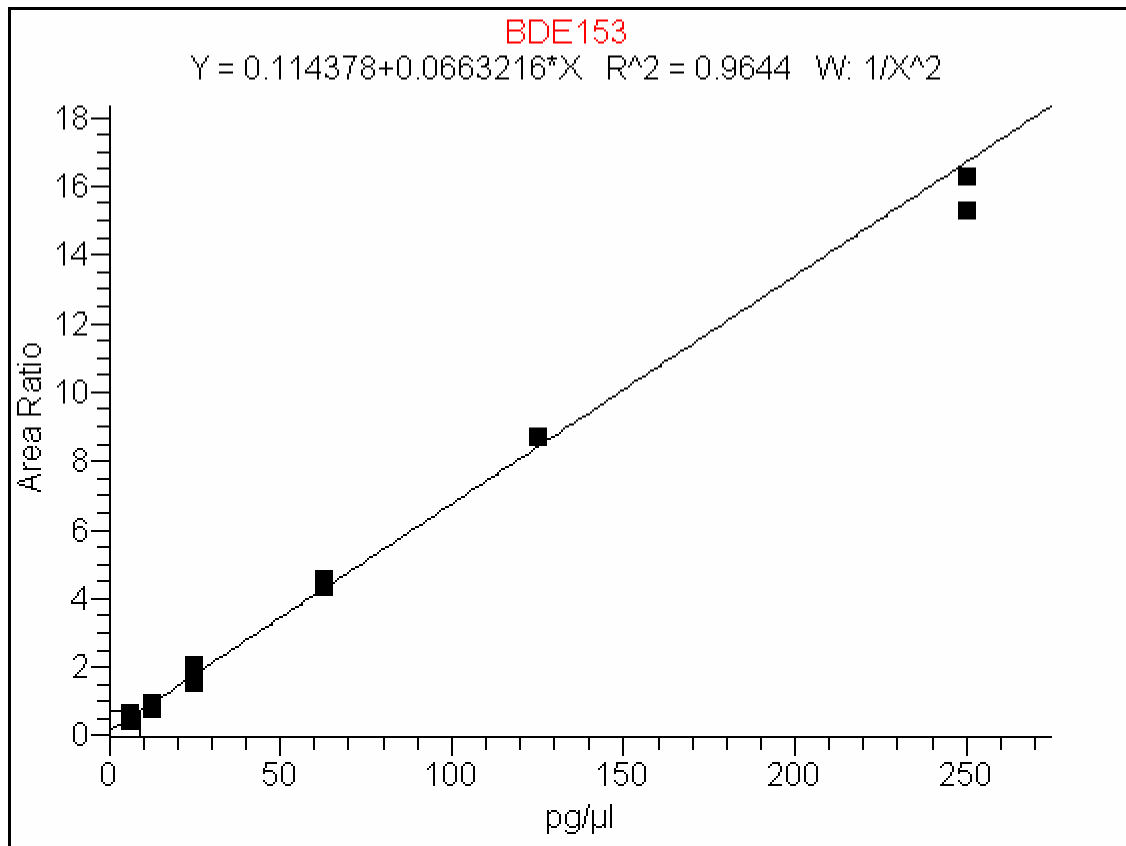


Figure 5.1 An example of a calibration curve, constructed using sets of standards of known concentrations run before and after sample runs and used to calculate the concentrations of samples.

Obviously, samples for which the concentrations observed fall within the measurements of the calibration curve are likely to be far more accurate than those for which the measurements are far more concentrated. At concentrations falling above the line of the calibration curve the accuracy of the calibration is lower and the accuracy of the calibration is likely to fall more steeply the greater the distance away from the end of the curve. Therefore, for highly concentrated congeners, such as BDE-47 and -153, a greater level of accuracy is obtained by diluting the samples and thereby bringing the

concentrations measured into the range, or in any case significantly closer to the range of the calibration curve measured.

For this reason diluted samples were run on the lower resolution machine in addition to the running of the more concentrated samples. Sample dilutions were obtained by the removal of approximately 1% of the sample before sample reduction for transfer to GCMS vials. Samples were weighed before and after the dilute sample removal in order for appropriate corrections to be made in the final BDE concentrations. The volume removed as a dilute sample was then reduced down into its own GCMS vial for analysis.

Chromatograms of the GCMS runs help to view the reasoning behind the use of sample dilutions for the analysis if some of the more concentrated BDE congeners. In figure 5.2 we can see the results for a concentrated (above) and dilute (below) run for the same sample extraction. In the dilute run, clearly visible is the peak at 13.83mins representing the internal standard BDE-69 used to standardise the measurement of other congeners. The fact that this peak cannot be identified in a similar view of the concentrated run is an indication of the increased height if the peaks in the concentrated run, particularly of the peak at 15.57mins representing BDE-47. The BDE-47 peak (15.34mins) is still relatively large in the dilute run. However, concentrations of internal standards, such as BDE-69, and concentrations of the standards run at the beginning and the end of each run and used to form the calibration curve for the measurement of BDE-47 are not diluted, and so the lower relative concentration of BDE-47 is likely to be measured more accurately in these dilute samples.

The peak for BDE-153, at 23.57 and 23.34mins, is also significantly reduced in the dilute sample and is similarly more likely to be accurately measured from the results of the dilute sample analysis than from the large peak seen in the concentrated sample. However, the results for BDE-153 varied with some sample concentrations below the detection limits. Therefore, results for BDE-153 were taken from the dilute samples, except in cases where the dilute samples were recorded as below the detection limit, in which case the results from concentrated samples would be used. All other congeners were found at concentrations reasonably measured using the concentrated samples.

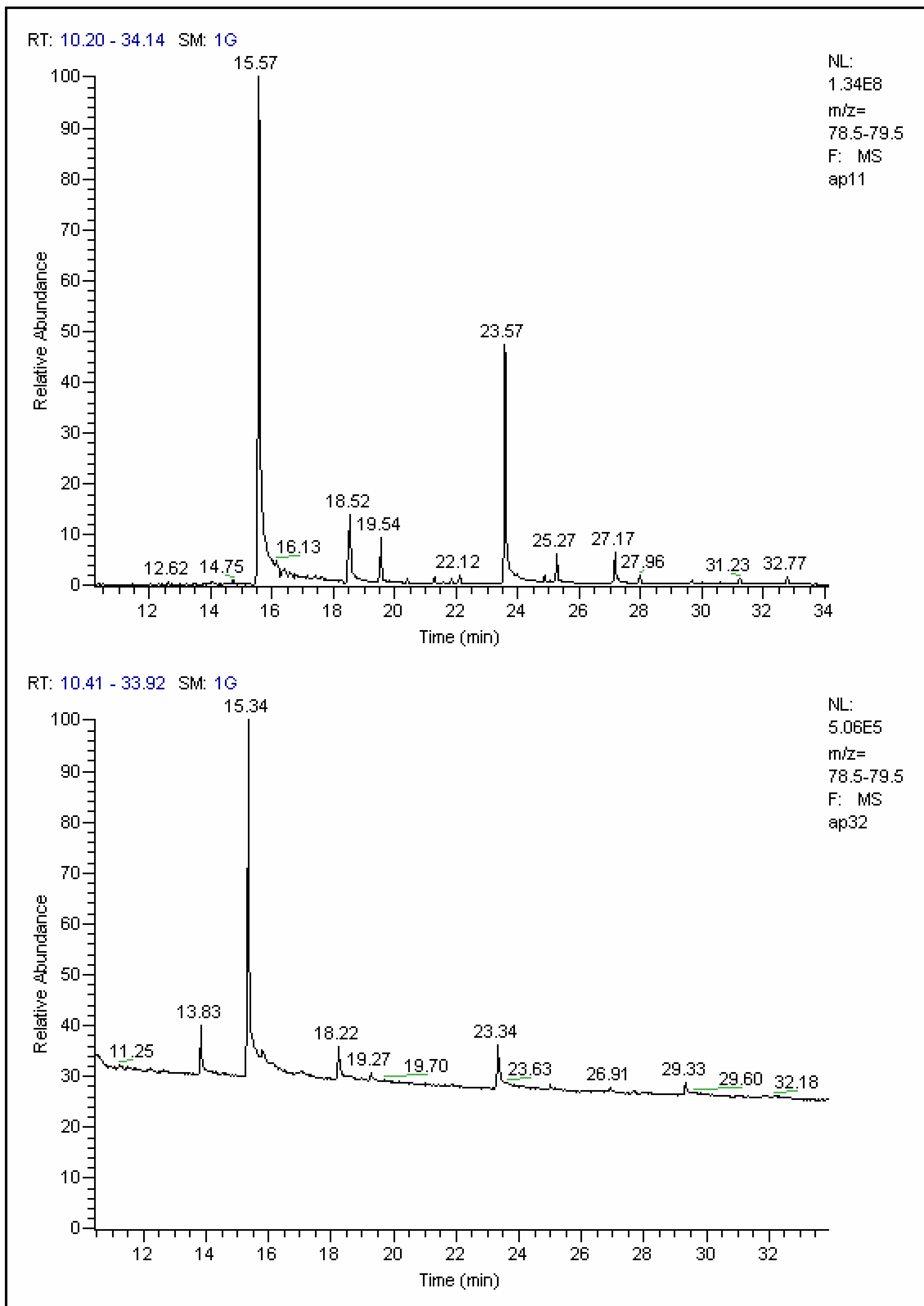


Figure 5.2 Chromatograms obtained from the lower resolution GCMS machine for the liver sample OTR148, as an example of a typical chromatogram obtained. The above trace is that obtained from the concentrated extract, the trace below being that obtained from the dilution of the sample.

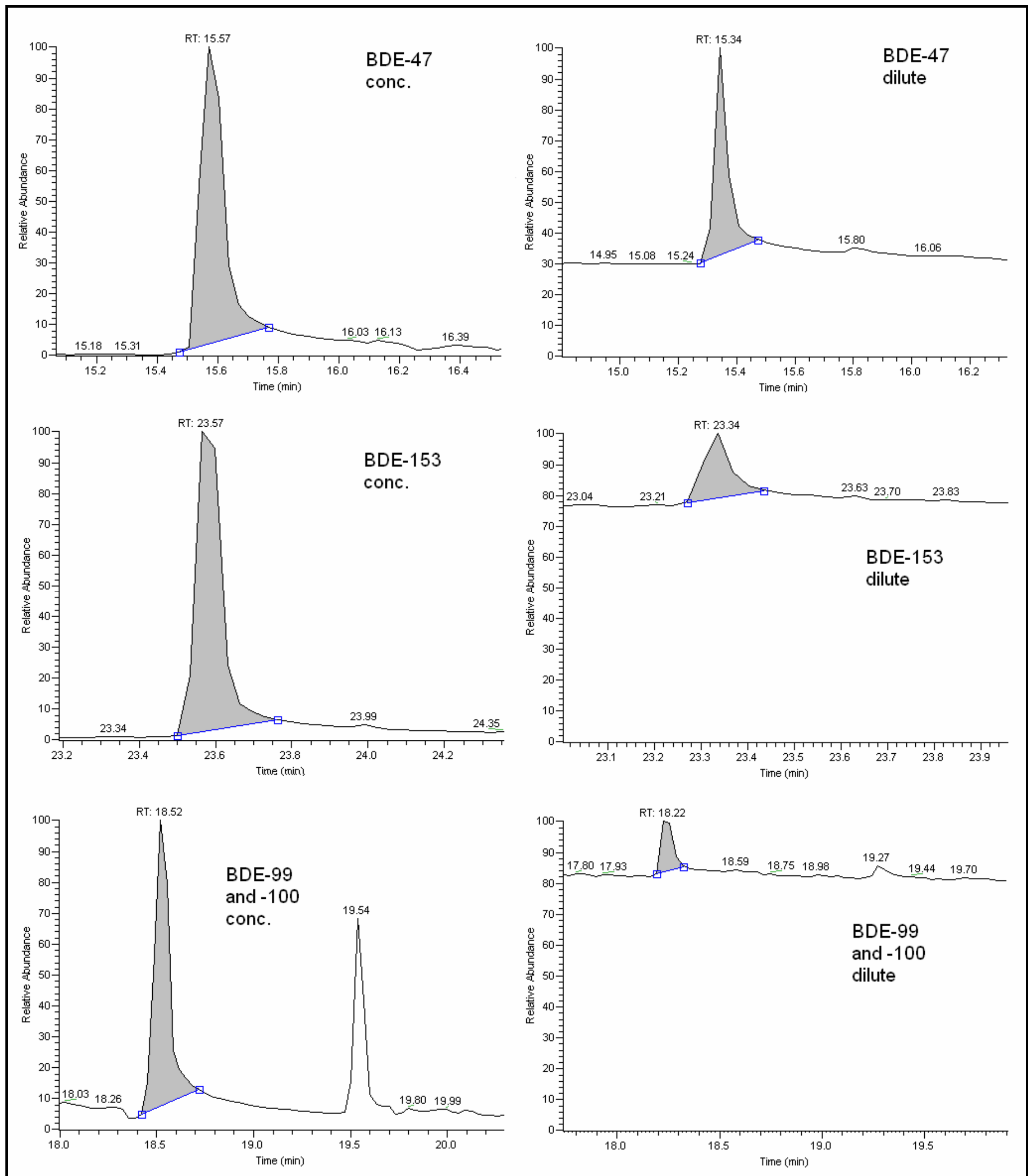


Figure 5.3 Congener profiles from runs of concentrated (left) and dilute (right) samples.

Figure 5.3 shows expanded chromatograph congener peaks for concentrated and dilute sample runs for the same sample (OTR048). The figure clearly shows that for congeners BDE-47 and BDE-153 concentrated runs produce large peaks which are relatively inaccurate to call, whereas peaks from dilute runs were more easily defined. The next most concentrated congeners, BDE-99 and BDE-100 are shown in the bottom pane. For these congeners the concentrated samples provided neat and reliable peaks whilst peaks produced from dilute samples tended to be small and somewhat harder to call and. For congeners with even lower concentrations peaks were often too weak to be visible in the diluted samples.

In summary, sample dilutions (approximately ×100) were used to obtain more accurate readings for concentrations of BDE-47. The concentrations for BDE-153 were also obtained from dilute sample runs, except in cases where concentrations were too low to be read, in which case the results from concentrated samples were used. All other congener concentrations were obtained from concentrated sample runs.

5.2.4 Blanks, Internal Standards and Sample Duplicates

5.2.4i) Blanks

It is likely that commercial mixtures of PBDEs will have been used in the construction of some of the laboratory equipment and building materials. Further atmospheric concentrations may have been brought into the laboratory via people traffic and particulate matter in the air exchange systems of the laboratory. It was therefore necessary to identify what the background concentrations of BDE congeners were in the laboratory in order to eliminate them from our analysis of liver sample concentrations. Blank samples were run in order to try to assess the background concentrations entering the samples during the extraction process.

Blank samples contained no liver or blubber tissue but were otherwise essentially treated the same as the other samples analysed. In total 33 blank samples were run. Two were removed from the final analysis, one due to a failure of the sample to express concentrations for any of the compounds analysed, including the spikes, the second was removed from analysis as it showed particularly high concentrations consistent with a contamination event. The remaining blank samples (n = 31) were used to negate the

effects of background PBDE concentrations, with the average concentration obtained from the blanks being taken away from the concentrations obtained for the liver samples. This process being repeated for each of the BDE congeners analysed.

The blank samples were also used to calculate limits of detection. With the limits of detection being calculated as three times the standard deviation of the blanks, standardized for each individual sample using the weight of liver/lipid extracted per sample.

5.2.4ii) Internal Standards (Seal Blubber Samples)

An internal standard was used in order to try to assess levels of variation in sample extraction success across the study. The material used was a sample of seal blubber, homogenised with a small amount of anhydrous sodium sulphate and stored under refrigeration throughout the period of study. This single sample of seal blubber was extracted and analysed with roughly every other batch of otter liver samples, the concentrations obtained being used to look for any major increases or decreases in the effectiveness of the extraction and measurement of BDE congener concentrations.

The only BDE congener to be consistently measured at concentrations above the limit of detection was BDE-47. The limits of detection were adjusted according to the weight of lipid in the sample analysed, the result was that the limits of detection for BDE-47 in the seal blubber ranged from 54 to 103 ng.g^{-1} lipid. Four out of the fifteen internal standard samples run expressed concentrations of BDE-47 below the limit of detection. For the remaining eleven internal standard runs BDE-47 concentrations ranged from 59.8 to 162.8 ng.g^{-1} lipid, with a mean of 117.5 (29.2) ng.g^{-1} lipid.

The only other BDE congener at concentrations above the limit of detection for a significant number of internal standard runs was BDE-154, where five of the fifteen samples were found to show concentrations just above the limit of detection, which ranged from 1.56 to 2.99 ng.g^{-1} lipid. For these five samples BDE-154 ranged from 1.87 to 4.80 ng.g^{-1} lipid, with a mean of 3.52 (±1.73) ng.g^{-1} lipid. Several of the other BDE congeners analysed showed concentrations marginally above the limits of detection for one or two samples only. As the concentrations measured were generally

close to the limits of detection they were not significantly different suggesting relatively little variation in the concentrations measured between samples.

In conclusion, the seal blubber sample was not particularly useful as an internal standard due to the low levels of BDE congeners present. Variation between the concentrations observed in duplicate extractions from otter liver tissue samples are likely to be a more accurate method for assessing levels of variation in concentration data attributable to variation in sample handling and/ or processing.

5.2.4iii) Duplicate Sample Runs

Further assessment of the accuracy of measures of BDE congener concentrations in otter liver tissue was made possible as 15 of the liver samples were extracted and analysed twice. Some of these samples were extracted a second time due to potential problems with the initial sample extractions. Details of this and indications of the ranges of BDE congeners analysed per sample duplication are provided in Table 5.1.

Table 5.1 Fifteen of the otter liver samples were extracted twice and were analysed in duplicate for at least some of the BDE congeners. Reasons for the second extraction are given in the notes section where appropriate.

Sample ID	Both sample extractions analysed for:			Notes
	Lower congeners (conc. sample)	BDE-47 and BDE-153 (dil. sample)	Higher congeners (high res. run)	
M833	Yes	Yes	Yes	Possible sample loss from first sample
M929	Yes	Yes	Yes	
M1026	Yes			
M1035	Yes			
M1080	Yes			
M1092		Yes		Conc. sample lost in first run - vial dropped
M1118	Yes	Yes	Yes	Sample loss (approx. half) from first sample
M1214	Yes			
M1215	Yes			Possible sample loss from first sample
C41	Yes	Yes	Yes	Possible sample loss from first sample
C259	Yes	Yes	Yes	Possible sample loss and/or contamination in first sample
C560	Yes	Yes	Yes	
C676	Yes	Yes	Yes	Two different liver duplicates sampled
C702	Yes	Yes	Yes	Sample loss (approx. half) from first sample
C743	Yes	Yes	Yes	High BDE-209 concentrations checked

Nine of the samples analysed in duplicate were assessed for the full range of BDE congeners analysed for both liver sample extractions. The ΣBDE concentrations for eight of these duplicate extractions are shown in Figure 5.4. The final sample, M743, gave significantly higher overall concentrations, but similar levels of accuracy, with 9524 $ng.g^{-1}$ lipid for the first extraction and 10,581 $ng.g^{-1}$ lipid for the second.

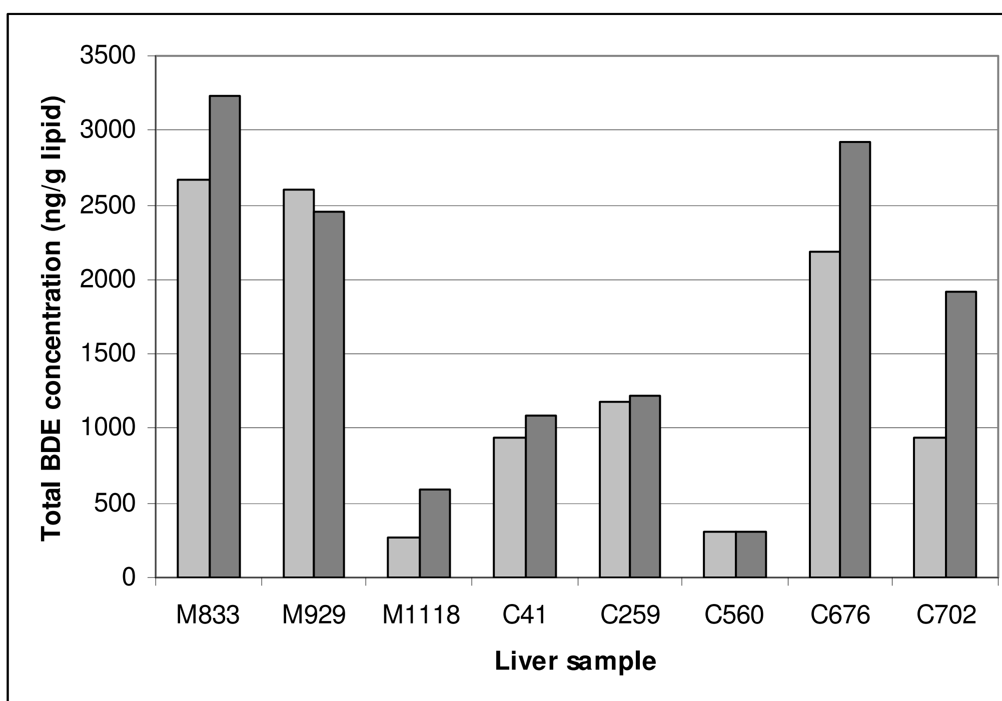


Figure 5.4 Variation in ΣBDE concentrations for otter liver samples analysed in duplicate. Light bars represent concentrations obtained from initial extractions and darker bars represent concentrations obtained from duplicate extractions.

In general, the first and second extractions from the same sample provided similar ΣBDE concentrations. For samples for which this was not the case there was usually a clear explanation for the disparity. It was noted that for samples M1118 and C702 approximately half the sample was lost from the initial extraction and this is mirrored in the results, with the concentrations obtained from the first sample run being approximately half those of the second (Figure 5.4; M1118, 266 vs 593 $ng.g^{-1}$ lipid and C702, 930 vs 1893 $ng.g^{-1}$ lipid for the first and second extractions respectively). Similarly, sample loss was recorded from the first extractions for samples M833 and C41, and this is visible in the resulting concentrations (M833, 2665 vs 3226 $ng.g^{-1}$ lipid and C41, 934 vs 1083 $ng.g^{-1}$ lipid for the first and second extractions respectively). Sample C676 also shows a significantly lower concentration from the first extraction compared to the second (2179 vs 2925 $ng.g^{-1}$ lipid). This was the only sample for which two liver samples were available from the same animal and the resulting two extractions were taken one from each of these liver samples. The results indicate that the two liver samples, although they were recorded as having originated from the same animal, contain significantly different concentrations of PBDEs.

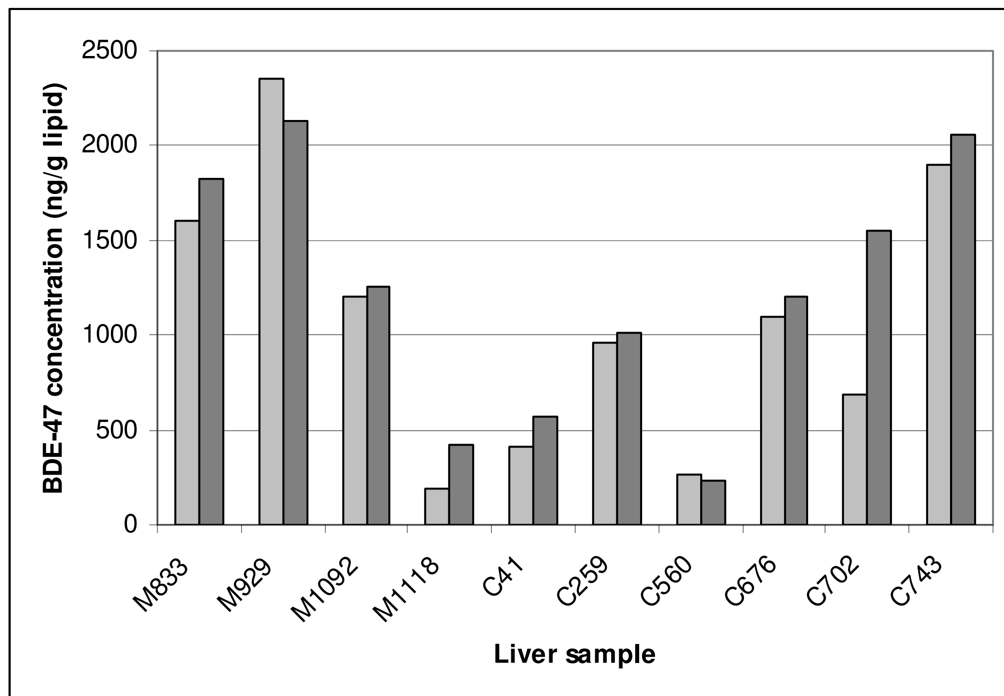


Figure 5.5 Concentrations of BDE-47 obtained from duplicate extractions of otter liver tissue. The concentrations obtained from initial extractions are represented by light bars, while the darker bars represent concentrations obtained from duplicate extractions.

A similar pattern was observed in the comparison of BDE-47 concentrations from duplicate extractions of the otter liver samples (Figure 5.5). Again the majority of samples show similar concentrations for both of the sample extractions analysed, with samples M118, C41 and C702 showing evidence in the results of the sample spillages which occurred during the initial extraction and analysis of the samples.

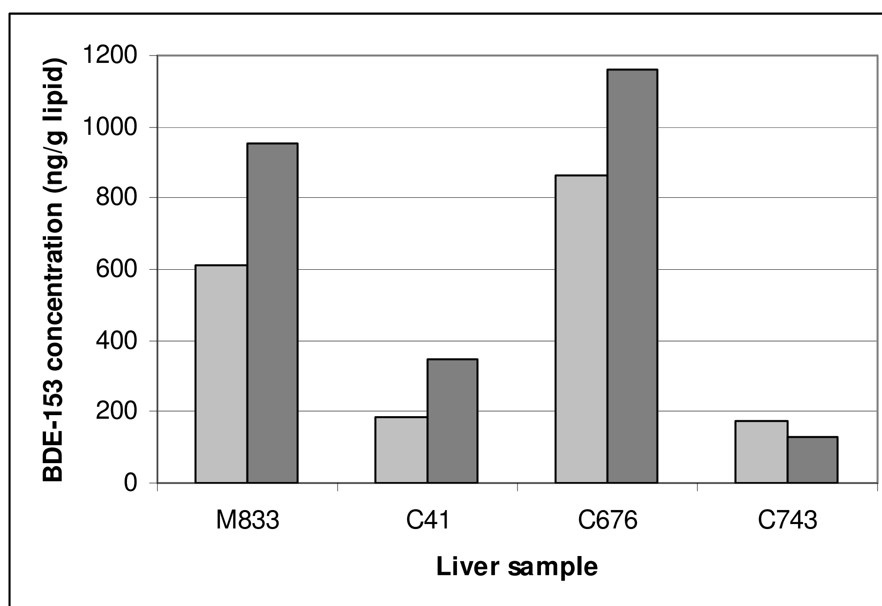


Figure 5.6 Variation in concentrations of BDE-153 obtained from duplicate otter liver sample extractions. The concentrations obtained from initial extractions are represented by light bars, while the darker bars represent concentrations obtained from duplicate extractions.

For BDE-153, it can be seen from Figure 5.6 that samples M833 and C41, as with BDE-47, showed significantly lower concentrations in the first extractions compared to the second. This was consistent with the patterns of sample loss from the first extractions from these liver tissue samples. For sample M1118, where again there was a large proportion of sample loss from the first extraction, BDE-153 concentrations were below the limit of detection for the first sample extraction but were measured at 107.3 $ng.g^{-1}$ lipid for the second. For a couple of other samples BDE-153 concentrations were measured at relatively low concentrations for one extraction whilst being below the limit of detection for the other. A concentration of 67.4 $ng.g^{-1}$ lipid was measured from the first extract from sample M929, whilst the second extract showed as being below the limits of detection. Similarly, for sample C259 the first extract was below the limit of detection while the second was measured at a concentration of 50.0 $ng.g^{-1}$ lipid. This suggests that for these samples BDE-153 concentrations were only successfully measured for one of the two extraction duplications because the relatively low concentration present in the sample was not detected in the both sample extractions.

Most of the samples analysed in duplicate for BDE-209 showed very little difference between the concentrations obtained from the initial extraction and the concentrations obtained from the duplicate extraction (Figure 5.7). Exceptions to this include M1118

where, despite approximately half the sample being lost during the first extraction, concentrations of BDE-209 were higher in the first extraction than the second (48.6 vs 32.1 $ng.g^{-1}$ lipid for the first and second extractions respectively). Also showing a relatively high level of disparity was sample C259, with concentrations of BDE-209 obtained from the first and second extractions being 73.7 and 35.8 $ng.g^{-1}$ lipid respectively. By far the most striking difference in concentrations of BDE-209 was for sample C676, where 10.5 $ng.g^{-1}$ lipid was obtained from the first sample while 271.6 $ng.g^{-1}$ lipid was obtained from the second. It is unclear whether this was due to the fact that different liver samples were used, both recorded as originating from the same animal, C676.

One further sample was assessed for reproducibility of BDE-209 concentration measurement. Sample C743 contained the highest concentration of any of the liver samples analysed and it was with the specific purpose of checking this high concentration that the sample was extracted for a second time. A BDE-209 concentration of 6487 $ng.g^{-1}$ lipid was obtained from the first extractions, whilst an even higher concentration of 7129 $ng.g^{-1}$ lipid was obtained from the second, the relative similarity of the measurements suggesting that BDE-209 concentrations were truly this high within the liver of this otter.

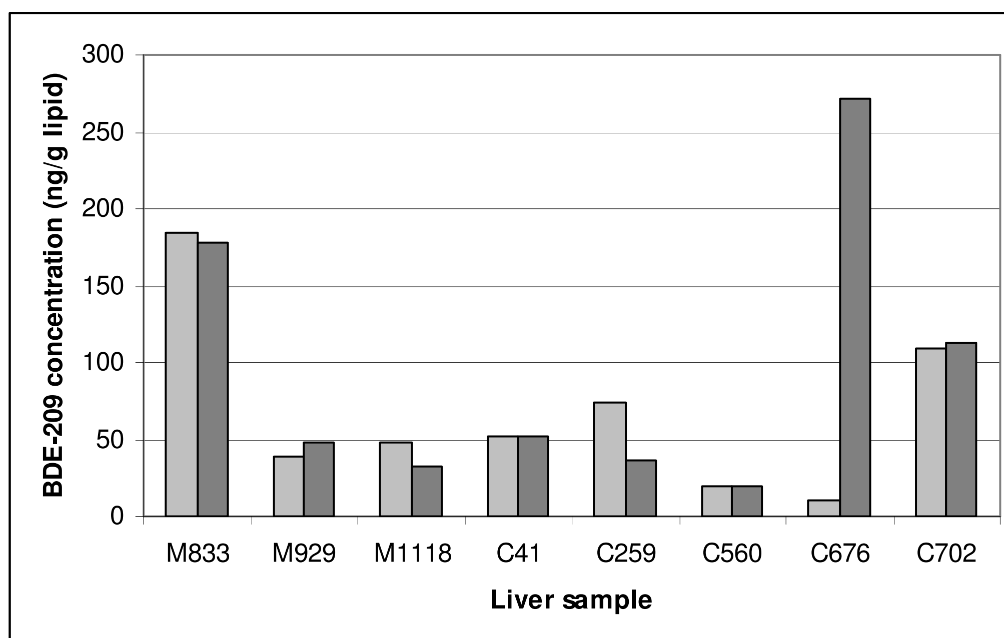


Figure 5.7 Variation in concentrations of BDE-209 obtained from duplicate otter liver sample extractions. The concentrations obtained from initial extractions are represented by light bars, while the darker bars represent concentrations obtained from duplicate extractions.

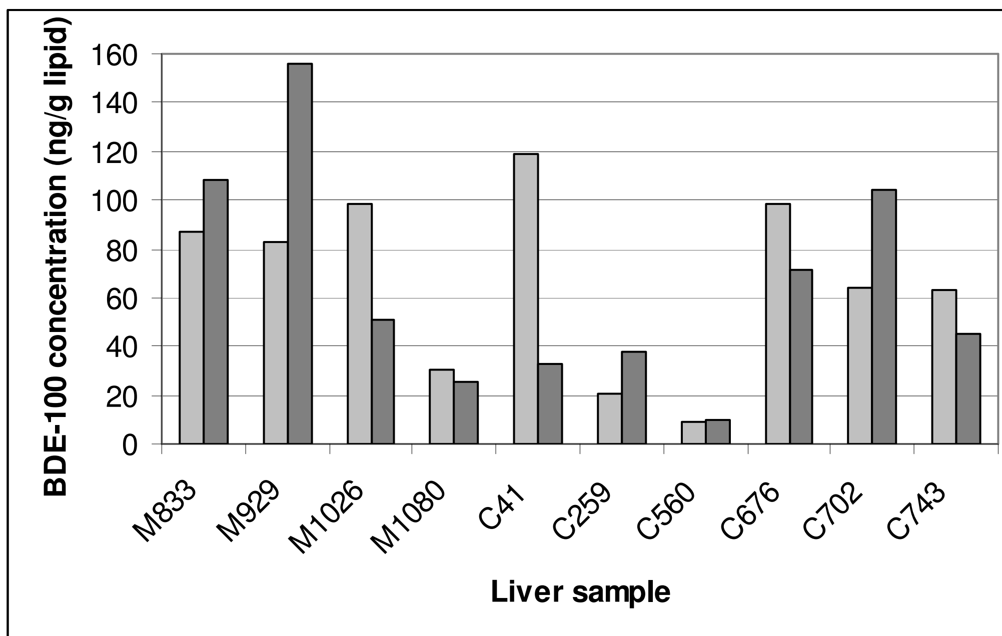


Figure 5.8 Variation in concentrations of BDE-100 obtained from duplicate otter liver sample extractions. The concentrations obtained from initial extractions are represented by light bars, while the darker bars represent concentrations obtained from duplicate extractions.

In general the results from duplicate sample extractions suggest that the accuracy of measurement is good, however this relative accuracy does appear to reduce as the concentrations measured reduce in intensity (for example see Figure 5.8). Where there are large differences in the concentrations obtained from duplicates this can normally be assigned a particular cause. For example, sample loss in the initial extraction occurred for samples M833, M1118, C41 and C702, resulting in significantly lower concentrations being obtained from the initial extractions in line with the loss experienced during the extraction process. For samples M1118 and C41 concentrations from the second extractions were used in the final analysis in order to negate the effects of sample loss from the first extraction.

One sample for which there was a large disparity not attributable to differences in the extraction process was C676. This was the only samples for which extractions were made from two separate liver tissue samples, recorded as having originated from the same otter. The sample showed disparity in the concentrations observed for the two liver tissue samples, particularly for the concentrations of BDE-153 and BDE-209. This

disparity in the concentrations obtained from the two samples of liver supposedly obtained from the same otter may have occurred for several reasons.

Firstly, the disparity may have occurred as a result of the sample extraction techniques. Although the fact that other samples extracted in duplicate did not express similar levels of variation suggests that the cause has something to do with the fact that two different liver tissue samples were extracted as opposed to the same liver tissue sample being sampled and extracted from twice. Although both the samples were recorded as being labelled C676 it may be that a mistake was made, either in the labelling of the sample or in the recording of the sample name. If this is the case then the two samples may have originated from two different otters, explaining the different congener concentrations observed. A further possible reason for the disparity in concentrations observed for C676 could be that the samples were stored and transported in separate batches. The storage of samples may have played a role in altering BDE congener concentrations, either via congener decomposition or contamination.

It may be that BDE congeners are not acquired and stored uniformly across otter livers and so samples taken from different areas of the liver may not contain comparable concentrations of BDE congeners. It should be noted though that for other liver tissue samples duplicate samples were taken from different areas and sides of the tissue mass. Therefore, if there was a disparity according to the area of the liver then it would be likely to show up for these samples also.

5.3 PBDEs in Otter Liver Tissue

5.3.1 PBDE Congener Concentrations

PBDEs were detected in all of the otter liver tissue samples analysed, with ΣBDE concentrations ranging from 12 to 69,883 ng.g^{-1} lipid. The mean ΣBDE concentration of 3242 ng.g^{-1} lipid and median ΣBDE concentration of 1564 ng.g^{-1} lipid were closer to the lower limits of this range. For individual congeners the highest concentrations were generally only observed for one or two individuals with the remaining samples containing concentrations closer to the lower limits of the concentrations ranges observed (Figure 5.9).

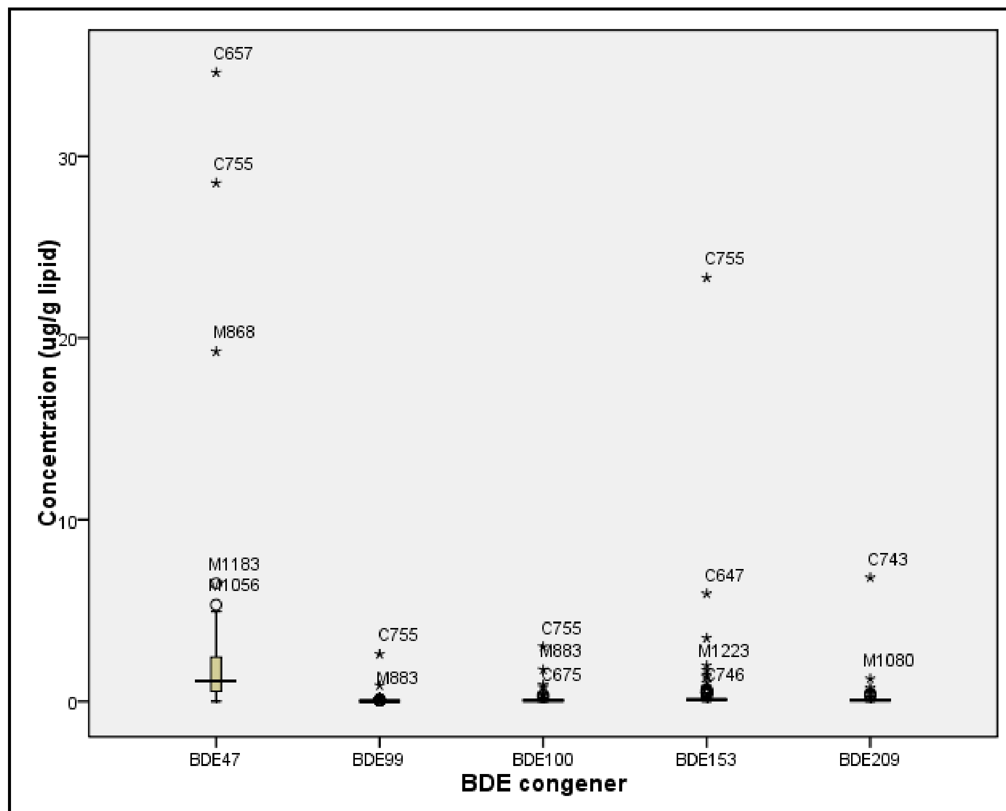


Figure 5.9 Box and wisper plot of BDE congener concentrations in the liver tissue of otters from England and Wales (n = 129).

A few otter liver tissue samples contained particularly high concentrations of individual congeners, differing significantly in their resulting congener profile. Sample C755, a sub-adult female collected in 2005 from Dyfed in south Wales (grid ref. SN379488) contained very high concentrations of many of the lower congeners, including BDE-47,

-99, -100 and -153. Samples C657, an adult female collected in 2003 from Leicestershire in the midlands (classified as East Anglia for the purposes of this study, grid ref. SP645957), and M868, an adult male collected from Bodmin in Cornwall (grid ref. SX037632) in 2005, both had very high concentrations of BDE-47. Interestingly, in both these individuals this high concentration of BDE-47 was not associated with significantly elevated concentrations of other congeners. An even more unusual case was that of otter C647, an adult female collected from the Wye Valley area (grid ref. SO541120) in 2005; this otter contained relatively high concentrations of BDE-153, but did not contain correspondingly elevated concentrations of other BDE congenrs. A final notable case was that of otter C743, an adult male otter collected from a location in Humberside, north east England (grid ref. TA200693) in 2005. The liver sample from this otter contained an especially high concentration of BDE-209, suggesting a high level of exposure to the deca-BDE commercial mixture.

These instances of individual otters containing particularly high concentrations of individual BDE congeners are likely to have occurred for one of two reasons. Firstly, the otters affected may have been exposed to point sources of BDE congeners in the environment leading to the uptake of large amounts of particular congeners over a relatively short period of time. A second possible explanation is that animals suffering from poor body condition due to disease or starvation may have subsequently built up higher concentrations of BDE congeners because their ability to metabolise and/or excrete the compounds was impaired and/or because a lower nutritional status lead to the mobilisation of PBDEs from adipose tissue resulting in the build up of these compounds in the liver, as can occur for other lipophilic organics (She *et al.*, 2002; Kannan *et al.*, 2007; Naert *et al.*, 2007).

Of the group of otters with particularly high BDE congener concentrations, only otter C647, containing particularly high concentrations of BDE-153, showed signs of low body condition ($K = 0.86$). This otter was also recorded as suffering from sepsis. In contrast, otters C657, C743 and M868 showed relatively high body condition indexes ($K = 1.29$, 1.13 and 1.18 respectively) compared to the general range of $K = 0.47$ to 1.37 (mean = 0.99, median = 1.02). In addition, all three of these otters, plus otter C755, were recorded as having died as a result of road traffic accidents (RTAs), suggesting that there is no direct link between the BDE concentrations in the liver of the otters and their mode of death.

Table 5.2 Individual congener concentration statistics for otters from England and Wales (n = 129).

Congener	Congener concentration (ng.g^{-1} wet wt)			Congener concentration (ng.g^{-1} lipid wt)		
	Mean	Median	Range	Mean	Median	Range
BDE-17	0.008	L.O.D.	<0.01-0.226	0.211	L.O.D.	<0.1 - 7.125
BDE-28	0.442	0.066	<0.04 - 39.0	9.138	1.965	<0.4 - 735.82
BDE-32	0.000	L.O.D.	<0.01 - 0.017	0.007	L.O.D.	<0.1 - 0.355
BDE-35	0.035	L.O.D.	<0.01 - 0.608	1.011	L.O.D.	<0.1 - 30.00
BDE-37	0.004	L.O.D.	<0.01 – 0.182	0.081	L.O.D.	<0.1 - 3.004
BDE-47	84.32	38.14	<1.1 - 1512.3	2228.53	1125.35	<10.4 - 34610.62
BDE-66	0.197	L.O.D.	<0.01 - 6.607	5.251	L.O.D.	<1.2 - 124.62
BDE-71	0.056	L.O.D.	<0.01 - 0.879	1.553	L.O.D.	<0.1 - 18.97
BDE-77	0.227	L.O.D.	<0.01 - 19.66	4.357	L.O.D.	<0.8 - 239.06
BDE-85	0.366	0.121	<0.04 - 16.52	8.173	3.312	<0.5 - 311.69
BDE-99	2.470	0.548	<0.1 - 137.62	51.01	15.59	<1.5 - 2595.77
BDE-100	4.857	1.845	<0.2 - 159.73	122.37	51.01	<2.5 - 3012.87
BDE-119	0.010	L.O.D.	<0.02 - 1.261	0.276	L.O.D.	<0.2 – 35.39
BDE-128	1.886	L.O.D.	<0.2 - 210.20	37.15	L.O.D.	<2.7 – 3964.79
BDE-138	1.486	0.173	<0.04 - 141.84	30.69	4.954	<0.7 – 2675.38
BDE-153	19.01	3.217	<0.3 - 1236.22	447.53	96.71	<5.9 – 23317.48
BDE-154	0.494	0.092	<0.03 - 39.73	10.34	2.375	<0.8 – 749.44
BDE-166	0.013	L.O.D.	<0.02 - 0.937	0.406	L.O.D.	<0.2 – 34.77
BDE-183	0.010	L.O.D.	<0.04 - 0.177	0.294	L.O.D.	<0.4 – 7.004
BDE-190	1.360	L.O.D.	<0.1 - 172.95	25.59	L.O.D.	<1.4 – 3262.24
BDE-196	0.004	L.O.D.	<0.05 - 0.241	0.075	L.O.D.	<0.5 – 3.937
BDE-197	0.271	L.O.D.	<0.06 - 31.63	5.309	L.O.D.	<0.6 – 596.53
BDE-206	0.086	0.045	<0.01 – 1.367	2.137	1.210	<0.3 – 24.90
BDE-207	1.381	0.753	0.080 – 30.85	39.58	19.32	1.526 – 858.45
BDE-208	0.254	0.146	<0.01 – 3.608	7.349	3.597	<0.17 – 101.16
BDE-209	5.852	2.252	<0.3 – 246.13	167.32	62.25	<8.5 – 6808.12
∑BDE	126.85	53.58	0.18 - 3704.96	3241.94	1564.48	12.18 – 69882.54

5.3.2 PBDE Congener Profile

The results show that the PBDE congener profile of the European otter in England and Wales was dominated by the BDE-47 congener, which constituted an average of 71.5% of the ΣBDE congener concentration. The concentrations of BDE-47 observed in the otter liver samples were significantly greater than the concentrations of any of the other BDE congeners measured (Figure 5.10), with a mean BDE-47 concentration of 2228.5 $ng.g^{-1}$ lipid and a median concentration of 1125.4 $ng.g^{-1}$ lipid.

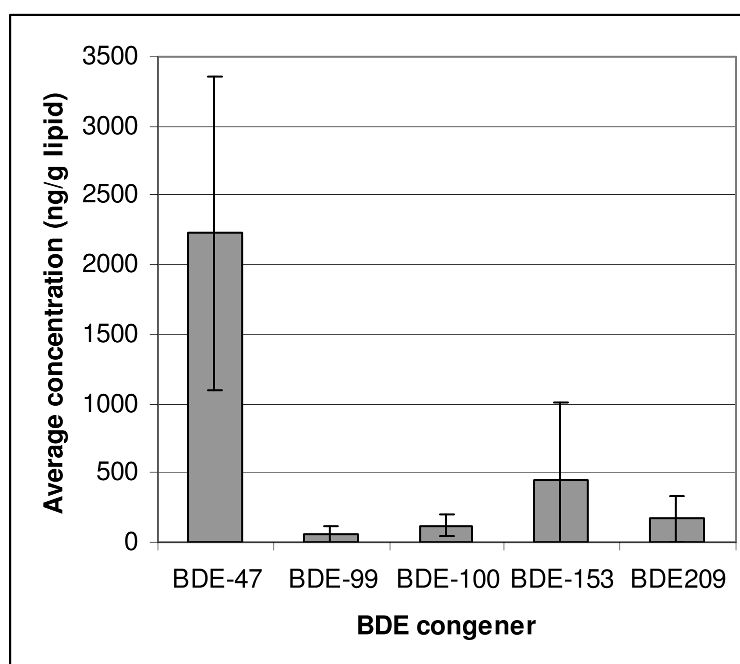


Figure 5.10 Mean concentrations of the five most concentrated PBDE congeners measured in the liver tissue of otters.

The second most concentrated BDE congener measured in otter livers was BDE-153 (Figure 5.10), with a mean of 447.5 $ng.g^{-1}$ lipid and a median concentration of 96.7 $ng.g^{-1}$ lipid and making up an average of 8.8% of the congener profile. Constituting 8.2% of the congener profile of otters, BDE-209 was the third most concentrated congener (mean = 167.3 $ng.g^{-1}$ lipid, median 62.3 $ng.g^{-1}$ lipid). The congener BDE-100 was found at the next highest concentration (mean = 122.4 $ng.g^{-1}$ lipid, median 51.0 $ng.g^{-1}$ lipid), with BDE-28, -99, -128, -138, -154, -190 and -207 also observed at significant concentrations in the liver tissue of otters (mean concentrations above 9.0 $ng.g^{-1}$ lipid and concentrations ranging to maximums over and above 700 $ng.g^{-1}$ lipid). The congener profile of the otter, in terms of the highest mean concentration to the

lowest was BDE-47 > BDE-153 > BDE-209 > BDE-100 > BDE-99 > BDE-207 > BDE-128 > BDE-138 > BDE-154> BDE-28.

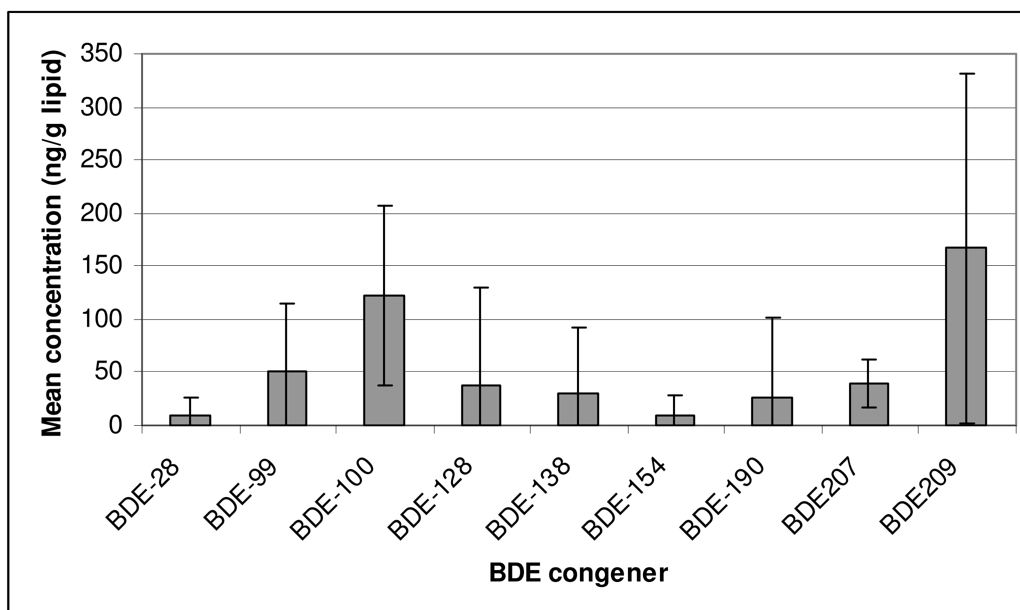


Figure 5.11 The PBDE congener profile of otters from England and Wales, showing the mean congener concentration of the most concentrated BDE congeners, bar the two most concentrated, BDE-47 and BDE-153.

5.3.3 PBDE Congener Correlations

5.3.3i) Pearson's Correlations

The PBDE congener concentrations present in environmental samples, including the otter liver samples, are likely to have originated from just a few distinct sources, i.e. from the three commercial mixtures. Therefore, patterns should be evident in the proportions in which these congeners are found according to which commercial mixtures the congeners originated from. For example, for an otter exposed to particularly high concentrations of the Penta-BDE mixture we would expect to see relatively high concentrations of congeners BDE-47, -99 and -100, all of which originate from this commercial mixture.

In this study the level of correlation between the concentrations of the congeners found in the otter liver tissue was initially assessed using Pearson's correlation coefficients.

The results are shown in a matrix in Table 5.3, where a value of one denotes perfect positive correlation between the concentrations of the two congeners, and a value of zero signifies that there is no correlation. Therefore, the closer the value is to one the more closely correlated the two congeners are and the more likely it is that they originated from the same source. The correlation matrix (Table 5.3) reveals a distinct pattern of correlations, with two main groups of correlated congeners.

The first group of correlated congeners consists of twelve tri- to octa-BDEs. High correlation coefficients ($r > 0.900$) are observed between BDE-28, -85, -99, -128, -138, -153, -154, -190 and -197. The congener BDE-100 correlates with this group, but to a slightly lesser extent, generally expressing correlation coefficients of approximately 0.800 and above. The congeners BDE-47 and BDE-66 correlate with this group to a lesser extent still, showing significant correlation with all of the above mentioned congeners, at correlations coefficients of approximately 0.550 and 0.700 respectively. The correlation of this suite of lower congeners suggests that they have all originated from the same source. Given the size and identity of the congeners the source is likely to be the release of Pent- and Octa-BDE commercial mixtures into the environment.

A second group of congeners showing significant levels of correlation included BDE-196, -206, -207, -208 and -209, with particularly high correlations observed between the nona- and deca-BDE congeners. As with the lower congeners, the high levels of correlation between these nona- and deca-BDEs suggests a shared source, in this case likely to be the Deca-BDE commercial mixture.

Of note is the fact that all of the congeners with high concentrations, i.e. with mean concentrations in the otter liver tissue above $5ng.g^{-1}$ lipid wt, correlate with one of theses groups of congeners, suggesting that there are unlikely to be any further sources of PBDE contamination.

Table 5.3 Pearson's correlation matrix for 18 PBDE congeners in otter samples from England and Wales. Highlighted values are significant at p <0.01.

	BDE -28	BDE -47	BDE -66	BDE -85	BDE -99	BDE -100	BDE -128	BDE -138	BDE -153	BDE -154	BDE -183	BDE -190	BDE -196	BDE -197	BDE -206	BDE -207	BDE -208	BDE -209
BDE-28	**1.000**																	
BDE-47	**0.609**	**1.000**																
BDE-66	**0.708**	**0.610**	**1.000**															
BDE-85	**0.911**	**0.558**	**0.656**	**1.000**														
BDE-99	**0.935**	**0.559**	**0.692**	**0.970**	**1.000**													
BDE-100	**0.821**	**0.598**	**0.604**	**0.910**	**0.867**	**1.000**												
BDE-128	**0.995**	**0.550**	**0.715**	**0.916**	**0.941**	**0.802**	**1.000**											
BDE-138	**0.993**	**0.549**	**0.720**	**0.924**	**0.944**	**0.802**	**0.997**	**1.000**										
BDE-153	**0.951**	**0.575**	**0.678**	**0.902**	**0.903**	**0.796**	**0.995**	**0.951**	**1.000**									
BDE-154	**0.989**	**0.559**	**0.703**	**0.950**	**0.961**	**0.839**	**0.993**	**0.994**	**0.959**	**1.000**								
BDE-183	-0.029	-0.071	0.047	-0.053	-0.049	0.003	-0.028	-0.030	-0.053	-0.036	**1.000**							
BDE-190	**0.995**	**0.544**	**0.707**	**0.916**	**0.942**	**0.800**	**0.999**	**0.998**	**0.951**	**0.994**	-0.027	**1.000**						
BDE-196	-0.016	-0.041	0.001	-0.034	-0.024	-0.035	-0.014	-0.018	-0.025	-0.020	0.083	-0.015	**1.000**					
BDE-197	**0.994**	**0.543**	**0.707**	**0.918**	**0.942**	**0.800**	**0.999**	**0.997**	**0.951**	**0.994**	-0.025	**0.999**	0.011	**1.000**				
BDE-206	-0.036	0.199	0.173	-0.050	-0.035	-0.011	-0.043	-0.052	-0.009	-0.055	0.095	-0.052	**0.681**	-0.029	**1.000**			
BDE-207	-0.032	0.081	0.103	-0.036	-0.037	-0.038	-0.034	-0.041	-0.018	-0.040	0.053	-0.040	**0.740**	-0.005	**0.834**	**1.000**		
BDE-208	-0.043	0.177	0.191	-0.054	-0.037	-0.050	-0.049	-0.056	-0.021	-0.056	0.080	-0.050	**0.575**	-0.026	**0.857**	**0.921**	**1.000**	
BDE-209	-0.017	0.029	0.065	-0.029	-0.028	-0.029	-0.020	-0.024	-0.017	-0.027	0.080	-0.023	**0.821**	0.009	**0.796**	**0.960**	**0.833**	**1.000**

5.3.3ii) Principal Component Analysis

The correlation between different BDE congeners was analysed using a principal component analysis (PCA). The PCA works by creating statistical entities, known as principal components, which explain the correlation between groups of congeners. The individual congeners are then assigned a value, known as a loading that evaluates the amount of variation in the concentrations of that congener explained by the principal component. The higher the loading the more accurately the principal component explains the variation in concentrations observed.

The PCA, carried out in this study using the varimax rotation method, produced six principal components with eigenvalues greater than one. The first two of these components, explained the relationships between the congeners most accurately, accounted for 41.6% and 17.6% of the total variance in the data respectively (Figure 5.12). The first principal component (PC1) relates to the correlation between congeners BDE-28, -85, -99, -100, -128, -153, -154, -190 and -197; congeners which are generally associated with the Pent- and Octa-BDE commercial mixtures. The correlation between them suggests that these congeners were released into the environment from a similar source. BDE-47 and BDE-66 express relatively high loadings for PC1, however not as high as the other congeners in this group, suggesting that there are likely to be secondary sources for these two congeners.

The second principal component (PC2) shows evidence of a significant correlation between the concentrations of BDEs with greater numbers of bromine molecules surrounding the central diphenyl ether structure, with BDE-196, -206, -207, -208 and -209 all showing strong loadings for this component (Figure 5.12). In this case the common source is likely to be the Deca-BDE commercial mixture, as this is the only mixture to contain BDE congeners of this size.

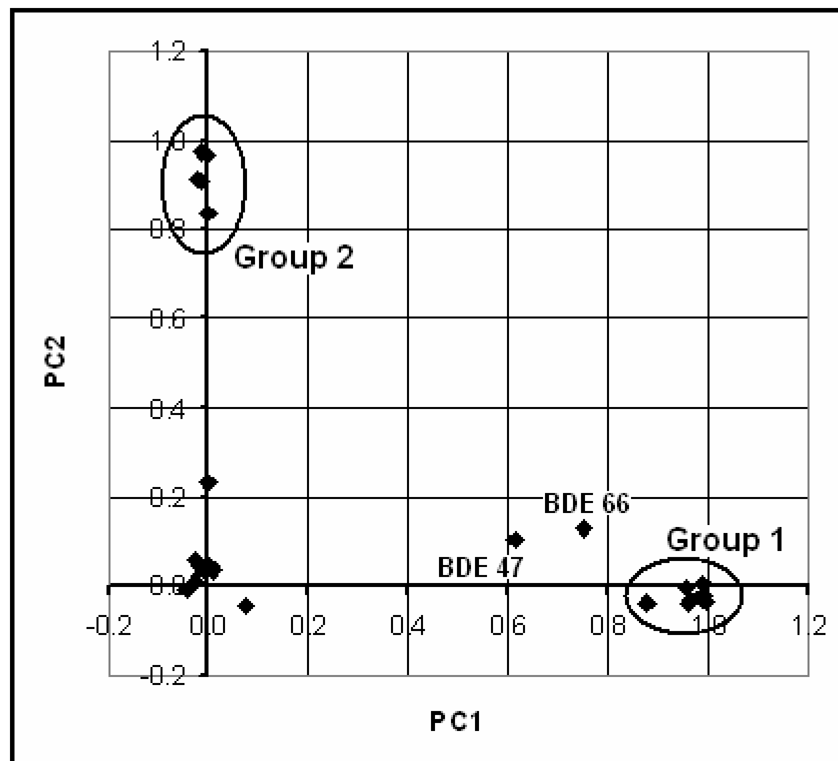


Figure 5.12 Plot of the principal component loadings for BDE congeners concentrations measured in the liver tissue of otters.

5.3.4 Potential Factors Affecting PBDE Congener Uptake

The effects of factors, such as geographical location, age and body condition, on the concentrations found in otter liver tissue were investigated using a multiple regression model in an effort to pinpoint possible causes for the variation in PBDE content observed in otter liver samples. As part of the principal component analysis introduced in section 6.1.2 principal component scores were assigned to individual otter liver samples based on the relative concentrations of the congeners correlating with the principal component in question. The scores for the two most influential principal components, PC1 and PC2, were then analysed for their correlation with other factors relating to the otters, such as the area of the country they were found or the approximate age of the animal, in order to try and find the cause of the variation in PBDE concentration observed.

The first factor included in the regression models was geographical location, for which samples were split into five areas - northeast England (n = 11), West Midlands (n = 4), East Anglia (n = 13), south Wales (n = 36) and southwest England (n = 64). Otters

were grouped in terms of their life stage - as adults, sub-adults/juveniles and cubs - analysed using body weight and signs of sexual maturity as a guide (Simpson, 2007; Chadwick, 2007). The gender of the animal was included, as well as the year of the otters death and whether the animal died as a result of a road traffic accident or from another cause. A final factor included in the model was body condition index (K) (Kruuk & Conroy, 1996), calculated using the formula:

$K = W / a L^n$

Where: W = body weight (kg)

L = total length (m)

a = 5.87 for males and 5.02 for females

n = 2.39 for males and 2.33 for females

The model was first run with all the factors included, the individual factors showing the least significance being removed sequentially until the model and the effects of all factors within it were significant. The model for PC1 scores, corresponding to the group of BDE congeners with a lower number of bromine molecules surrounding the central diphenyl ether structure, did not show correlation with any of the factors entered. Therefore, either the statistical model was not powerful enough to detect an effect, or none of the factors analysed explain the variation in the concentrations of the lower congeners, represented by the PC1 scores. For the regression model of PC2 scores, corresponding to the group of BDE congeners with a lower number of bromine molecules surrounding the central diphenyl ether structure, the only factor of significance was geographical area (t = 2.508, df = 117, p = 0.014) explaining 5.1% of the variation ($R^2 = 0.051$). With both a box and whisper plot of PC2 scores (Figure 5.13) and a plot of mean BDE-209 concentrations (Figure 5.14) showing higher levels in northeast England, the West Midlands and East Anglia, and lower levels in south Wales and southwest England, it would appear that otters in the north and east of England contain, on average, significantly higher concentrations of the higher PBDE congeners than do otters from southwest England and south Wales.

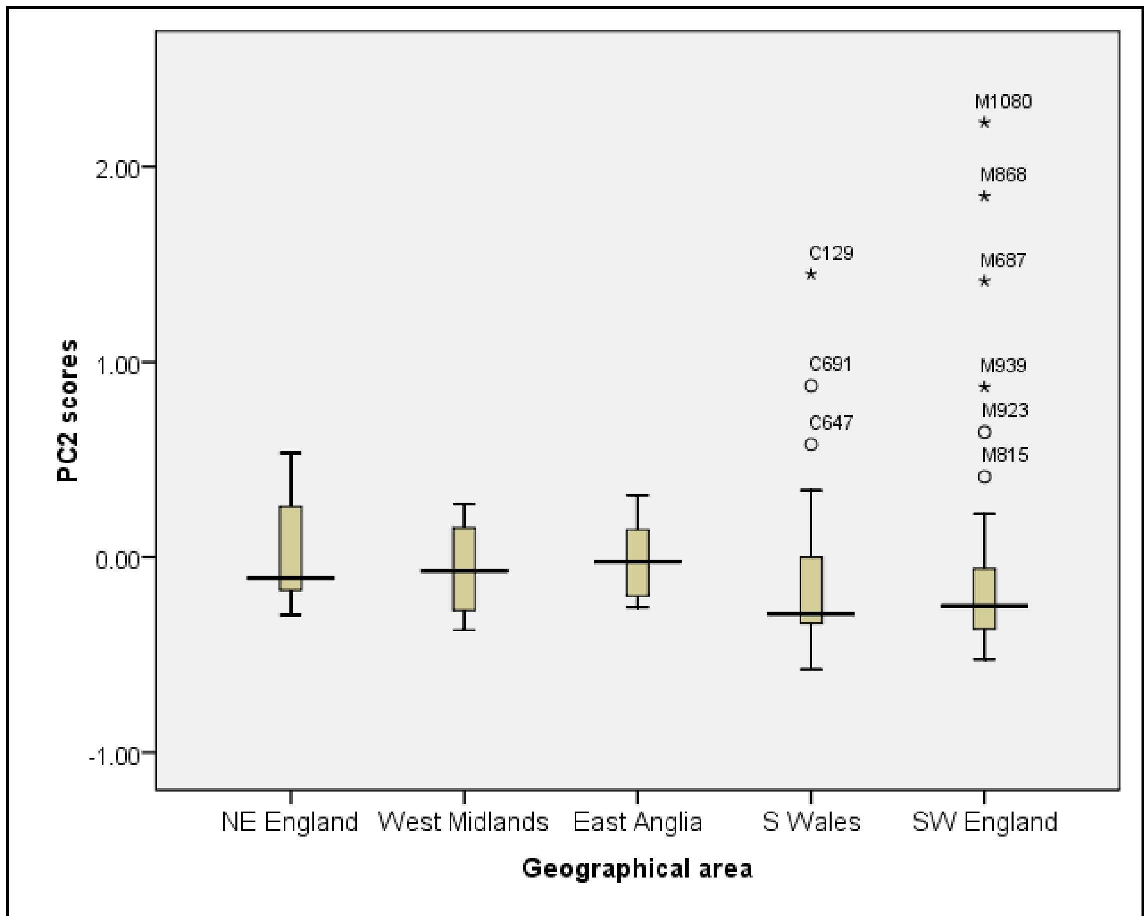


Figure 5.13 Box and whisper plot of PC2 scores for otters collected from different locations in England and Wales.

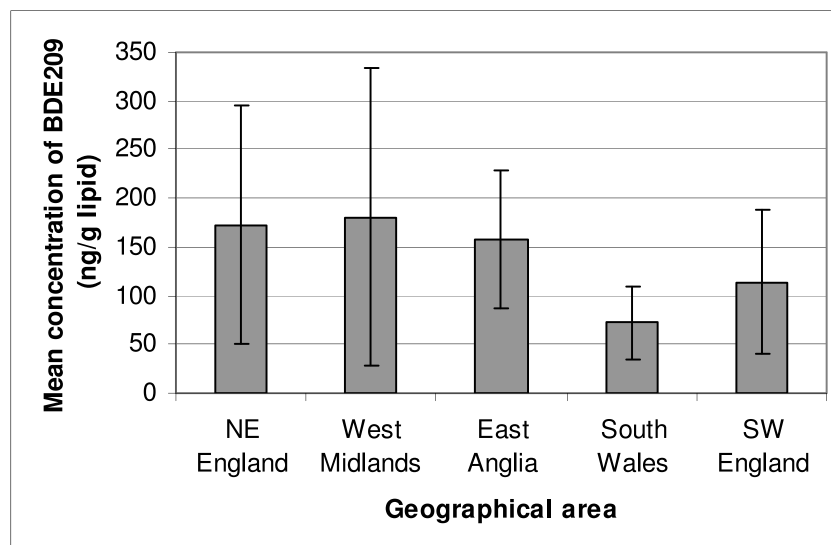


Figure 5.14 Mean concentrations of BDE-209 for otters collected from different locations in England and Wales.

5.4 Comparisons of PBDE concentrations with PCBs and OCs

Polychlorinated biphenyls (PCBs) and DDTs have both been banned from manufacture and use for several decades. However, due to the compounds environmental persistence significant concentrations of several PCB congeners and DDT breakdown products are still present in the environment today (Walker, 2001; Storelli *et al.*, 2005; Braune *et al.*, 2007; Jamshidi *et al.*, 2007). The concentrations of PCBs and OCs observed in the liver tissue of otters are relatively high, but trends generally show a decrease in concentrations over the last few decades (Simpson, 1998, 2007; Chadwick, 2007).

Despite the fact that many of the liver samples used in this study had previously been analysed for PCB and DDT content, samples were re-analysed for experimental rigor to address the question of how body burdens of PBDEs relate to those of PCBs/OC . A subset of otter liver sample extracts, initially analysed for PBDE content, were run through a second analysis to measure PCB, DDT and HCB congener concentrations. This enabled a direct comparison of PBDE, PCB and DDT concentrations without bias from variations in the sample set analysed or the methods of extraction used.

5.4.1 Contaminant Concentrations

A summary of contaminant concentrations measured in the otter liver tissue samples is presented in table 5.4. The mean concentration of ΣBDE (3.2 $\mu g.g^{-1}$ lipid) was approximately a quarter that of ΣPCB (12.9 $\mu g.g^{-1}$ lipid), indicating that the concentrations of PBDEs measured in the liver tissue of otters were comparaple to those of PCBs. The mean concentration of ΣBDE (3.2 $\mu g.g^{-1}$ lipid) was only slightly below that of ΣDDT (3.9 $\mu g.g^{-1}$ lipid), with the maximum ΣBDE concentration (69.9 $\mu g.g^{-1}$ lipid) being significantly greater that the maximum ΣDDT concentration (46.9 $\mu g.g^{-1}$ lipid). This again indicates that PBDEs are a major contaminant in otters, in this case in comparison to DDT burdens. It should be noted that only four DDT breakdown products were analysed, however, suggesting that on an individual congener basis DDT breakdown products are generally more significant contaminants, in terms of the concentrations measured, than individual BDE congeners. A summary of the concentrations of individual congeners is presented in Table 5.5.

Table 5.4 Otter liver tissue contaminant concentration summary data.

	Sample nos.	Nos. of congeners	Mean (ng.g^{-1} lipid wt)	Range (ng.g^{-1} lipid wt)
∑PCB	86	25	12928.2	1.8 – 141761.1
∑DDT	86	4	3859.0	L.D. – 46922.5
∑BDE	129	26	3241.9	12.2 – 69882.5
HCB	86	1	260.5	L.D. – 1557.8

L.D. = below the limit of detection

PCB-138 was by far the most concentrated congener measured in the liver tissue of otters, at a mean concentration of 3.92 µg.g^{-1} lipid and a median concentration of 1.96 µg.g^{-1} lipid. Other significant PCB congeners, in order of diminishing concentration, were PCB-153 > PCB-180 > PCB-170 > PCB-187 > PCB-118, with mean concentrations ranging from 2.75 to 0.55 µg.g^{-1} lipid. Overall the second most concentrated congener found in otters was *pp*-DDE (mean = 3.00 µg.g^{-1} lipid, median = 1.46 µg.g^{-1} lipid), the most environmentally persistent breakdown product of DDT (Storelli *et al.*, 2005; Braune *et al.*, 2007).

Table 5.5 Summary data for the top 16 most concentrated (by mean) congeners measured in the liver tissue of otters from England and Wales (n = 86).

Congener [a]	Concentration in µg.g^{-1} lipid [b]		
	Mean	Median	Range [c]
PCB-138	3.922	1.955	37.982
pp-DDE	2.990	1.455	33.650
PCB-153	2.753	1.409	37.043
BDE-47	2.591	1.161	34.611
PCB-180	1.782	0.673	24.098
PCB-170	1.301	0.534	13.876
PCB-187	1.043	0.587	10.353
PCB-118	0.548	0.299	9.413
BDE-153	0.526	0.090	23.317
pp-DDD	0.494	0.229	11.049
PCB-194	0.364	0.117	5.258
PCB-203	0.334	0.158	4.359
HCB	0.262	0.199	1.558
PCB-156	0.252	0.117	4.516
PCB-99	0.201	0.116	2.308
BDE-209	0.199	0.064	6.808

[a] in descending order of mean concentration
[b] 1 µg.g^{-1} lipid being equal to 1000 ng.g^{-1} lipid
[c] from below limit of detection to the upper limit shown

Several BDE congeners were observed in the liver tissue of otters at concentrations similar to some of the high concentrations observed for PCB and DDT congeners. The most concentrated of these was BDE-47, found at a mean concentration of 2.60 $\mu g.g^{-1}$ lipid and a median concentration of 1.16 $\mu g.g^{-1}$ lipid. In terms of the mean and median concentrations observed BDE-47 was the forth most concentrated congener analysed in this study, suggesting that BDE-47 was a significant contaminant in the otters analysed.

The second most concentrated PBDE congener, BDE-153, was also positioned among the top most concentrated congeners observed in the otter liver tissue. The mean concentration of BDE-153 (0.53 $\mu g.g^{-1}$ lipid) was almost as high as the mean concentration observed for the 6th most concentrated PCB congener, PCB-118 (0.55 $\mu g.g^{-1}$ lipid). The median concentration of BDE-153 (0.09 $\mu g.g^{-1}$ lipid) was low in comparison to the medians of many of the top PCB and DDT congeners, however the maximum concentration of BDE-153 (23.32 $\mu g.g^{-1}$ lipid) was relatively high in comparison to the maximum concentrations observed for many of the other congeners analysed (see Table 5.5). This indicates that the concentrations observed for PCB and DDT congeners were generally more evenly spread throughout the range of concentrations. In comparison, the majority of samples expressed a BDE-153 concentration closer to the lower end of the range observed for this congener

Concentrations of the third most concentrated BDE congener, BDE-209, were significant when considered in comparison to concentrations of PCB and DDT congeners. With a mean of 0.20 $\mu g.g^{-1}$ lipid, median of 0.06 $\mu g.g^{-1}$ lipid and range of up to 6.81 $\mu g.g^{-1}$ lipid BDE-209 concentrations were only an order of magnitude (approx. x20 based on means, approx. x30 based on medians) lower than the most concentrated PCB congener (PCB-138, mean 3.92 $\mu g.g^{-1}$ lipid, median 1.96 $\mu g.g^{-1}$ lipid).

5.4.2 Congener Correlations

A principal component analysis (PCA) was carried out to analyse for correlations between concentrations of PBDE, PCB, DDT and HBC congeners. The top five principal components (PCs) extracted by the PCA, together explaining 67.3% of the variance in the data, grouped the most concentrated congeners from each of the compound groups (Table 5.6). The results indicate that there is little correlation between concentrations of congeners from different compound groups, but high levels of correlation between groups of congeners from the same compound group.

It is generally considered that PCs with eigenvalues greater than 1.0 are significant in terms of the identification of correlations in the data (Field, 2005). However, for this analysis the first five PCs, all with eigenvalues greater than 2.5, explained the majority of variation in the data and formed the most comprehensive groups of correlated congeners. The remaining PCs with eigenvalues between 1.0 and 2.5 generally showed high loadings for just one or two of the congeners, these congeners generally being ones which expressed relatively low concentrations in the otter liver tissue. Therefore, it was the first five PCs that were considered of most relevance in terms of explaining the variation in the congener concentrations observed (see Tables 5.5 and 5.6).

Table 5.6 Summary data, including lists of the contaminants expressing high loadings (>0.50), for the five most relevant principal components by the PCA.

PC [a]	Eigenvalues	% of variance explained	Cumulative %	Corresponding congeners (those with values over 0.5 for the corresponding principal component)
1	14.778	26.869	26.869	PCB-87 [b], -99, -105, -118, -138, -153, -156, -157, -158, -167, -170, -180, -183, -187, -189, -194, -203, HCB
2	10.484	19.062	45.931	BDE-28, -47, -66, -85, -99, -100, -128, -138, -153, -154, -190, -197
3	5.006	9.102	55.033	BDE-196, -206, -207, -208, -209, PCB-151
4	4.217	7.667	62.700	PCB-28, -49, -52, -74, -110
5	2.553	4.642	67.342	*pp*-DDD, *pp*-DDE, *pp*-DDT, BDE-35

[a] Principal component
[b] not exclusively assigned to this principal component

The first PC extracted by the PCA explained 26.9% of the overall variation in the data by accounting for the correlation between the main group of PCB congeners measured in the otter liver tissue samples. All of the most highly concentrated PCB congeners (see Table 5.5) were assigned to this group, along with HCB (the only organochlorine pesticide included in the analysis besides DDT).

PCs 2 and 3 explained the variance in BDE congener concentrations and generally mirrored the groupings observed for the PBDE based PCA carried out in section *5.3.3ii, Principal Component Analysis*. The group of lower BDE congeners, which correlated via PC1 in the PCA carried out for PBDEs alone, were correlated via PC2 in this second PCA analysis carried out using concentrations from all contaminants analysed. This group consisted of the tri- to octa-BDE congeners, with all of the most concentrated congeners, including BDE-47, in this size range showing high loadings for this principal component (PC2). In this second PCA 19.1% of the total variation in the data was explained by this second principal component (PC2). PC3 described the correlation between congeners BDE-196, -206, -207, 208 and -209, explaining 9.1% of the total variation in the contaminant concentration dataset. PCB-151 showed a relatively high loading of 0.592 for PC3. However, concentrations of this congener were low (mean = 0.18 (SD 1.148) $ng.g^{-1}$ lipid), with few of the samples expressing concentrations above the limit of detection, suggesting that the correlation of this congener may have been due to the fact that the few samples with measurable PCB-151 concentrations by chance were the same samples which express high nona- and deca-BDE concentrations.

Accounting for a further 7.7% of the variance in the contaminant data, PC4 explains the correlation between five further PCB congeners. Of note is the fact that all of these congeners were observed in the otter liver samples at relatively low concentrations, ranging from a mean of 1.04 (SD 3.79) $ng.g^{-1}$ lipid for PCB-28 to 10.81 (SD 12.63) $ng.g^{-1}$ lipid for PCB-74. In comparison, the PCB congeners corresponding to PC1 ranged from a mean concentration of 19.89 (SD 51.05) $ng.g^{-1}$ lipid for PCB-158 to a mean of 3534.37 (SD 4893.72, n = 83) $ng.g^{-1}$ lipid for PCB-138.

The final principal component to signify correlations in the concentrations of a significant proportion of the congeners was PC5, accounting for 4.6% of the variation in the data. PC5 explained the correlation between concentrations of the DDT breakdown products, with *pp*-DDD, *pp*-DDE and *pp*-DDT all expressing high loadings for this

component. Interestingly BDE-35 also showed a high loading for PC5, signifying correlation of this congener with concentrations of DDT products, although concentrations of BDE-35 were generally low (mean 1.01 $ng.g^{-1}$ lipid, range <0.1 - 30.0 $ng.g^{-1}$ lipid), suggesting that this correlation with PC5 may have occurred by chance.

In summary, the PCA extracted five principal components which between them explained the majority of the variation in congener concentrations, splitting the congeners into the five principal component groups according to relative concentrations in the different otters. Two of the principal components correlated PCB congeners. PC1 consisting of a large group of PCB congeners, included all of those at the highest concentrations. The second group of correlated PCB congeners, represented by PC4 and numbering only five, were generally far less concentrated in the otter tissue than those congeners correlated via PC1. A further two principal components explained the correlations of PBDE congeners in the otter liver tissue; PC2 representing the correlation of the most concentrated tri- to octa-BDEs and PC3 representing the correlation of the higher nona- and deca-BDE congeners. The final principal component considered to be significant given its eigenvalue (2.55), the number of congeners correlated (4) and the percentage of the total variance explained by the component (4.64%) was PC5, which correlated the breakdown products of DDT. PCB-151 and BDE-35 were the only congeners not to fit the general pattern of principal component assignment, assigning to PC3 and PC5 respectively. Both of these congeners were found at relatively low concentrations it the otter liver tissue with relatively high instances of non-detects, suggesting that the respective correlations of these two congeners may have occurred by chance rather than due to true correlations.

5.5 PBDE Project Discussion

5.5.1 PBDE Concentrations in Otters from England and Wales

The ∑BDE concentrations measured in the liver tissue of otters (mean = 3241.9 $ng.g^{-1}$ lipid, median = 1564.5 $ng.g^{-1}$ lipid) were similar in magnitude to those observed in the blubber and liver tissues of many marine mammal species (She *et al.*, 2002; Law *et al.*, 2002; Kannan *et al.*, 2007). The highest ∑BDE concentration measured in otter liver tissue samples, 69,882.5 $ng.g^{-1}$ lipid, was almost three times the upper limits seen in some of the most contaminated marine mammal species studied. For example, in Californian sea lion (*Zalophus californianus*, n = 26) concentrations ranged from 570 to 24,240 $ng.g^{-1}$ lipid wt (Stapleton *et al.*, 2006), and in southern sea otters (*Enhydra lutris nereis*, n = 80) concentrations ranged from 10 to 26,800 $ng.g^{-1}$ lipid wt (Kannan *et al.*, 2007).

In summary, the ∑BDE concentrations observed in otters were within the range seen for other aquatic/semi aquatic species. In particular, the concentrations found in the otters were similar to concentrations observed for many marine mammal species, suggesting that otters are as susceptible to PBDE uptake as other aquatic top predator species are.

5.5.2 PBDE Congener Uptake and Storage

5.5.2i) Lower Congeners

Correlations of PBDE congeners in otters showed two main groups of congeners suggesting two different sources in the environment. The first correlated congener group consists of the more prominent and concentrated lower congeners: BDE-28, -85, -99, -100, -128, -138, -153, -154, -190 and -197. The fact that these congeners correlate so similarly with each other suggests that they are all taken up and treated similarly from one otter to another. In other words the congener profile of these congeners will remain similar from one otter to the next even if there are large variations in overall BDE uptake. This implies that the rules and factors that govern the intake, metabolism and excretion of these congeners in otters do not change, only the availability of the congeners themselves and/ or the overall uptake of BDEs.

The congeners observed in this first correlated group of BDE congeners are all found in the Penta- and Octa-BDE commercial mixtures. The correlation of these tri- to octa-BDE congeners suggests that these congeners have all originated from a similar source, likely to be the release of Penta- and Octa-BDE mixtures into the environment. BDE-47 only partially correlated with this group of lower congeners, suggesting that there is a second source for this particular congener. The comparatively high concentrations observed would suggest that the second source of BDE-47 contamination plays a significant part in the overall PBDE contamination burden of otters.

5.5.2ii) Higher Congeners

The second group of congeners to correlate were the nona-BDEs (BDE-206, 207 and -208) and BDE-209. These congeners were likely to have originated from the deca-BDE industrial mixture, the smaller congeners resulting from debromination of BDE-209, either through metabolism in the otter or through some other breakdown mechanism in the environment or the laboratory. Nona-BDE congeners make up just 0.3 – 3% of the decaBDE industrial mixture, whereas in this study they represent 31.5% of the total nona- and deca concentration, proving that there must be some reason for this disparity in relative concentrations. BDE-196 was also significantly correlated with this group, suggesting that this congener may result from the breakdown of BDE-209. However, BDE-196 was only observed at low concentrations, measurable at concentrations over the detection limit in just three otters, suggesting that it is not a major factor in the contaminant burden of otters. The main reason for the correlation between BDE-196 and BDE-209 was probably the fact that the sample with the highest concentration of BDE-196 was also the sample with the highest concentration of BDE-209.

A range of BDE congeners were observed in the otter liver tissue samples. BDE-47 was by far the most concentrated BDE congener measured in the otter liver tissue, however, it was not the only BDE congener present in concentrations which would be considered significant in comparison to concentrations observed in other studies. Within the top six most concentrated congeners observed in otter liver tissue were congeners from both congener correlation groups, with BDE-209 being the third most concentrated congener and BDE-207 being, on average, the fifth most concentrated congener observed in otters. The fact that BDE-209 and BDE-207 are within this group

of highly concentrated congeners suggests that Deca-BDE is a major contaminant of otters.

5.5.2iii) Aquatic Environmental Influences

The congener profile of the otter shows distinct similarities with those of marine mammals, with the congener BDE-47 as the most dominant congener and relatively high concentrations of the congeners BDE-99, -100, -153 and -154 (Boon *et al.*, 2002, Law *et al.*, 2002; Kajiwara *et al.*, 2006; Stapleton *et al.*, 2006), although other congeners, such as BDE-128 and -138 are found at higher relative concentrations in otters. The congener profile of fish has important implications for many aquatic predators, including otters, for whom fish are the main food source (Clavero *et al.*, 2003; Sales-Luís *et al.*, 2007). As many fish species exhibit BDE-47 as the dominant congener (Boon *et al.*, 2002; Ikonomou *et al.*, 2002; Covaci *et al.*, 2004; Schlabach *et al.*, 2004), we would expect that this would also be the dominant congener in otters. We would also expect congeners BDE-99, -100, -153 and -154 to be found at relatively high concentrations, as they are in species of fish (Ikonomou *et al.*, 2002; Covaci *et al.*, 2004; Schlabach *et al.*, 2004). Indeed, the congener patterns observed in the otter livers fit with this, with BDE-47, -99, -100 and -153 amongst the most concentrated congners measured.

The lower BDE congeners are lighter in mass and so are generally more mobile, travelling greater distances in air and water transport systems and therefore becoming widely distributed throughout the environment (de Wit *et al.*, 2006). As a result, lower congeners tend to be found at much higher concentrations than higher congeners in marine environments and in the water columns of freshwater environments. This higher availability of these lower congeners means that they tend to build up to higher concentrations in aquatic species such as aquatic invertebrates and fish (Boon *et al.*, 2002; Ikonomou *et al.*, 2002; Covaci *et al.*, 2004), which not only absorb contminants via their diet but also via the gills or skin. It is not clear however, how the different uptake pathways and metabolism play a part in the congener concentration patterns observed in these species.

In biota from UK environments, or locations such as the North Sea that are significantly influenced by the industrial activities of the UK (Boon *et al.*, 2002), congener patterns tend to follow the trend most commonly observed in biota from across the globe. BDE-47 is often the most dominant congener, followed by BDEs-99 and -100 and then BDE-153 and -154, consistent with a pattern of contamination from the pentaBDE mixture. In a study of cormorants (*Phalocrocorax carbo*, n = 47) and harbour porpoise (*Phocoena phocoena*, n = 60) sampled from around England and Wales, BDE-47 was the most dominant congener observed, ranging from 39 – 88% of BDE content in porpoise and from 24 – 100% in cormorants (Law *et al.*, 2002). In a separate study of harbour porpoise in the North Sea congeners BDE-99,-100 and -154 were present at concentrations approximately half to one third those of BDE-47 (Figure 5.15; Boon *et al.* 2002). In harbour seal (*Phoca vitulina*) sampled from the North Sea BDE-47 was the dominant congener, with BDE-99 and -153 also making up significant proportions of the total (Figure 5.16; Boon *et al.* 2002).

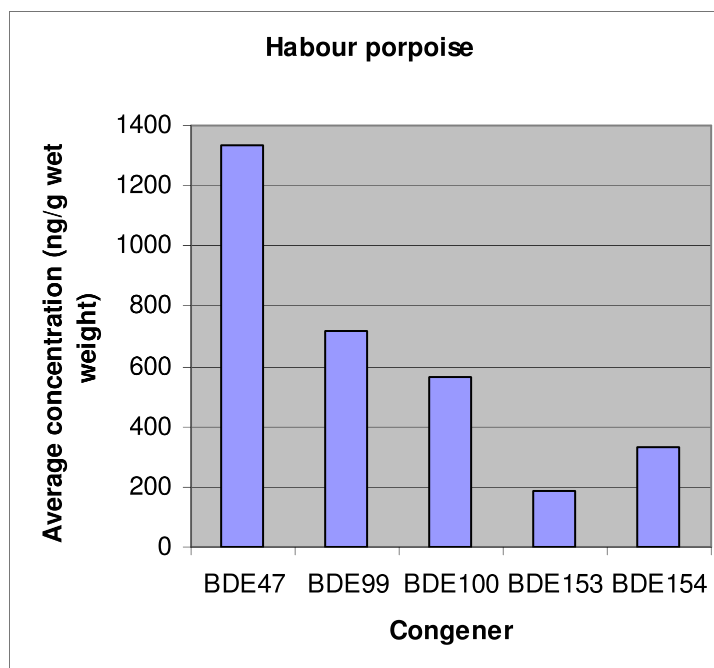


Figure 5.15 Relative concentrations of the five most prominent congeners measured in harbour porpoise from the North Sea, drawn using data from Boon *et al.* (2002)

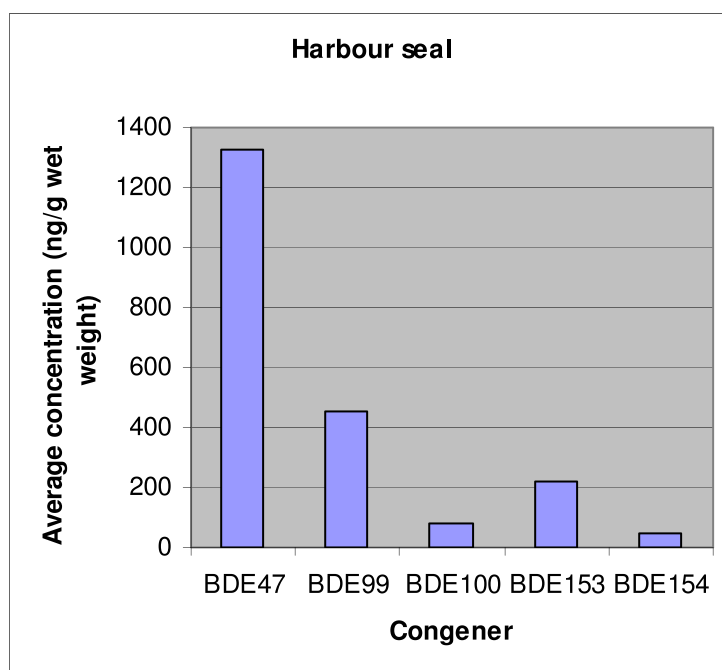


Figure 5.16 Relative concentrations of the five most prominent congeners measured in harbour seal from the North Sea, drawn using data from Boon *et al.* (2002)

A comparison of the ΣBDE concentrations observed in otters to concentrations found in a range of marine species (Figure 5.17) showed that the otters analysed in this study showed similar, but generally higher concentrations to those found in many marine mammal species. The proportions of BDE-47 to ΣBDE in the different species indicate that for the majority of marine mammal species, like the otter, BDE-47 is the major BDE congener present, constituting a significant proportion of the ΣBDE concentration (Figure 5.17).

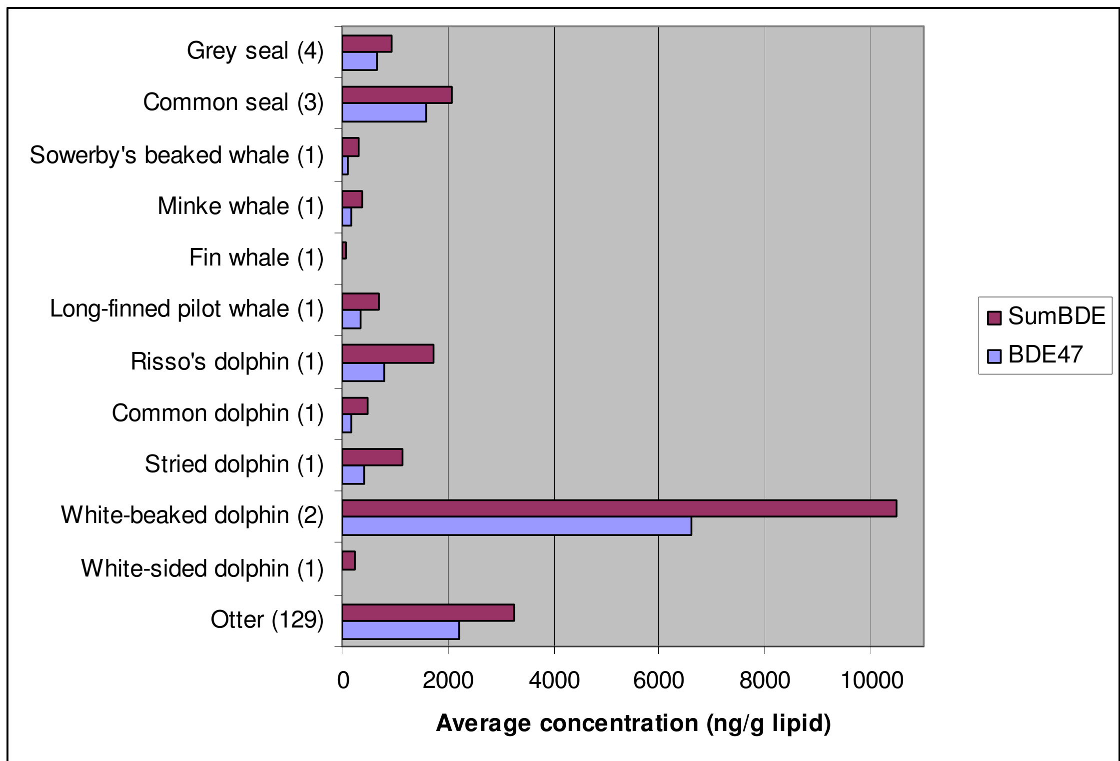


Figure 5.17 Concentrations of ∑BDE and BDE-47 in marine mammals and otters from England and Wales. Sample numbers given in parenthesis. Concentrations calculated using data from Law *et al.* (2002) (data originally obtained from CEFAS, unpublished data)

A look at concentrations of some other lower congeners reveals that otters contain similar concentrations of BDE-99 and BDE-100 to many marine mammal species, although generally the relative concentrations of these congeners are more variable between species (Figure 5.18). In comparison, concentrations of BDE-153 are significantly higher in otters, being almost four times higher than the concentrations observed in any of the other marine mammal species presented here (Figure 5.18).

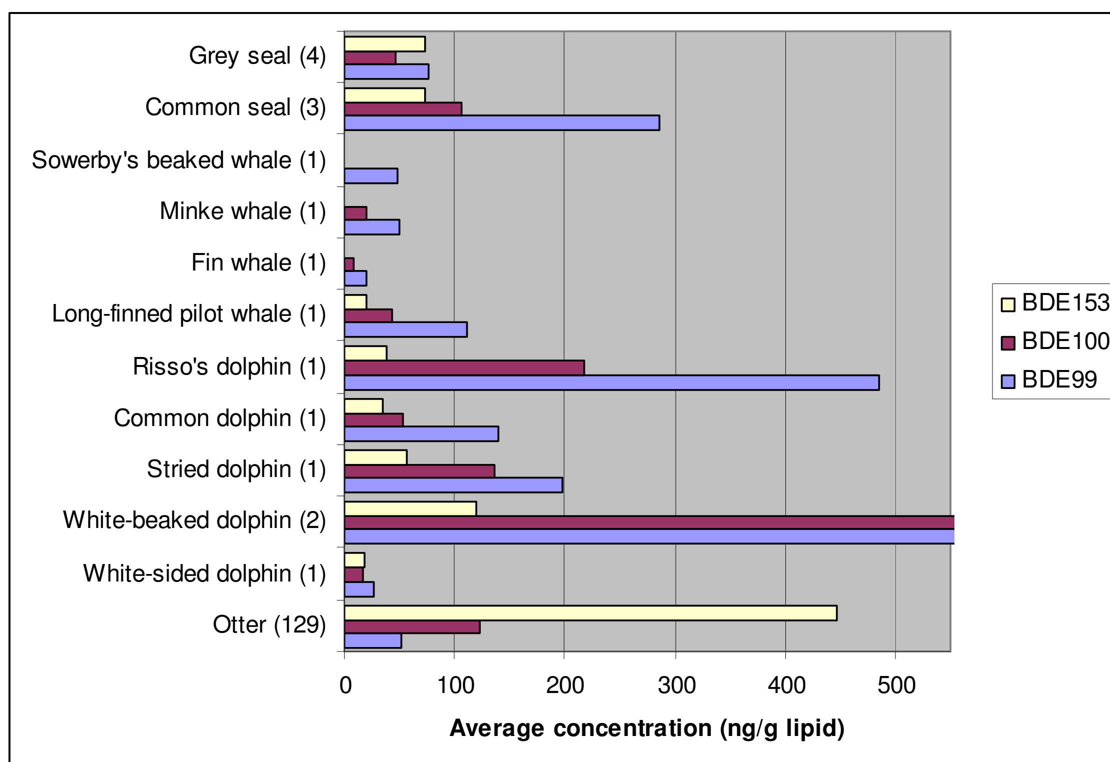


Figure 5.18 Concentrations of BDE-99, 100 and -153 in marine mammals and otters from England and Wales. Concentrations for White beaked dolphin, BDE-99 = 1676 ng.g^{-1} lipid, BDE-100 = 1890 ng.g^{-1} lipid. Sample numbers given in parenthesis. Concentrations calculated using data from Law *et al.* (2002) (data originally obtained from CEFAS, unpublished data)

In summary, lower congeners tend to be more prevalent in aquatic environments due to the greater mobility of these lighter mass compounds (de Wit *et al.*, 2006). The prevalence of BDE congeners in the environment generally reflects the concentrations of the various congeners in the commercial mixtures released. The concentrations of BDE congeners found will also be influences by the extent of leaching of these commercial mixtures into the local environment (Boon *et al.*, 2002). However, there may be other mechanisms, such as preferential uptake/excretion or metabolism, resulting in variations in the patterns of BDE congener concentrations observed in different species (Covaci *et al.*, 2004; Schlabach *et al.*, 2004). Concentrations in marine mammals generally follow similar patterns, with BDE-47 dominating the profile, followed by significant concentrations of BDE-99, -100 and -153. The high concentrations of lower congeners, in particular of BDE-47, observed in otter liver tissue would suggest that the otters mostly aquatic based diet has a significant effect on the PBDE congener uptake of the species.

5.5.2iv) Terrestrial Environmental Influences

As with many terrestrial bird species (Sellström *et al.*, 2001; Jaspers *et al.*, 2006), BDE-153 concentrations are comparatively high in otters, suggesting that the prevalence of BDE-153 may be linked to the terrestrial environment. However, some of the most striking similarities are those with studies of terrestrial mammals. Racoon dogs, foxes and Grizzly bears all contain relatively high concentrations of BDE-209 (Table 5.7), suggesting that this congener is readily bioavailable to these top mammalian, terrestrial predators (Christensen *et al.*, 2005; Voorspoels *et al.*, 2006; Kunisue *et al.*, 2008). Concentrations of BDE-209 in otter were high in comparison to those found in other terrestrial mammals, but low in comparison the concentrations of the congener BDE-47, suggesting influences from both the freshwater and the terrestrial environments.

Table 5.7 Concentrations of BDE-209 in terrestrial mammal species.

Species	Median	Range	Reference
European otter, *Lutra lutra*	62.2 $ng.g^{-1}$ lipid wt	<8.5 – 6808 $ng.g^{-1}$ lipid wt	This study
Red fox, *Vulpes vulpes*	<0.9 $ng.g^{-1}$ lipid wt	<9.1 – 760 $ng.g^{-1}$ lipid wt	Voorspoels *et al.* (2006)
Racoon dog, *Nyctereutes procyonoides*	6.7, 19 and 20 $ng.g^{-1}$ lipid wt *	<0.1 - 160 $ng.g^{-1}$ lipid wt	Kunisue *et al.* (2008)

* medians for the three geographical regions studied

The high concentration of BDE-153 observed in otters, combined with the correlation of this congener with other prevalent tri- to octa-BDE congeners was a particularly notable finding. In a study of polar bear (*Ursus maritimus*), another species which is principally terrestrial, but relies on a largely aquatic based diet, BDE-153 was found to be the most concentrated and persistent BDE congener studied (Jenssen *et al.*, 2004). Higher proportions of BDE-153 were present in the congener profile of the polar bear than in their marine prey (Jenssen *et al.*, 2004), suggesting that there may be, as yet unknown, reasons for the retention of this congener over others in terrestrial species.

The correlation of BDE-153 with concentrations of other BDE congeners in otters would suggest that BDE-153 originate from the same industrial source as these other lower congeners. The relatively high concentrations of BDE-153 in comparison to

other congeners, i.e. its position as the second most concentrated congener in the BDE congener profile of otters, suggests that, in common with many terrestrial top predator species (Mariussen et al., 2008; Voorspoels *et al.*, 2006; Kinisue *et al.*, 2008), otters have a tendency to retain the BDE-153 congener to a greater extent than many of their prey species.

Otters showed relatively high concentrations of the higher congeners, with BDE-209 and BDE-207 in particular making up significant proportions of the congener profile. In the few studies which have looked at concentrations of PBDEs in terrestrial top predators BDE-209 is often one of the most prominent congeners. BDE-209 dominated the profile of racoon dogs (*Nyctereutes procyonoides*, n = 39) from Japan (Figure 5.19; Kunisue *et al.*, 2008), and in foxes (*Vulpes vulpes*, n = 33) from Belgium Voorspoels *et al.* (2006) found that in the 40% of animals which contained measurable concentrations of BDE-209 the congener made up approximately 70% of the congener profile.

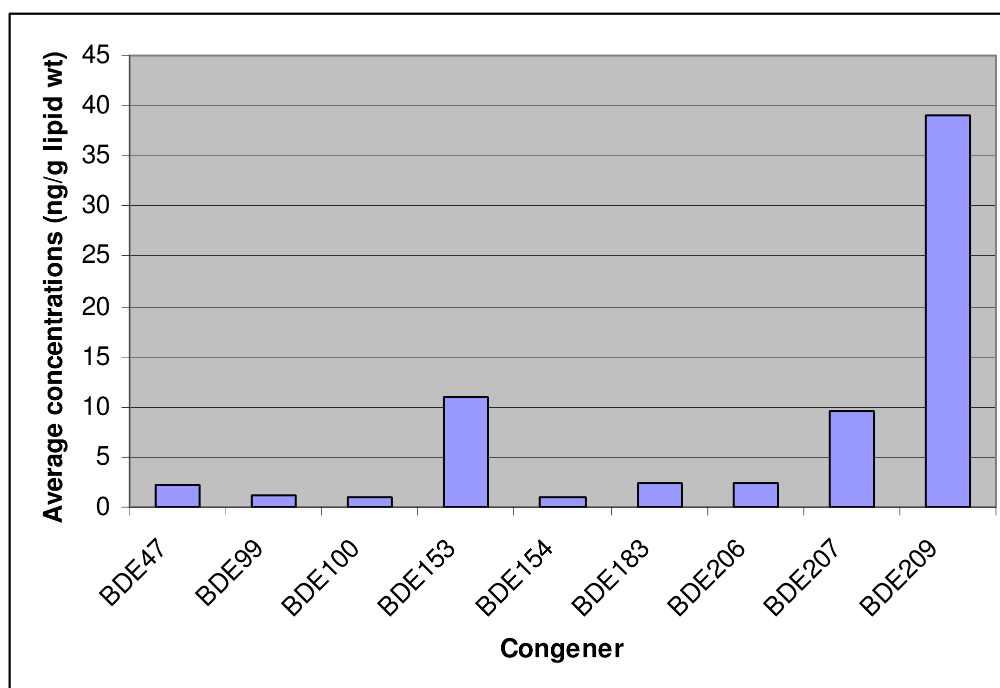


Figure 5.19 Congener profile of most prominent congeners found in racoon dogs (n=39) from Kanagawa, Japan, drawn using data from Kunisue *et al.* (2008)

Terrestrial mammals are likely to be more exposed to the uptake of larger congeners, such as BDE-209, that are less mobile and less water soluble and therefore tend to build

up in soils and sediments. Voorspoels *et al.* (2006) suggested that high concentrations of BDE-209 in foxes may be the result of ingestion via the contamination of prey with soil and other particulate matter. Otters do not tend to take a large amount of terrestrial prey, however due to their semi-aquatic lifestyle the otters themselves are likely to be exposed to large volumes of sediment particles from the aquatic systems as well as soil particles from the time they spend ashore. An otter's lifestyle requires for them to groom regularly in order that their coat remains waterproof and warm, therefore increasing the amount of particulate matter they are likely to ingest from their own coats. The congener concentration data would suggest that lower congeners, that are found to correlate well with each other, are most likely to be taken up by otters through their diet. The relatively high concentrations of BDE-209, the absence of this congener from many prey species and its lack of correlation with the lower congeners suggests that this congener is likely to be taken up through more direct environmental exposure.

Thomas *et al.* (2005) found that during the experimental exposures of three captive grey seals (*Halichoerus grypus*) a high proportion of BDE-209 (57-97%) was retained in the body. Before exposures took place BDE-209 was not observed in any of the seals. The seals were exposed to BDE-209 through their diet for a period of a month. BDE-209 concentrations in blood and blubber samples rose significantly during the exposure period and continued to rise for several days after the last exposure before dropping again steadily. The half life of BDE-209 in the blood of seals was calculated to be 8.5 to 13 days. At 29 days after exposure blubber concentrations ranged from 3.4 to 7.4 $ng.g^{-1}$ lipid wt, representing, when taken on a whole body basis, an estimated 11 to 15% of the cumulative amount ingested (~320 µg per seal) still present in the body one month after exposure (Thomas *et al.*, 2005).

Given the concentrations of BDE-209 in otters and the short half-life of the congener in mammals, it would suggest that otters are exposed continually to high concentrations of BDE-209. Alternatively, certain species, including otter, may be more pre-disposed to absorbing and/or retaining BDE-209. Without extensive study into levels of uptake, storage, metabolism and excretion of this congener in otters themselves, or studies to better understand the overall mechanisms behind variations in these factors in mammals, it is not possible to know for certain which factors are specifically leading to the relatively high concentrations of BDE-209 observed in some species but not others.

5.5.3 Variation in PBDE Concentrations between Otters

A large amount of variation was observed in the PBDE congener concentrations in the different otter liver tissue samples, raising questions over the potential causes of this variation. After a principal component analysis was carried out to determine the correlation patterns of the BDE congener concentrations in otter livers each sample was assigned a score for the two main BDE congener correlation groups, PC1 and PC2 (principal components 1 and 2). The principal component scores of the liver samples were assigned based on the relative concentrations of the BDE congeners which correspond to that principal component. For example, a liver sample containing relatively high concentrations of deca- and nona-BDEs would be assigned a high PC2 score as this principal component corresponds to the correlations of these higher congeners.

Statistical models were used to look for correlations between the principal component scores of liver samples and potential variables relating to the otter sampled which may have influenced the congener concentrations observed. The only factor found to have a significant effect on PBDE concentration in otter liver samples was geographical area, for which a geographical split was observed in the relative concentrations of the higher BDE congeners (correlating with PC2).

5.5.3i) Effects of Geographical Location

Geographical location was the only factor extracted by the statistical model to show a significant relationship with BDE congener concentrations. No relationship was observed for PC1 scores, suggesting that there was no significant geographical split in lower BDE congener concentrations in otters from different locations in England and Wales. However, the results show that otters from the north and east of England and Wales contained, on average, higher concentrations of the higher BDE congeners (i.e. the congeners which corresponded to PC2) than otters from south Wales and southwest England. This suggests either lower exposure to the Deca-BDE commercial mixture in the south west of England and south Wales, or that otters in these regions bioaccumulate these congeners to a lesser extent than do otters in more northerly and easterly regions. Without more in depth analysis of environmental PBDE levels it is difficult to ascertain whether it was increased exposure or increased susceptibility that caused this pattern of

concentrations across different geographical ranges. However, several studies have linked geographical variation in PBDE concentrations back to industrial or residential sources, suggesting that the more likely explanation for the higher concentrations of nona- and deca-BDE congeners in otters from further north and east is a higher level of release of the Deca-BDE commercial mixture across this area of the country.

This is not the first study to recognise that PBDE concentrations can vary significantly according to the geographical region of collection (table 5.8; Boon , 2002). In a study of fish (*Barbus* sp.) from five Portuguese rivers a significant difference was observed between average BDE-47 liver concentrations observed in fish from the rivers Douro (16.8 $ng.g^{-1}$ dw) and Mondego (29.54 $ng.g^{-1}$ dw) in the north of the country, compared to concentrations observed in fish from the rivers Tejo (1.58 $ng.g^{-1}$ dw), Sado (6.68 $ng.g^{-1}$ dw) and Guadinana (4.33 $ng.g^{-1}$ dw) in the south (Gama *et al.*, 2006). Gama *et al.* (2006) argued that higher concentrations in the north of the country were likely to be a reflection of higher human population density and greater levels of industrial activity compared to the south.

Table 5.8 Concentrations ($ng.g^{-1}$ dw) of PBDEs in fillets of bream from locations in the Netherlands (de Boer *et al.*, 2003).

Location	No.	BDE-47	BDE-99	BDE-153
Amsterdam	(5)	19	0.2	0.9
Eijsden (Meuse)	(12)	110	<0.1	2.9
Haringvliet	(1)	80	<0.5	<3.2
Lobith (Rhine)	(3)	90	<0.2	<2.5
Bergumermeer, Bergum	(13)	1.9	<0.04	0.1
Vrouwenzand (Ijssel lake)	(10)	22	<0.8	<1.6
Apeldoorns Canal (Epe)	(22)	15	<0.09	0.4
Dommel, near STP Eindhoven	(18)	130	<0.6	4.1

Covaci *et al.* (2004) studied PBDE concentrations in several species across a variety of locations in Belgium. For zebra mussels (*Dreissena polymorpha*) concentrations varied from 0.15 (SD 0.04) $ng.g^{-1}$ ww (n = 3) in one location to 1.84 (SD 0.20) $ng.g^{-1}$ ww (n = 4) in another. In fish species concentrations varied from 1.98 (SD 1.08) $ng.g^{-1}$ ww (n = 11) to 14.0 (SD 14.1) $ng.g^{-1}$ ww (n = 10) in juvenile eel (*Anguilla abguilla*), <0.10 $ng.g^{-1}$ ww (n = 10) to 6.0 (SD 3.26) $ng.g^{-1}$ ww (n = 6) in carp (*Cyprinus carpio*), and 0.62 (SD 0.31) $ng.g^{-1}$ ww (n = 8) to 3.75 (SD 0.68) $ng.g^{-1}$ ww (n = 4) in gibel carp (*Cyprinus*

auratus gibel carpio). Unfortunately, fish species were often not sampled from corresponding locations and so between species comparisons were not possible. However, the results for individual species do suggest high levels of variation between sampling site in terms of the ∑BDE concentrations observed (Covaci *et al.*, 2004).

Clear instances of contamination from point sources were observed in a study of biota from coastal British Columbia clear (Ikonomou *et al.*, 2002). Five Dungeness crab (*Cancer magister*) sampled from sites close to areas of industrial activity (principally pulp and paper mills) contained concentrations ranging from 200 to 480 $ng.g^{-1}$ lipid, whereas the one crab sampled from a reference site away from the sites of industry contained just 4.2 $ng.g^{-1}$ lipid, representing an almost 80-fold difference between the industrial and reference sites. In the same study eleven English sole (*Pleuronectes vetulus*) sampled from relatively uncontaminated sites each contained less than 150 $ng.g^{-1}$ lipid. In contrast the two English sole sampled from sites close to a large industrial port contained 280 and 340 $ng.g^{-1}$ lipid respectively. One harbour porpoise (*Phocoena phocoena*) sampled from a site close to a sewage outlet contained three times the BDE concentration of any of the other four porpoise sampled in the study (Ikonomou *et al.*, 2002)

In racoon dogs (*Nyctereutes procyonides*, n = 39) from Japan there was significant variation with location, with animals from the area of Kanagawa containing significantly ($p < 0.05$) higher concentrations for the congeners BDE-47, -153, -154, -183, -197, -206 and -207 (Kunisue *et al.*, 2008). These results suggest therefore a higher usage of PBDE industrial mixtures in this region than in the other two regions studied. However, concentrations of BDE-209 and ∑BDE did not vary significantly according to geographical region (Kunisue *et al.*, 2008).

Looking more specifically at geographical splits in the United Kingdom, Allchin *et al.* (1999) found high concentrations of PBDEs in sediments form the River Skerne and Tees, downstream from the PBDE manufacturing site at Newton Aycliffe in county Durham (Law *et al.*, 2002). High concentrations were also found in fish sampled from the Tees bay, with BDE-47 found at particularly high concentrations (Allchin *et al.*, 1999; Law *et al.*, 2002).

Boon *et al.* (2002) found that concentrations of ∑BDEs were significantly greater in marine invertebrates, sea star (*Asterias rubens*), hermit crab (*Pagurus bernhardus*) and whelk (*Buccinum undatum*), sampled from locations of the North Sea situated close to the English coast. The Tyne and Tees estuary situated in the North East of England appears to be a particular source of PBDEs, with high local concentrations and the possibility that contaminants are carried south from this location by winter currents, providing the elevated concentrations observed in invertebrates along the east coast of Britain (Boon *et al.*, 2002).

Concentrations in cormorants (*Phalocrocorax carbo*, n = 47) and harbour porpoise (*Phocoena phocoena*, n = 60) sampled from around England and Wales ranged from 1.8 to 140 ng.g^{-1} ww and from below detection limits to 6900 ng.g^{-1} ww, respectively (Law *et al.*, 2002). No attempt was made to resolve a geographical pattern in the concentrations obtained, however the highest concentration in harbour porpoise was obtained from an animal picked up close to Tynemouth, Tyne and Weir, just north of the river Tees (Law *et al.*, 2002).

5.5.3ii) Temporal Variation

No significant relationship was observed between the year of death and scores for PC1 or PC2, corresponding to the lower and higher BDE congeners respectively. It should be noted though that, despite a temporal range spanning 1995 to 2005, very few of the samples analysed (13 out of the 129) originated from otters that died between the years of 1995 and 2000.

Previous studies of PCB concentration in otter liver samples have shown a significant decrease in concentrations over time (Simpson, 1998). PCBs have been banned from use for a much longer period of time and so are more likely to be decreasing in concentration in the environment. PBDEs have only recently been banned from manufacture and use in Europe and as materials manufactured over the last few years are still in use and the resulting leaching of PBDEs into the environment still remains a problem. This, combined with the fact that PBDEs are environmentally persistent compounds, means that the concentrations of PBDEs in the environment may continue to increase over the next few years (de Wit *et al.*, 2006; Braune *et al.*, 2007).

5.5.3iii) Seasonal Variation

No seasonal variation was observed for concentrations of PBDEs in otters. This is perhaps not surprising as otters in England and Wales do not tend to exhibit a yearly cycle of feeding and breeding activity as they do in some other areas of their range where fluctuations in the weather conditions and food availability favour a more cyclic breeding season.

Very few species exhibit a seasonal change in PBDE concentrations, however when they do it is usually linked to changes in body condition through the seasons. For example, concentrations of PBDEs in bream (*Abramis brama*) collected from across Holland varied according to the season of collection. Fish sampled in April contained significantly lower concentrations than those collected in September (Table 5.9). de Boer (2003) suggest that this is due to the increased body condition of fish collected in September, which have built up greater body fat reserves than fish collected in April at the end of the spring spawning season.

Table 5.9 BDE-47 concentrations (ng.g^{-1} dw) in bream collected from locations around the Netherlands in April (period 1) and September (period 3) of the same year (de Boer *et al.*, 2003).

Location	Collection period 1	Collection period 3
Amsterdam	4.4	19
Eijsden (Meuse)	9.1	110
Haringvliet	16	80
Lobith (Rhine)	37	90
Bergumermeer, Bergum	0.2	1.9
Vrouwenzand (Ijssel lake)	13	22

5.5.3iv) Gender

The results of this study show that there no significant differences in the BDE congener concentrations found in male and female otters. Several papers suggest that the different sexes are likely to express different concentrations of contaminants, such as PBDEs, because breeding females can pass proportion of their contaminant burden on to their offspring. Indeed analysis of concentrations in pregnant marine mammals and

their foetuses have shown that PBDE congeners are passed from mother to offspring during pregnancy (Law *et al.*, 2002, mother 612 $ng.g^{-1}$ ww: foetus 353 $ng.g^{-1}$ ww; She *et al.*, 2002, mother 782 $ng.g^{-1}$ lipid wt: foetus 430 $ng.g^{-1}$ lipid wt). The author suggests that other factors, such as the increased dietary requirement of pregnant or lactating females or the increased stress on the body of a pregnant female, may result in an increase in the contaminant burden of breeding females, cancelling out the effects of contaminant loss due to transfer to the developing young.

Harbour porpoise (*Phocoena phocoena*, n = 60) collected from the coasts of England and Wales showed no significant differences in congener concentrations between the two sexes (Log (Ln) transformed data, t-test; $t = 0.112$, $df = 58$, $p = 0.912$; calculated using data from Law *et al*, 2002). Similarly, no significant difference was observed between the two sexes in harbour seal (*Phoca vitulina*) from the San Fransisco Bay area in North America ($p > 0.05$; She *et al.*, 2002), although a contributing factor could be the low sample numbers used (6 males vs. 4 females)

Hartmann *et al.* (2007) looked at PBDE concentrations in the bile and liver tissue of brown trout (*Salmo trutta*) from Switzerland. The only congener found in consistently quantifiable concentrations was BDE-47, which was found to be partitioned differently in the different sexes. Male trout (n = 98) tended to build up higher concentrations than of BDE-47 in the bile, whereas females (n = 119) tended to build up higher concentrations in the liver. It should be noted that this trend was only observed when the concentrations observed were relatively high, and if anything the trend was reversed at lower concentrations. Due to the limits of the data no statistical significance was applied. Despite this, the trends do hint that different tissue partitioning may be carried out by the different sexes of brown trout (Hartmann *et al.*, 2007), and perhaps the same phenomenon is possible in other species.

5.5.3v) Life Stage

For the purposes of this study otters were split into groups according to life stage, to investigate any trends associated with the age of the otters. The results showed that there was no statistically significant difference in the concentrations of lower (PC1) or

higher (PC2) BDE congeners found in adult otters (n = 52), sub-adult/juvenile otters (n = 36) or cubs (n = 3).

In studies of other species possible links with the age of the animal have been found. For example, at a site in Carbo do Mundo, in Portugal mussels with an approximate weight of 2.0g contained an average BDE-47 concentration of 2.03 ng.g^{-1} dw, in comparison mussels with a weight of approximately 1.0g contained an average BDE-47 concentration of 1.47 ng.g^{-1} dw (Gama *et al.*, 2006). As the mussels continue to increase in size with age it can be understood that BDE-47 concentrations increase with the age of the mussel (Gama *et al.*, 2006).

In a study of coho salmon (*Oncorhynchus kisutch*, n = 16) and chinook salmon (*Oncorhynchus tshawytscha*, n = 5) from Lake Michigan in North America, Manchester-Neesvig *et al.* (2001) found significant correlations between PBDE concentrations and the length (slope = 1.45, r^2 = 0.39) and mass (slope = 65.0, r^2 = 0.32) of the fish. In studies of fish the length and mass are often used as am measure of the age of a fish, and so Manchester-Neesvig *et al.* (2001) interpreted this result as a significant increase in PBDE concentration with the increasing age of the fish. A significant relationship between body length and BDE concentrations in Arctic char (*Salvelinus alpinus*; n = 20; Log transformed data, P = 0.033, r^2 = 0.23) indicates that this species also expresses age related accumulation of PBDEs (Vorkamp *et al.*, 2004).

In all these studies the organisms size has been used to assess the age of the animal. This means that any effects observed could arguably be a result of variation in the size, in terms of body mass or for aquatic organisms in terms of volume to surface area ratio (Boon *et al.*, 2002) rather than due to the age *per say*.

Vorkamp *et al.*, 2004 found higher concentrations and a greater range of concentrations of $\sum$BDE in adult (n = 5; mean 38 ng.g^{-1} lipid wt; range 21-74 ng.g^{-1} lipid wt) verses sub-adult (n = 14; mean 35 ng.g^{-1} lipid wt; range 21-74 ng.g^{-1} lipid wt) ringed seals (*Phoca hispida*). Similarly, Hall *et al.* (2003) found that $\log_{10}$ $\sum$BDE were significantly higher in juveniles (mean = 2.655 0.235) than in post-weaned pups (mean = 2.273 0.251)(t-test; p <0.0001). However, they also point out that this could be due to a loss in body condition of juveniles as seal pups were shown to loose significant body condition in the months following weaning (Hall *et al.*, 2003).

5.5.3vi) Body Condition

No significant relationship was observed between BDE congener concentrations in the liver samples (PC1 and PC2 scores) and the body condition (*K*) of the otters. Interestingly, the otters with outlying high concentrations of particular congeners tended to be individuals expressing high (BDE-47, -99 and -209) or low (BDE-153 and -209) condition index measures. Although the significance of this is not clear it does indicate that there may be issues related to high and low body condition which also relate to the uptake of PBDE congeners.

Studies of PCB contamination in otters have found that the relationship between concentrations of PCBs and body condition (*K*) can be variable, depending it appears, on another unidentified variable. Kruuk and Conroy (1991) and Chadwick (2007) found no correlation between PCB concentrations and body condition (*K*) in otters. However, Kruuk and Conroy (1996) found a significant correlation between body condition (*K*) and PCB concentrations ($r = -0.45$, $p = 0.001$) in otters from Scotland. Ruiz-Olmo *et al.* (2002) recorded significant correlation between PCB concentrations and body condition (*K*) in otter from France, but no such correlations in otter from Spain.

Correlations between measures of body condition and concentrations of PBDEs have been found for many species of bird and mammals. For example, in harbour seals (*Phoca vitulina*) collected from the San Fransisco Bay area in North America, out of eleven seals sampled the three with the highest ∑BDE concentrations (8325, 1944 and 2986 $ng.g^{-1}$ lipid wt) were also the three individuals with the lowest fat content (28%, 40% and 48%)(She *et al.*, 2002). Kannan *et al.* (2007) found that PBDE concentrations where significantly lower in southern sea otters (*Enhydra lutris nereis*) that were well nourished compared (n = 20) to those that were in a poorly nourished (n = 20) or emaciated (n = 27) condition.

Naert *et al.* (2007) found that buzzards (*Buteo buteo*) which contained only a limited amount of adipose tissue, ie considered in lower body condition, contained significantly higher concentrations of PBDEs in their brain tissue than buzzards considered to be in good bodily condition ($t = 2.162$, $p = 0.037$). This they argued, was due to mobilisation of PBDE congeners after the loss of adipose mass, these contaminants resettling in areas such as the brain – similar to the behaviour observed for PCBs (Naert *et al.*, 2007). In

theory animals that are starving are in the process of using up fat reserves, and as they do so the contaminants that have been stored in those fat reserves become mobilised and end up concentrating in the remaining fat reserves and in organs and tissues such as the liver and brain tissue (Kannon *et al.*, 2007; Naert *et al.*, 2007).

Otters generally contain comparatively little adipose tissue, even when in healthy bodily condition (Vic Simpson, pers. comm.). This may mean that contaminants, such as PBDEs, are more concentrated in the adipose tissue which is present. However, it may also mean that the lack of fat reserves result in a naturally high concentration in other tissues, such as the liver tissues analysed in this study. As a result, increases in liver concentrations may be less severe when individuals become emaciated as concentrations are already high.

5.5.3vii) Cause of Death

The results showed that PBDE contaminants contained in the liver tissue of otters killed in road traffic accidents (RTAs) were not significantly different from concentrations observe in otters dying by other means. The theory behind this analysis was that otter dying in RTAs were a better representative of healthy otters and that those dying from other causes were more likely to be suffering from the effects of starvation or disease. If otters dying in poor health express higher concentrations of PBDE this would indicate that either a high PBDE burden is detrimental to the health of otters, or that otters in poor health are more susceptible to building up high concentrations of PBDEs in the liver tissue.

The analysis itself was flawed due to the fact that not all otters recorded as non-RTAs were in poor health when they died, for example, otters which were shot or which died as a result of fighting were recorded as non-RTAs even if they were otherwise healthy at the time of death. This means that the lack of a relationship cannot be considered as proof that BDE congener concentrations are not influenced by, or do not have an influence on the health of otters. However, the method of separating otters into categories according to health can provide useful in situations where a relationship with contaminant burden is observed. For example, Roos *et al.* (2001) found that otters that were traumatically killed contained significantly lower ($p < 0.01$) ∑PCB concentrations

than otter that died from unknown causes (traumatically killed: n = 19, median = 67 ng.g$_{-1}$ lipid wt, range = 5 – 217 ng.g$_{-1}$ lipid wt; unknown causes: n = 12, median = 240 ng.g$_{-1}$ lipid wt, range = 5.7 – 970 ng.g$_{-1}$ lipid wt;). However, Kannon *et al.* (2007) found no significant difference in the PBDE concentrations of southern sea otters (*Enhydra lutris nereis*) classified as suffering from infectious disease (n = 26) at the time of death those considered healthy (n = 27; note that otters dieing in an emaciated condition with no obvious cause of death were classified into a third 'emaciated' classification).

5.5.4 Comparisons of PBDE concentrations with Concentrations of Other Contaminants

5.5.4i) Contaminant Concentrations

Concentrations of PBDEs, both in terms of the sum concentrations of all the congeners and in terms of individual congener concentrations, were compared to those of PCBs and DDTs. The results showed that PBDEs were a significant contaminant of the otter liver tissue samples analysed. The mean ΣBDE concentration of 3242 ng.g^{-1} lipid (26 congeners analysed) was almost as high as the mean ΣDDT concentration (3859 ng.g^{-1} lipid; 4 congeners analysed) and approximately a quarter of the mean ΣPCB concentration (12,928 ng.g^{-1} lipid; 25 congeners analysed).

In terms of individual congeners, several of the BDE congeners were measured at concentrations paralleling those of some of the most concentrated PCB and DDT congeners. In particular, the most concentrated BDE congener, BDE-47, was the forth most concentrated congener (by mean and median) measured in the otter livers. BDE-47 should therefore be considered one of the most prominent organohalogen contaminants in otters and should be regarded as a concern in terms of relative concentrations.

The congener BDE-153 was observed at a similar mean concentration (526 ng.g^{-1} lipid) to that of the sixth most concentrated PCB congener, PCB-118 (548 ng.g^{-1} lipid). Overall, when mean concentrations are considered, BDE-153 was the ninth most concentrated congener. Also found at significant concentrations was BDE-209. By mean BDE-209 concentrations (199 ng.g^{-1} lipid) were approximately twenty times

lower than those of PCB-138 (mean = 3922 $ng.g^{-1}$ lipid), the most concentrated congener measured in the otter liver tissue. PCB concentrations were relatively high in otters, with a mean ΣPCB concentration of 12,928 $ng.g^{-1}$ lipid, compared to, for example: a mean ΣPCB concentration of 4877 $ng.g^{-1}$ lipid in ivory Gull (*Pagophila eburnea*, n = 6) eggs collected from the Canadian Arctic in 2004 (Braune *et al.*, 2007), mean ΣPCB concentrations of 3032 (SD 1074) $ng.g^{-1}$ lipid and 2403 (SD 855) $ng.g^{-1}$ lipid in the blubber of sub-adult male norther fur seals (*Callorhinus ursinus*) on the Alaskan islands of St. George (n = 10) and St. Paul (n = 10) respectively (Loughlin *et al.*, 2002), or a mean ΣPCB concentration of 51.42 (SD 21.85) $ng.g^{-1}$ lipid in striped dolphin (*Stenella coeruleoalba*, n = 31) collected from the western Mediterranean in 2001 (Aguilar & Borrell, 2005). Therefore, the fact that PBDE congener concentrations were comparable to those of PCBs indicated that PBDEs should also be considered a significant concern in term of the contamination of otters.

For both BDE-153 and BDE-209 median concentrations were relatively low (BDE-153 = 90 $ng.g^{-1}$ lipid; BDE-209 = 64 $ng.g^{-1}$ lipid), indicating a bias towards the lower end of the concentration ranges. Higher means and significantly higher maximum concentrations (BDE-153, mean = 526 $ng.g^{-1}$ lipid, maximum = 23, 317 $ng.g^{-1}$ lipid; BDE-209, mean = 199 $ng.g^{-1}$ lipid, maximum = 6808 $ng.g^{-1}$ lipid) indicate that comparatively few individuals expressed particularly high concentrations, but that these individuals may be crucial in assessing the overall impact of these congeners purely because of this significantly higher concentration. This pattern of bias towards a few extremely high concentrations in a population expressing mostly relatively low concentrations suggests that a certain percentage of individuals may be significantly more at risk from the potential negative effects of these congeners than most. This was in contrast to PCB congener data where means and medians were more similar to each other, indicating a more even spread throughout the concentration range (see Table 5.4).

5.5.4ii) Congener Correlations

In a principal component analysis (PCA) of concentration data, congeners from different contaminant groups assigned to different principal component (PC) groups. The results suggest that there were two sources of PCB contamination. The first, corresponding to PC1, resulting in the majority of PCB contamination observed in the otter livers, with all of the most concentrated PCB congeners correlating with this principal component. The second set of correlating PCB congeners corresponded to PC4. These congeners were generally observed at much lower concentrations, and this combined with the lower number of congeners suggests that this group of congeners have less of an impact, in terms of the concentrations observed, on the otter population of England and Wales.

PCBs were banned from manufacture and use several decades ago (Walker, 2001) but are known to remain at significant concentrations in the environment due to the compounds high environmental persistence. The concentrations observed in otters will have originated from this high environmental background concentration or from materials manufactured before the ban leaching contaminants into the environment. In a similar fashion DDTs have been banned from use in the UK for several decades and the concentrations observed in the present day otter liver tissue samples were likely to have resulted from residual levels still present in the environment due to the compounds environmental persistence. In particular *pp*-DDE, one of the main breakdown products of DDT, is known to have a long half life in the environment (Storelli *et al.*, 2005).

As identified in the PCA of PBDE congeners alone (see section *5.3.4 Principal Component Analysis*), the PCA results from the multiple contaminant analysis suggest that BDE congeners originate from two main sources. The more concentrated lower tri- to octa-BDE congeners correlated with PC2, suggesting that all of these congeners originated from the same source, likely to be the release of Penta- and Octa-BDE commercial mixtures into the environment. The nona- and deca-BDE congeners analysed all correlated via PC3, suggesting that these higher congeners originated from a second PBDE source, likely to be the leaching of the Deca-BDE commercial mixture into the environment.

5.5.5 Overall Conclusions – PBDE Concentrations in Otters

The results show that PBDE concentrations in otter liver tissue were relatively high, both in terms of comparison with other species and in terms of comparison to concentrations of other organic contaminants in the otter liver tissue. The ΣBDE concentrations observed in the otter liver tissue (mean 3242 $ng.g^{-1}$ lipid, median 1565 $ng.g^{-1}$ lipid, range 12 - 69,883 $ng.g^{-1}$ lipid) were similar, and in many cases higher than ΣBDE concentrations observed in marine mammal species (see section *1.3.2v, Marine Mammals*). In contrast concentrations of ΣBDEs were much higher in the otters than in terrestrial predatory mammal species (see section *1.3.2vii, Terrestrial Mammals*), suggesting that the high ΣBDEs concentrations observed in otter reflect the influence of a semi-aquatic lifestyle and a diet dominated by fish species.

PBDEs were found to be one of the major organohalogen contaminants measured, with ΣBDE concentrations found to be almost as high as ΣDDT concentrations and approximately a quarter of the concentration of ΣPCBs. On an individual congener basis the most concentrated BDE congener, BDE-47, was the forth most concentrated congener of all the contaminants analysed. Other BDE congeners, in particular BDE-153 and BDE-209, were also amongst the top most concentrated congeners measured in the otter liver samples.

The impact that these relatively high concentrations were likely to have had upon the wild otter population of England and Wales is difficult to assess. The mechanisms of toxicity of PBDE congeners have been shown to be similar to those of PCBs (see section *1.3.4 Toxicity*). In general PBDEs have been observed to be less acutely toxic than PCBs (Hallgren *et al.*, 2001). However, more study is needed into the varying toxicological effects of different congeners and toxicity can vary significantly from one species to another (Legler & Brouwer, 2003; Viberg *et al.*, 2004; Tseng *et al.*, 2006; Lema *et al.*, 2007), making assumptions based on laboratory study species potentially miss-leading and inaccurate. In addition, little is known about the potential additive effects when organisms are exposed to PBDE contamination in addition to exposure to PCBs and other organic contaminants (Hallgren & Darnerud, 2002).

The BDE congener patterns and correlations observed in the otter samples suggests that there were two main sources of PBDE contamination, with the lower congeners originating from use of the Pent- and Octa-BDE commercial mixtures and the nona- and

deca-BDE congeners originating from sources of the Deca-BDE commercial mixture. Comparison to congener concentrations observed in other species indicated that the otters studied were affected by exposure via both the aquatic and terrestrial environments in terms of PBDE congener uptake. High concentrations of BDE-47, -99, -100 and 153 mirror congener patterns observed in many marine predators (Vorkamp *et al.*, 2004; Kajiwara *et al.*, 2006). However, the particularly high concentrations of BDE-153 in comparison to other congeners, such as BDE-99 and BDE-100, combined with a relatively high concentration of BDE-209, suggested that the otters had also been subjected to significant influence from the terrestrial environment (Christensen *et al.*, 2005; Voorspoels *et al.*, 2006; Mariussen et al., 2008).

Chapter 6 Overall Discussion

The overall aim of the project was to better understand the impact that human activities have had upon the otter population of England and Wales and to predict factors which may negatively impact upon populations in the future. This remit was split into three main areas of study. The first was to improve upon our knowledge of the way in which local otter populations are structured by surveying them using non-invasive microsatellite genotyping to identify individual otters. The second area of study was to look more directly at the population genetic aspects of the decline in otter numbers and subsequent release of captive bred otters. To this end the River Itchen otter population, one of the most severely impacted populations, as determined using demographic data collected via presence/absence surveys, was used as a case study population for comparison with other less severely affected populations. Evidence suggests that one of the main reasons for the initial decline in otter numbers is likely to have been contamination by organohalogen compounds, in particular PCBs and dieldrin (Chanin & Jefferies, 1978; Simpson, 1998; Ruiz Olmo *et al.*, 2002; Barbosa *et al.*, 2003). The final area of study was aimed at analysing organohalogen contamination levels in the present day otter population, with a particular focus on a relatively new suite of environmental contaminants, PBDEs.

6.1 Obtaining Population Information on a Local Scale

The first area of study was aimed at improving knowledge of local otter populations in terms of population size and the ways in which individual otters use particular locations in the study area. This was done by carrying out a non-invasive census of the otter population present on the River Camel in Cornwall as a case study for the technique.

6.1.1 Improving Techniques for Genotyping Otter Spraint

6.1.1i) Microsatellite Loci

Previous studies have indicated that the use of nine microsatellite loci plus the SRY is sufficient to successfully distinguish between the majority of genotypes obtained (Dallas *et al.*, 2003; Arrendal *et al.*, 2007). Calculations suggest that this number of

loci are sufficient to produce a low probability that two closely related individuals will share an identical genotype at all of the loci analysed ($PI_{sib} = 5.6 - 6.8 \times 10^{-3}$, Dallas *et al.*, 2003; $PI_{sib} = 2.33 \times 10^{-3}$, Arrendal *et al.*, 2007). However, microsatellite loci differ in their variability according to the population being studied and the loci used. Under circumstances where there are low levels of allelic variation at multiple loci increasing the number of microsatellite loci used (within the bounds of feasibility, practicality and cost) will be beneficial in the accurate identification of individual animals.

The SRY loci was not analysed in this study due to potential contamination and reproducibility problems (see section *1.1.6iii, Sexing Faecal Samples*). The total number of microsatellite loci used in this study was increased to ten to compensate. This in theory will have increased the accuracy of individual separation and identification. However, the loci used showed relatively low levels of genetic diversity in the River Camel population, with eight out of the ten loci expressing only two alleles. Despite low levels of genetic diversity, the relatively high number of loci used will have gone some way to maintaining good levels of individual identification and distinction. The fact that none of the samples showing identical genotypes were collected from sites particularly distant from each other adds confidence to the fact that the ten loci used were sufficient to identify the individual otters using the River Camel catchment at the time of survey.

6.1.1ii) Causes of Variation in Genotyping Success Rates

Studies utilizing DNA extracted from spraint to genotype otters often experience low rates of success (Dallas *et al.*, 2003; Arrendal *et al.*, 2007; Hansen *et al.*, 2008). This study has proved to be no exception with only 18.1% of samples producing a DNA extract of suitable quality to genotype. Potential reasons for this low success rate were investigated by comparing various collection and sample parameters with genotype success rate.

A statistical model of the effects that field collection parameters had upon extraction success rates revealed that the weather conditions and sample type both had a significant effect upon the chances of obtaining a genotype from a sample. The results revealed that samples collected under cold weather conditions, recorded as snow or

frost, were significantly more likely to produce a genotype than those collected under other weather conditions. This is in agreement with other studies in which the effects of weather conditions have been considered. For example, Hájková *et al.* (2006) found that, for unfrozen samples, genotyping success rates were significantly reduced by increasing temperature at the time of sample collection (ANOVA; $F = 26.24$, $df = 1, 8947$, $p < 0.001$). Under colder conditions the metabolic function of bacteria slows and the resulting breakdown of DNA in the spraint material will also slow down, resulting in greater levels of DNA preservation and a higher chance of obtaining a genotype.

The second factor extracted by the field based model involved the sample type analysed, with anal jelly samples being significantly more likely to produce a genotype than spraint. The same phenomenon has been observed in previous studies (Hung *et al.*, 2004; Hájková *et al.*, 2006; Arrendal *et al.*, 2007), with samples of anal jelly producing a higher genotyping success rate than samples of spraint. The fact that samples recorded as containing both anal jelly and spraint were no more likely to produce a genotype than spraint alone suggests that the reason for the disparity is likely to be a negative impact from the spraint material, rather than a significantly greater DNA content in the anal jelly samples. As spraint contain many different chemicals, enzymes and bacteria picked up in the gut or after defecation, it would be logical that this has a negative impact upon the DNA present, therefore reducing the quality and quantity of DNA extracted from the spraint.

A second statistical model was carried out to look at the potential effects, in terms of genotyping success, of factors regarding the spraint material itself and the collection medium in which it was stored. The results showed that the volume of collection material in comparison to the volume of spraint material collected significantly affected the chances of obtaining a successful genotype. The lower the volume of collection medium in comparison to volume of spraint material (classified as a 'poor IMS:spraint ratio') the greater the chances of obtaining a genotype. The results also showed that samples of spraint which coloured the collection medium amber or darker were extremely unlikely ($p < 0.001$) to produce a genotype. This relationship with IMS colour suggests that the IMS collection medium interacted with something in the spraint sample to reduce the success rate of DNA extractions, either by reducing the concentrations of DNA or by producing a product which reduces the efficiency of the DNA extraction kits or inhibits PCR success. Together these findings suggest that the

use of IMS as a collection medium may in fact have had a negative impact upon the DNA storage, extraction or genotyping success rate. A more tried and tested DNA storage medium, such as biological grade ethanol or a specifically designed commercial DNA storage medium, would be suggested for any future spraint genotyping studies.

6.1.1iii) Coverage of the River System

Important to any population census study is the level of coverage of the population system. This is particularly true for species, such as the otter, that are territorial, with strict home range areas. If an area of the catchment is not sufficiently sampled this could result in the failure to detect the otters inhabiting that particular area. In the census of the River Camel otter population there was significant disparity between the numbers of otters observed using different areas of the river. Relatively high numbers of otters were observed using the lower half of the catchment and the estuary (n = 13), with a reasonable number of otters found using the upper reaches of the river (n = 3). In contrast, no otters were genotyped using the middle reaches of the River Camel or the River Allen tributary, despite the fact that there were signs of otter activity in both of these areas.

There were two main reasons for the gaps in the coverage of the catchment. Firstly, collections were carried out by volunteers and so the amount of time and effort that was put into collections was largely down to the ability and willingness of the volunteers assigned to that particular area of the catchment. Secondly, spraint samples were significantly easier to locate and collect in certain areas of the catchment, with certain volunteers investing large amounts of time and effort for relatively little gain in terms of sample numbers collected. This variation in ease of sample collection was due in part to the physicality of the river system, with some sprainting sites being easier to access than others. Variation in level of otter activity also made sample collection difficult in some locations, with significantly fewer samples available to find and collect.

6.1.2 Numbers of Otters

More traditional population census methods, involving techniques such as the counting of otter signs (tracks and spraint), are useful in terms of assessing otter presence or absence in an area (Crawford, 2003). However, accurately calculating the number of otters occupying an area can be difficult using this method as levels of sprainting can be affected by many factors, including the breeding status of individuals, topography of the riverbank, as well as the weather and levels of local flooding at the time of the census (Kruuk, 1992). Otter tracks are often difficult to come by and are easier to find and identify in areas of heavy snowfall (Erling, 1967).

The use of non-invasive genetic surveys to count the number of otters using an area is a more accurate way to census a population (Dallas *et al.*, 2003; Hung *et al.*, 2004; Arrendal *et al.*, 2007). In this study 16 different genotypes were obtained from the spraint collected. A total of nine otter were observed using the river during the first period of study, while twelve were observed in the second. Five of the otters were observed at least once during both seasons of collection, meaning that a total of sixteen otters were observed using the Rivel Camel catchment over the two seasons of study.

Information about the date and location of spraint collection was linked to the genotype obtained to provide information on time periods and locations for the individual otters identified. This provided information about the movement of the different otters within the catchment area. As a result four main areas of otter occupancy were identified, each area supporting several otters at some point over the course of the collection period. None of the otters genotyped were observed in more than one of these four areas of otter activity, though location data does indicate that many of the otters had individual home ranges within one of the main areas. This indicates that, as has been suggested in other studies of otter behaviour (Erlinge, 1967; Ruiz-Olmo *et al.*, 2005), there may have been two or three females with territories which overlap, not only with each other but also with that of at least one male. However, without information regarding the age and gender of the otters surveyed, this observation is purely speculative. Females with cubs were known to be present on the river during the period of study (Jon Evans, pers. comm.) and so this will increase the instances of several individuals being observed using the same territory.

6.1.3 Measures of Genetic Relatedness

Data regarding the dates and locations of individual otter observations can provide information regarding the number of otters using a particular location at any one time. However, without information about the age and gender of the otters it is difficult to ascertain the relationship between the individuals observed. One way in which genotype information can aid this is by providing a probability that two individuals were genetically related to each other.

Relatedness and parentage statistics indicate that at the time of sampling there were three groups of otters present on the river system showing levels and patterns of genetic relatedness consistent with those of a family group structuring. The first family group consisted of five otters related to each other to varying degrees. This pattern of varying strengths of pariwise relatedness would be expected given an extended family tree containing secondary relationships, such as grandparents or aunts, as well as more primary relationships, such as parent-offspring and full siblings. All of these five otters were located geographically in relatively close proximity to each other, either on the estuary or the lower section of the River Camel just above the estuary, as might be expected in a closely related family group. The other two family groups also showed patterns of genetic relatedness consistent with what might be expected given an extended family situation. Related individuals tended to be found in similar locations to each other, with a few instances of geographical separation consistent with short range migration patterns away from the natal range.

6.1.4 Conclusions

The main hypothesis for this section of the project was that:

H_1 Significant increases in our understanding of the numbers of otters and the way in which otters are using a particular geographical location can be achieved by using the microsatellite genotyping of DNA extracted from spraint material.

The technique of microsatellite genotyping spraint material was successfully used to confirm that at least sixteen otters used the River Camel catchment over the two seasons of study. The study revealed information about the minimum number of otters that

different areas of the catchment were capable of supporting. Groups of otters appeared to occupy specific areas of catchment, with possible individual territories within these main areas, but little or no overlap with the territories of otters in neighbouring areas. This suggests that there are barriers to otter movement, imposed by natural structuring of the river, otter population structuring or by man-made structures or pressures. Levels of otter movement would suggest that these barriers are crossed by otters during dispersal, but not during normal home range use.

The main drawbacks of the spraint genotyping technique are that it only provides a minimum count and that the genotyping of spraint samples is expensive. It would never be possible using this technique to be absolutely certain that every otter using the area had been accounted for, particularly with the existence of highly mobile transient animals. To increase the chances of sampling all individuals present it would be logical to genotype as many spraint samples as possible. However, the high cost of genotyping, particularly in light of the multiple repeat PCR and fragment analysis runs required to genotype spraint DNA extracts, limits the number of samples it is possible to genotype.

Despite uncertainty over whether all otters present had been counted, the spraint genotyping technique provides a significant improvement in terms of population census over a river catchment area. In the past otter census have been carried out using differences in track sizes to identify individual otters (Erling, 1967; Prigioni *et al.*, 2007), however, this technique is better suited to areas of heavy snowfall where tracks are laid more often and are generally better preserved. Even under favourable climatic conditions, a study by Arrendal *et al.* (2007) showed that the microsatellite genotyping of spraint samples was a much more accurate method of distinguishing between individual otters than analysis of otter tracks.

In England otter population census has traditionally been carried out using signs of otter activity, in particular sprainting and tracks, as confirmation of otter presence in an area. This technique has provided important information regarding the presence and absence of otters on catchments across the country, with nationwide surveys helping to document the decline and recovery of otter populations across England (Crawford, 2003). Despite the usefulness of the technique on a wider scale, on a more local scale signs of otter activity are less informative. The finding of signs of otter activity proves that there are otters present in the area, but does not provide much evidence to suggest

how many otters are likely to be present. Therefore, on a more local, river wide scale the census of populations using genotypes obtained from spraint is a significant improvement in terms of accurately counting the number of otters present, even given that it is a minimum count over a period of several months and not an accurate point census, as would be ultimately desired.

In terms of providing information about individual otter movement and home range use within the river catchment, a significant amount of information was gained using the spraint genotyping method. By connecting genotypes to the date and location of spraint sample collection it was possible to gain an idea of how many otters were using a particular area of the catchment. For otters observed more than once it was also possible to gain an idea of minimum home range use and the minimum amount of time the otter was present on the river. This, while not providing a complete picture, did significantly increase our understanding of how otters use the River Camel catchment. The results show that otters on the River Camel inhabit distinct home ranges. In several cases more than one otter was observed using the same locations over the same or similar time periods. Whether these represent mothers with cubs or adult individuals sharing resources is not clear, although it is likely that both scenarios occurred.

The use of genetic information to calculate probable genetic relationships also significantly increased our knowledge of the dynamics of the River Camel otter population. Three potential family groups were identified inhabiting different stretches of the river system. This not only indicates that the river supports a healthy breeding population, but also suggests that many of the offspring born on the river remain on the river system later in life. Several pairs of otters showing a high probability of being closely related to each other were found in similar areas of the river, but far enough apart to suggest that short range migration had occurred into a neighbouring territory. This pattern of migration is common in territorial mammals, such as the otter, where males generally undergo greater levels of dispersal, while females often remain close to their natal range. It is likely that the short range migration witnessed here, where closely related individuals are observed on neighbouring territories, is representative of the short-range migration of female offspring.

Over recent years several studies have used the technique to non-invasively census otter populations (Coxon *et al.*, 1999; Dallas *et al.*, 2003; Arrendal *et al.*, 2007), with the

information being used not only to calculate number of otters, but also to study behavioural factors such as home range use (Coxon *et al.*, 1999) and levels genetic relatedness (Hung *et al.*, 2004). In this study several of these endpoints were used in order to gain as much information about the otter population inhabiting the River Camel as possible. The major drawback of the method is that it provides only a snapshot of what is going on in a population, with limits to resources limiting the number of spraint collected and genotyped and therefore limiting the amount of information gained. Each piece of information should therefore be treated as a minimum, for example, a minimum otter count on a particular otter being present for a minimum amount of time. Despite this the amount of information about the local otter population which can be gained using this method is a significant improvement on that which is gained via other methods. In summary, the use of microsatellite genotyping DNA extracted from spraint has significantly increased our understanding of the River Camel otter population, in terms of otter number, behaviour and population dynamics.

6.2 The River Itchen Case Study

The second aim of the project was to assess the impacts of the severe population decline experienced in the 1950s to 1980s and the subsequent release of captive bred otters on the genetic structure of the present day otter population. The River Itchen in Hampshire experienced a severe population bottleneck, as the numbers of otters reduced down to just a few individuals and otters essentially became extinct from surrounding river systems (Crawford, 2003). During the early 1990s at least four otters were released from the Otter Trust captive breeding programme (Crawford, 2003). Given this demographic history, the River Itchen lends itself as an ideal case study population for the study of the effects of population decline and subsequent augmentation using captive bred otters. The River Itchen otter population was compared to other otter populations in order to assess the levels of genetic differentiation which could be attributed to the demographic history of the population.

6.2.1 Microsatellite Analysis

Being neutral markers with relatively high mutation rates, microsatellite loci are useful tools for providing information about population dynamics and histories (Jeffreys *et al.*, 1988; Kelly *et al.*, 1991; Van de Zande *et al.*, 2000). In this study the microsatellite genotypes of otters from the River Itchen were compared to those of otters from east Cornwall and south Dorset in an effort to assess the genetic impact of differences in the demographic histories of the different populations.

East Cornwall is situated in the heart of the south west of England, an area known to be an otter population stronghold. The population did suffer during the period of national otter population decline, but to a much lesser extent than most otter populations in England. In the south west of England, even at the height of the declines over 20% of sites deemed suitable for otter habitation showed signs of otter activity (Crawford, 2003), indicating that significant numbers of otters survived in the area. Many rivers in east Cornwall retained relatively healthy otter populations (Crawford, 2003), and these are likely to have maintained healthy levels of migration between river systems, effectively sustaining a healthy overall population structure.

South Dorset is situated along the coast to the west of the River Itchen. The otter population here experienced a similar, if less severe, demographic history. As on the River Itchen, levels of otter occupancy decreased dramatically during the period of national otter population decline, with only small isolated pockets of otter activity remaining (Crawford, 2003). Also in common with the River Itchen population, Dorset received several captive bred otters which are likely to have integrated, both behaviourally and genetically, into the wild population. One main difference between the south Dorset and River Itchen populations was that Dorset is positioned closer to the otter population stronghold in the south west of England. As the otter population recovered so the margins of the otter stronghold spread, enveloping the south Dorset population along the way. In contrast, it is thought that the margins of this otter population expansion are only just reaching the River Itchen in Hampshire.

6.2.1i) Genetic Diversity

The results show that the east Cornwall otter population was the least genetically diverse of all three populations analysed. Despite the fact that significantly fewer samples were analysed from the River Itchen (n = 9) and south Dorset (n = 5) populations, both these populations showed greater levels of allelic richness and heterozygosity than the east Cornwall otter population sample set (n = 16). In terms of the potential effects of variation in sampling, samples from each of the three sites were collected from across similar geographical spreads, suggesting that the differences in genetic diversity observed were not due to differences in sampling. The one exception may be for the River Itchen samples, which were collected across a smaller region of study than samples for the other two regions.

The demographic histories of the three populations indicate that the Cornish population remained relatively healthy while the Dorset and River Itchen populations both experienced severe bottlenecks. This demographic occurrence alone would have led to the Cornish population retaining significantly higher levels of genetic diversity than the other two populations. The fact that the Dorset and River Itchen populations were found to be more genetically diverse than the Cornish population suggests that there was a secondary source of genetic material entering these two populations; the most likely explanation for this being the introduction of captive bred otters. The higher genetic diversity of these populations, suggests that the captive bred otters released into the River Itchen and Dorset populations successfully integrated into the local populations and bred. The otters sampled in this study, given the time lapse between the releases of captive bred otters in the early 1990s and the sampling in the early 2000s, will represent otters several generations after the release of the captive bred otters. The genotypes are therefore likely to represent the genetic integration of native and captive bred otters.

The results suggest that the release of captive bred otters led to a large increase in genetic diversity, to the extent that the highly bottlenecked Dorset and River Itchen populations show significantly greater levels of genetic diversity than the Cornish population which experienced significantly lower levels of bottlenecking. This relatively large disparity, given the population histories, suggests that the otters used to found the captive breeding population were from a source distinct from that observed in the south west of Britain.

In terms of comparisons between the River Itchen and south Dorset otter populations, the south Dorset population was shown to be more genetically diverse. This difference may be an artefact of several different factors. Firstly, the Dorset population did not suffer as greatly during the declines and so less genetic diversity is likely to have been lost compared to the more severely bottlenecked River Itchen population. Secondly, the geographical position of the Dorset population, significantly closer to the otter stronghold in the south west of England, meant that as the otter population expanded out of the south west, the Dorset population was engulfed significantly sooner than the River Itchen. The high levels of genetic diversity observed for the Dorset population is likely to be the result of the mergence of otters from the original Dorset population with otters from the captive bred population, as well as otters from the south west population expansion.

6.2.1ii) Genetic Differentiation

Levels of genetic differentiation (F_{ST}) mirrored geographic separation, with the River Itchen and Dorset populations showing a lower level of genetic differentiation between them than either population did with the Cornish population. As the geographic distance between populations increases then the level of genetic differentiation detected also increases, the most likely reason for this being the natural process of genetic drift. As the River Itchen and Dorset populations are significantly closer to each other than either is to the Cornish population it follows that levels of genetic differentiation match this pattern.

Patterns of genetic distance observed between individual otters from the three different sample locations were viewed using an allele-sharing neighbour-joining tree. All sixteen otters sampled from east Cornwall grouped together on one relatively tightly packed branch of the tree, suggesting that there was relatively little genetic separation between otters from this location. Otters from the River Itchen and south Dorset grouped together on a second branch of the tree. This second branch generally had longer and more spread out branches, suggesting that the genetic distances between otters in this clade were significantly greater than the genetic distances between otters in the Cornish clade.

The fact that otters from the River Itchen and south Dorset were intermingled in the tree may indicate that the otters from these regions originated from similar sources. The longer and more highly separated branches observed for otters from these locations was an artefact of the greater levels of genetic diversity observed in these populations. The results of the tree show the Cornish population as one distinctive group. The greater relative spread of the south Dorset and River Itchen otters suggests greater genetic diversity. Given that these populations are known to have experienced bottlenecks, this would suggest genetic input from an outside source, the most likely explanation being the introduction of captive bred otters. The large spread of otters originating from these locations, i.e. with some otters being grouped with or close to the Cornish clade and some situated a large distance away, and the River Itchen and Dorset otters being intermingled with each other, would support this finding. The otters placed in or close to the Cornish clade are likely to represent the genetic input from the expansion of the otter population from the stronghold in the south west of England. Of note is the fact that analysis of the otter populations of the south west of England by Dallas *et al.* (2002) suggested that otters from the Cornwall area are genetically distinct from populations further east. The bottlenecking of the Itchen and Dorset populations is likely to have distanced them from the populations of the south west of England, with some alleles being lost during the bottleneck and other alleles becoming significantly more common. However, the otters situated the greatest distance from the Cornish clade are likely to represent a genetic input from an external source, the most likely explanation being the introduction of captive bred otters.

6.2.2 Mitochondrial Haplotypes

The results of microsatellite analysis show the River Itchen population to be more genetically diverse than the Cornish otter population. This indicates that novel genetic material has been introduced into the bottlenecked River Itchen population, the most likely source being the introduction of captive bred otters. Further analysis to ascertain the extent to which the genetic integrity of the River Itchen population is likely to have been altered by the introduction of captive bred otters was carried out, including investigations into the potential origins of the otters used to found the captive breeding programme. This was carried out using sequences from a 300bp section of the 5′ end of

the mitochondrial control region to haplotype otters from different geographical regions representing native British populations, populations augmented by the release of captive bred otters and populations likely to have been donors of founders of the captive breeding programme.

6.2.2i) Origins of the River Itchen Otters

The first aim of this section of study was to use the mitochondrial control region haplotypes to ascertain whether the present day River Itchen otter population contains descendants from the captive bred otters released onto the river in the early 1990s. This was done by studying the haplotypes of otters from different regions of England and Wales. In particular focusing on populations of presumed native origins and comparing them to populations which have been largely influenced by the release of captive bred otters.

The otter populations of Cornwall and south Wales were studied as examples of British populations not receiving high numbers of captive bred otters. All samples analysed from Cornwall expressed the haplotype Lut1, while the haplotypes Lut1, Lut6 and Lut7 were all found to be present in the Welsh population (see also Stanton *et al.*, 2008). Therefore, it can be concluded that the haplotypes Lut1, Lut6 and Lut7 are all native to the British otter population.

East Anglian otters were analysed as representatives of a population containing a large proportion of otters released from the Otter Trust captive breeding programme. The native East Anglian population became extremely small and fragmented during the period of national decline and only began to recover after the release of relatively large numbers of captive bred otters (Crawford, 2003). It can therefore be surmised that a high proportion of the present day East Anglian otter population are descendant from captive bred otters. A proportion of the otters analysed from East Anglia expressed either the haplotype Lut1 (18.8%) or Lut6 (12.5%), haplotypes known from analysis of Cornish and Welsh otters to be native British haplotypes. However, the majority of East Anglian otters (68.8%) expressed the haplotype Lut3.

The high presence of the Lut3 haplotype in East Anglia, a population known to be composed of a high proportion of captive released otters, and the absence of Lut3 from other UK populations, suggests that this haplotype is synonymous with the captive bred otters. This confirms what has been observed in other studies of captive bred otters. Mucci *et al.* (1999) analysed two captive otters transferred to Denmark from Britain and found that both expressed the Lut3 haplotype (Pérez-Haro *et al.*, 2005). In addition, reanalysis of sequences obtained from otters transferred from the Norfolk Otter Trust breeding programme to Italy shows that these sequences match the Lut3 haplotype (Ketamier & Bernardini 2005, Genbank accession no. AY860321-AY860328). One exception to this is the finding by Pérez-Haro *et al.* (2005) that a family of otters held in Spain, but recorded as being of English origin, expressed the haplotype Lu6. In conclusion, the Lut3 haplotype appears not to be a native haplotype to Britain, but is likely to be a signature of the captive bred otters released from the Otter Trust captive breeding programme.

Analysis of otters from the River Itchen revealed that the majority (77.8%) express the haplotype Lut1, a haplotype native to British otter populations. However, a small but significant proportion (22.2%) of the otters analysed from the River Itchen expressed the Lut3 haplotype, suggesting that there are otters present on the Itchen that are direct descendants of the otters released from the captive breeding programme.

6.2.2ii) Origins of the Founders of the Captive Breeding Programme

Our analysis of East Anglian otters supports the findings of previous analysis of captive bred otters (Mucci *et al.* 1999; Ketamier & Bernardini 2005; Pérez-Haro *et al.*, 2005), indicating that the haplotype Lut3 can be considered synonymous with captive bred otters. Little is known about the origins of otters used in the Otter Trust captive breeding programme, however, it is known that at an otter brought to England from Indonesia as part of the pet trade was the first female to successfully breed as part of the programme (Paul Chanin, pers. comm.). It is not known whether the descendants of this otter were amongst those released into the wild, or if any further otters of Indonesian origin were integrated into the Otter Trust breeding programme.

The Lut3 haplotype was not observed in any of the populations not thought to have received captive bred otters, suggesting that Lut3 is not a native British haplotype. It is not possible to say for certain that the Lut3 haplotype is not a native British haplotype and that otters used to found the captive breeding programme were not of British descent. However, it is likely that the otters used would have been removed from one of the more populous locations of Britain, namely Scotland, Wales or the south west of England and analysis of animals from each of these regions has so far shown no instances of the Lut3 haplotype. Therefore, based on this analysis, it is unlikely that the otters used to found the Otter Trust breeding programme were of British descent.

It is feasible that the otters used to found the breeding programme may have originated from Europe. Although otter population declines occurred throughout Europe, there were areas where otter populations remained relatively healthy and it is feasible that one of these populations may have been used as a donor population for the captive breeding programme. The results of haplotype analysis indicate that there are at least two locations in Europe in which the haplotype Lut3 is present. Analysis of otters from eastern Germany showed that a large proportion expressed the Lut3 haplotype (Cassens *et al.*, 2000). The Lut3 haplotype is likely to have become more prevalent in this region due to a period of recent population bottlenecking.

One of three otters analysed from Belarus, in Eastern Europe, expressed the Lut3 haplotype (Ferrando *et al.* 2004). The fact that the Lut3 haplotype has been shown to be present in two locations in Eastern Europe suggests that this haplotype may have been preserved in a second refugium in Eastern Europe during the last ice age, before spreading back across Europe after the last glacial period (Cassens *et al.*, 2000; Ferrando *et al.* 2004).

The presence of the haplotype Lut3 in Eastern Europe suggests that this could have been a source of otters used to found the Otter Trust captive breeding programme. However, evidence suggests that at least one of the otters used in the breeding programme originated from Indonesia in Southeast Asia (Paul Chanin, pers. comm.). In order to try and verify this, investigations were made into the likelihood of the haplotype Lut3 being present in otters from Southeast Asia. Samples of Indonesian origin were not readily available. As a substitute, sequences were obtained from otters

of South Korean origin, in order to assess whether the Lut3 haplotype was present in the region.

The analysis of the South Korean otters revealed two novel haplotypes. A minimum-spanning network (see Figure 4.4) indicates that the South Korean haplotypes were most similar to the European haplotypes Lut3 and Lut7, with a minimum of two and three single base mutations between them. This suggests that it is feasible that these haplotypes have arisen through the mutation of the Lut3 haplotype, indicating that there is a significant possibility of Lut3 being present in otters from Southeast Asia.

In conclusion, it is not possible to ascertain from the control region haplotypes that the otters used to found the Otter Trust captive breeding programme did not originate from a native British population. However, the fact that the Lut3 haplotype has not been found in any of the extant native populations and only in those populations influenced by captive releases, suggests that the otters used in the breeding programme were not of British origin. The Lut3 haplotype has been found in two Eastern European populations, suggesting that this location could feasibly have been a source of the otters used to found the captive breeding programme. However, the similarity between South Korean haplotypes and the Lut3 haplotype means that Southeast Asia cannot be ruled out as the origin of the otters used.

6.2.3 Conclusions

The main hypothesis for this section of the project was that:

H_1 The River Itchen otter population shows significant genetic distinction from other local otter populations, in terms of genetic distance, diversity and composition, in a pattern which is indicative of population bottlenecking, the introduction of novel genetic material via the release of captive bred individuals, or a combination of both of these demographic factors.

Evidence of genetic bottlenecking was not evident in the River Itchen otter population. However, the genetic signature of a bottleneck is likely to have been masked by the introduction of novel genetic material via the introduction of captive bred otters. In

addition, if the expansion of the otter populations from the otter stronghold in the south west of England has reached the Itchen population, then the genetic input from these otters may have masked the signature of a bottleneck.

The River Itchen case study has produced genetic evidence to suggest that captive bred otters released onto the River Itchen successfully integrated and bred with the native population, introducing novel genetic material from a distant source. This is evidenced by the higher levels of genetic diversity in the River Itchen population than in the Cornish population, a population native to the south west of England. These high levels of genetic diversity suggest that there has been some kind of genetic input into the previously bottlenecked River Itchen population. Similar patterns of increased genetic diversity were observed for the south Dorset otter population, a population which is also known to have undergone a population bottleneck before receiving releases of otters from the captive breeding programme.

Analysis of mitochondrial control region haplotypes provides evidence that otters descendant from captive bred stock were present in the River Itchen population. The Lut3 haplotype, indicative of captive released otters, was observed for two out of the nine otters analysed. As mitochondrial DNA is maternally inherited the relatively low prevalence of the Lut3 haplotype is likely to represent an underestimate of the true genetic impact of the release of captive bred otters into the river system.

The extent of genetic impact that the release of captive bred otters has had upon the River Itchen population will depend to a large extent on the origins of the otters used to found the captive breeding programme. If the otters used to found the captive breeding programme originated from a source that was too genetically distinct from the native Brittish populations then this may result in the negative impacts of factors such as outbreeding depression in the resulting integrated populations. Investigation into the potential origins of the founding individuals suggests that they were unlikely to have originated from British populations. Given the haplotypes observed, the otters used in the captive breeding population may have originated from Europe, in particular from Eastern Europe. However, analysis of otters from South Korea indicates that the otters used to found the captive breeding programme could have originated from Southeast Asia, as has been suggested by anecdotal information (Paul Chanin, pers. comm.).

6.3 PBDEs in Otters from England and Wales

One of the main reasons for the national decline in otter numbers in the 1950s to 1980s is thought to have been contamination from organohalogens, in particular, dieldrin and PCBs (Chanin & Jefferies, 1978; Simpson, 1998; Ruiz Olmo *et al.*, 2002; Barbosa *et al.*, 2003). PCBs, dieldrin and another major organohalogen contaminant, DDT, have all been banned from use in the UK for several decades. However, due to the environmental persistence of the compounds PCBs and DDTs in particular are still present in relatively high concentrations in the environment.

PBDEs are a more modern organohalogen compound, used to flame retard a wide range of man-made materials. Concerns over environmental persistence, bioaccumulation potential and toxicity have led to recent bans on the manufacture and use of PBDE commercial mixtures. PBDEs are similar in structure to PCBs and they are just as mobile and persistent in the environment, being found in all environmental compartments, including air, water, soils and sediments, as well as in many species of biota (see section *1.3.2 Levels in the Environment*).

The aim of this section of the project was to assess levels of PBDE contamination in otters from across England and Wales. This enabled the assessment of potential sources of contamination, as well as study into possible reasons for variation in the concentrations observed. Concentrations of PCBs and DDTs were also analysed in otter tissue in order that comparisons could be made between body burdens of congeners from different contaminant groups.

6.3.1 PBDEs in Otters

The PBDE concentrations measured in the liver tissue of otters rivalled some of the highest concentrations measured in marine mammal species - species known to build up high concentrations of contaminants due to their position at the top of the marine food chain.

Congener correlation patterns suggest two main sources for BDE congeners. A group of tri- to octa-BDE congeners, including all of the most concentrated lower congeners correlated together, indicating that commercial mixtures of Penta- and Octa-BDE were

the main source of these lower BDE congeners. The second group of congeners to correlate were the nona- and deca-BDE congeners, consistent with the release of the Deca-BDE commercial mixture into the environment. Of note are the relative amounts of these higher BDE congeners, with nona-BDEs generally found in a higher proportion to deca-BDE in the liver tissue of otters compared with the Deca-BDE commercial mixture. This suggests that otters may preferentially take up nona-BDE congeners over the BDE-209 congener. Alternatively, debromination of BDE-209 may be occurring to form nona-BDE congeners, either in the environment, via metabolism in the prey species or in the otters themselves. Althernatively this process may occur in the laboratory during sample processing.

In a pattern consistent with many aquatic species, the otter liver tissue samples showed extremely high concentrations of BDE-47, with comparatively high concentrations of BDE-99 and BDE-100. However, otters also had high concentrations of BDE-153 and BDE-209, a profile that is more consistent with body burdens found in many terrestrial species of top predator. This indicates that otters are picking up PBDE contamination from both the aquatic and terrestrial environments, suggesting that their semi-aquatic lifestyle results in exposure to two intake pathways of PBDE contamination.

6.3.2 Comparison with PCBs and DDTs

Comparison suggests that the PBDE concentrations observed in otters are amongst the highest observed for any species. Mean ΣBDE concentration measured in the liver tissue of otters was approximately equal to the mean ΣDDT concentration and was approximately a quarter of the mean ΣPCB concentration. This would suggest that PBDEs are one of the major organohalogen contaminants in the present day otter population inhabiting England and Wales. In terms of individual congeners, BDE-47, the most concentrated BDE congener measured in the otter liver tissue, was in fact the fourth most concentrated congener overall, with PCB-138, *pp*-DDE and PCB-153 being the only congeners to show higher average concentrations in the otter liver tissue samples analysed. Other BDE congeners, in particular BDE-153 and BDE-209, were also measured at concentrations in the otter liver tissue that were comparable to concentrations measured for PCB and DDT congeners.

Over the last couple of decades PCB congener and DDT breakdown products have plateaued or slowly decreased in concentration in the liver tissue of otters from England and Wales (Simpson, 1998, 2007; Chadwick, 2007). Concentrations of PBDEs in the present day otter population match those of PCBs and DDTs. Without time trend data it is not possible to assess whether PBDE concentrations are still increasing or if they have also plateaued at the concentrations observed. However, given that PCB and DDT concentrations have only reduced marginally over the years, and PBDE concentrations are likely to be close to the maximum concentration that they are likely to have ever been, then it is likely that the concentrations observed in the present day population are close to the most concentrated that organohalogen compounds have even been in otters.

6.3.3 Conclusions

There were two main hypothesis associated with this section of study:

H_1 The otter liver tissues analysed contain significant concentrations of PBDEs, particularly of the BDE congeners most commonly observed in high concentrations in aquatic top predators, at concentrations approaching those observed for other organic pollutants in otter liver tissue, as well as concentrations of PBDEs observed in other aquatic predators.

H_2 The otter liver tissue samples analysed contain significant concentrations of PBDEs, but in a different congener profile to that expected for an aquatic top predator.

The results suggest that in fact both hypothesis are true, with otters expressing a congener concentration pattern typical of an aquatic top predator, as well as an overlying secondary pattern more typical of a terrestrial predator. Otter are likely to have obtained high concentrations of the congeners BDE-47, -99 and -100 from their diet. Most species of fish, the main prey of otters (Clavero *et al.*, 2003; Sales-Luís *et al.*, 2007), contain BDE-47 as the most dominant PBDE congener (Boon *et al.*, 2002; Ikonomou *et al.*, 2002; Covaci *et al.*, 2004), with BDE-99 and BDE-100 also often present at significant concentrations (Boon *et al.*, 2002; Ikonomou *et al.*, 2002; de Boer *et al.*, 2003; Schlabach *et al.*, 2004).

Relatively high concentrations of a range of higher mass BDE congeners, in particular BDE-153 and BDE-209, were observed for the otter liver tissue samples. These are congeners that are not commonly observed for fish and aquatic invertebrate species, suggesting that diet is unlikely to be the source of these contaminants in otters. High BDE-153 and BDE-209 concentrations are more commonly observed in terrestrial top predators (Christensen *et al.*, 2005; Jaspers *et al.*, 2006; Mariussen *et al.*, 2008), suggesting that the otters semi-terrestrial lifestyle is a major factor in the absorption of these congeners. The most likely explanation for the high concentrations of BDE-153, BDE-209 and other higher mass BDE congeners is the direct ingestion of particulate matter of soils and/or sediments (Voorspoels *et al.*, 2006). Heavier congeners tend to be less mobile in the environment and build up to high concentrations in soils and sediments, where the hydrophobic molecules bind to the particulate matter. An otters lifestyle, with the use of holts and couches for resting up during the day and time spent in contact with river beds and river banks, is likely to bring them into regularly contact with soils and sediments. This, combined with the need for them to maintain a healthy, waterproof coat, is likely to lead to a high instance of ingestion of particulate matter, and therefore the ingestion of associated BDE congeners.

The results show that the PBDE concentrations observed in otters are amongst the highest observed for any mammal and are found at similar concentrations in the present day otter population as those of PCBs and DDTs. Therefore, PBDEs are a major contaminant in otters, found at concentrations which should be considered significant in terms of being some of the highest concentrations observed for any species.

6.4 Overall Conclusions

The overall hypothesis of the project was that:

H_1 The genetic diversity of English and Welsh otter populations has been significantly altered by human activities, and concentrations of organic pollutants, including PBDEs, in present day otter populations are such that they should be considered of concern in terms of the future health of English and Welsh otter populations.

6.4.1 Alterations to Genetic Diversity

The first part of this hypothesis is concerned primarily with the impact that human activities have had upon the genetic diversity of English and Welsh otter populations. From demographic data it is known that humans have had two main impacts upon the otter populations of England and Wales. The first was to cause a severe population crash that lasted from the 1950s and only saw populations recovering as late as the 1980s (Chanin & Jefferies, 1978; Crawford, 2003). This is likely to have resulted in a significant loss of genetic diversity from populations across England and Wales, particularly in areas of mainland England which experienced the greatest levels of population decline. In contradiction to this, the results of this study showed the populations most severely affected by the decline in number to be more genetically diverse. This is likely to be due to the signature being masked by more recent population genetic alterations.

The captive breeding programme was set up by the Otter Trust in order to increase otter population sizes in areas of England where otter populations had become particularly small and fragmented. In terms of bolstering numbers the captive release programme was particularly successful, with evidence that several local populations were actually saved from extinction by the releases (Crawford, 2003). However, little is known about the origins of the otters used to found the captive breeding programme and it may be that the introduction of these captive bred otters has undermined the integrity of the resulting wild populations.

Evidence from this study indicates that captive bred otters released into wild populations have successfully integrated and bred with the native otters. This appears to have led initially to a significant increase in genetic diversity of the populations. On the face of it this would appear to be a good result, with greater genetic diversity in theory denoting a healthier population. However, the extent of the increase in genetic diversity suggests that the otters used to found the captive breeding population were from a population genetically distinct from the native British populations receiving the releases. This raises questions over whether factors such as outbreeding depression are likely to become an issue if problems with genetic incompatibility arise. This is a fundamental issue in conservation genetics - how distant is too distant and at what point do populations start to become genetically incompatible?

Information regarding the origin of the otters used in the captive breeding programme is not freely available, however, it is thought that at least one of the breeding females originated from Indonesia in Southeast Asia (Paul Chanin, pers. comm.). Analysis of DNA sequences from the mitochondrial control region suggests that the founders of the captive breeding population were unlikely to have been of British origin, but may have originated from Eastern Europe, or from Southeast Asia.

The results of population genetic analysis indicate that human activities have significantly impacted upon populations in England in particular, with those populations which suffered greatest during the declines also being affected by the release of captive bred otters. This has led to significant alterations to the genetics of populations, with those populations greatest affected actually showing increased levels of genetic diversity, but at an unknown future cost to the genetic integrity and health of future generations.

6.4.2 Effects of Organic Pollutants

The second part of the hypothesis concerns the potential threat to English and Welsh otter populations from organohalogen pollutants. A suite of contaminants, including PCBs, DDTs and other organochlorine pesticides are already routinely monitored in English and Welsh otters (Simpson, 1998, 2007; Chadwick, 2007). As yet PBDEs have not been measured, yet these persistent pollutants are fast becoming major contaminants in many environmental samples and species of biota (see section *1.3.2 Levels in the Environment*).

Analysis of PBDE concentrations in the liver tissue of otters from England and Wales indicates that they are present at concentrations which should be considered a significant concern. The ΣBDE concentrations observed in the otter liver tissue samples rival some of the highest concentrations observed for any species. It is perhaps not surprising that as a top aquatic predator otters contain particularly high concentrations of the range of congeners commonly observed in fish and aquatic invertebrate species. What is perhaps more surprising is that otters show high levels of congeners more commonly associated with terrestrial top predators, suggesting that the otters semi-terrestrial lifestyle is having a significant influence upon levels of PBDE uptake. As

only a small percentage of an otter's diet is made up of terrestrial prey, the high BDE-153 and BDE-209 concentrations observed indicate that the otters are picking these congeners up from a more direct source. One possible explanation is that otters may be absorbing PBDE contamination via the intake of particulate matter, whilst grooming or through some other manor of ingestion.

It is difficult to make predictions regarding the implications of this high level of PBDE contamination on the health of otter populations. Studies of toxicity indicate that PBDEs are generally less toxic that PCBs. However, little is known about the additive effect when biota are exposed to doses of a range of organohalogen compounds. As PCBs and PBDEs target similar biological systems and elicit similar toxicological responses it is likely that PBDE contamination will affect otter health by increasing the effective toxicity of PCB contamination.

Analysis of otter liver tissue samples for a range of organic pollutants indicated that ΣBDE concentrations are similar to those of ΣDDT concentrations and are approaching those of ΣPCB concentrations. In terms of individual congeners, several BDEs were found among the top most concentrated congeners measured in otters. PBDEs are now one of the most concentrated organic pollutants measured in liver tissue samples from the present day otter population of England and Wales. Although less toxic that PCBs, the PBDE concentrations observed have the potential to negatively impact upon the health of the otter population of England and Wales, particularly if they act in an additive manor with other organic pollutants found in the tissue of otter.

6.4.3 Combined Impact

Lack of genetic diversity in a population and the levels of persistent organic pollutants (POPs) it is exposed to can both potentially impact upon the health of the population. However, these factors also have the potential to interact with each other to further complicate the overall effect observed in a population. The interactions can be viewed either in terms of the impacts that population genetic changes will have upon the ability of a population to cope with organohalogen pollutants, or in terms of the impacts that these POP challenges have upon the genetics of a population, but more likely as a combination of both.

6.4.3i) Effects of POPs on Genetic Diversity

Contamination by POPs can have significant impacts at the population level, leading to reductions in genetic diversity. In the most severe cases, individuals in a population will die as a result of contaminant burdens, negating any future breeding potential of those individuals. If a large enough proportion of a population is lost as a result of contamination, then this will lead to a wide-scale loss of genetic diversity within the affected population.

Contamination events do not always consist of point sources of pollution leading to the death of the individuals affected. In terms of contamination by many POPs it is more likely that concentrations build up gradually in the environment, gradually increasing the toxicological pressures on populations. Many such compounds cause endocrine disruption and other toxic effects at a scale which may cause problems with fecundity, without necessarily causing large-scale loss of life. Reductions in the fecundity of individuals can be caused by direct alterations to the reproductive system itself (Meerts *et al.*, 2001; Tseng *et al.*, 2006), or by alterations to systems, such as the neurological systems, which lead to alterations in behaviour and/or development (Viberg *et al.*, 2003, 2004; Branchi *et al.*, 2005). All of these effects have the potential to limit reproductive output, either in terms of breeding or in terms of being capable of successfully raising young to adulthood. The population level effects of a loss in fecundity are generally more gradual, but over time can lead to a loss of genetic diversity in the population as fewer individuals manage to breed successfully.

The decline in otter numbers observed across areas of the UK and Europe in the 1950s to 1980s have been linked both to levels of dieldrin (Chanin & Jefferies, 1978; Simpson *et al.*, 2000) and to levels of PCB contamination (Roos *et al.*, 2001; Ruiz Olmo *et al.*, 2002; Barbosa *et al.*, 2003), suggesting that one or both of these pollutants was responsible for the widespread population declines observed. The declines in otter numbers were severe and were therefore likely to have resulted in a significant reduction in genetic diversity. This suggests that pollution by POPs has already led to large-scale population declines and resultant reductions in genetic diversity in many of

the otter populations across mainland England, as well as in other locations around Europe.

6.4.3ii) Effect of Genetic Diversity on a Population's Ability to Cope with POPs

The toxicological and endocrine disrupting potential of persistent organic pollutants (POPs), such as PCBs, PBDEs and DDTs, means that exposure to these compounds can be considered a challenge to the health of a population. It is therefore necessary, when considering the health of a polluted population, to consider the impacts that variation in genetic diversity will have upon the ability of the population to cope with the challenge of the contaminant burden.

During the period of national otter population declines many otter populations, across mainland England in particular where populations declined to the greatest extent, are likely to have experienced a severe decline in genetic diversity. This lowering of levels of genetic diversity may lower a population's ability to respond effectively to challenges, such as those posed by high concentrations of POPs. In other words, if the genetic information required to help otters survive contamination is lost from a population, then none of the otters from that population will be able to survive given an occurrence of high levels of POPs in the environment. Alternatively, as the genetic input from those individuals less able to cope with the challenge of POPs is lost, this may lead to selection of genetic material predisposing individuals in the resulting population to better cope with future contamination burdens.

The results of this study suggest that the introduction of captive bred otters significantly increased the genetic diversity of recipient populations. In theory this will have increased the chances of otters in these populations being able to withstand the challenge of high concentrations of POPs. However, the evidence suggests that the otters used in the captive breeding programme did not originate from a British source population and may even have come from as far away as Southeast Asia. If the otters used to found the captive breeding population were too genetically distinct from the English otter populations which were recipient of captive bred otters, this may have led to problems with outbreeding depression. This is particularly likely to be the case given

the fact that both populations, the captive bred and the recipient wild populations, are likely to have been suffering from the effects of low genetic diversity and inbreeding depression. Problems will arise if the genetic information from the two populations is incompatible, reducing the fitness of the resulting merged populations.

In summary, the reduction in genetic diversity is likely to have reduced the ability of otter populations to cope with the challenges of POP contamination. Increases in genetic diversity resulting from the introduction of captive bred otters may have gone some way to redress this balance, increasing the likelihood that a population will contain the genetic information necessary to meet the challenges. However, potential problems of genetic incompatibility and outbreeding may ultimately decrease the genetic health of populations further and reduce the ability of a population to meet the demands of exposure to pollutants such as PCBs, PBDEs and DDTs.

6.4.3iii) Conclusions

The decline in otter numbers observed in the 1950s to1980s is likely to have resulted from exposure of populations to the toxic effects of organic pollutants, in particular PCBs and/or dieldrin (Chanin & Jefferies, 1978; Roos *et al.*, 2001; Barbosa *et al.*, 2003). This reduction in the numbers of otters present in the populations will in turn have led to a loss of genetic diversity. In theory this loss of genetic diversity will have made the populations more susceptible to future POP contamination. However, it should be noted that the selective pressures of the original POP challenge may have led to the selection in the surviving otters of traits enabling them to better survive future contamination. In other words, the otters lost from the population are likely to be those most susceptible to the toxicological effects of organic pollutants, resulting in a surviving population which is more robust in the face of high levels of contamination. This process may have included the selection of genetic traits associated with the increased survival of organic pollutant challenges.

The introduction of captive bred otters has increased the genetic diversity of the resulting otter populations. However, it is unclear whether problems with outbreeding depression and genetic incompatibility will ultimately lead to problems with the genetic integrity and ultimate survival of these populations.

High concentrations of PBDEs were observed in liver tissue samples collected from present day English and Welsh otter populations. These high concentrations of PBDEs, in addition to high remnant concentrations of other organic pollutants, such as PCBs and DDTs, indicates that otter populations of England and Wales may face future population reductions due to toxicological responses to the high combined organic pollutant concentrations observed.

The ultimate aim of this study was to assess whether there is a link between low levels of genetic diversity and the concentrations of POPs, in particular PBDEs, in the present day otter population of England and Wales. Unfortunately, it was not possible to obtain the sample numbers required for such a comparison, as is often the case with studies of mammals which generally have small population sizes inhabiting a relatively large area. This study has shown that the genetic integrity of the present day otter populations, in particular those inhabiting areas of mainland England, may have been compromised by the introduction of otters from an outside source through the release of otters from the captive breeding programme. In addition, high concentrations of a range of PBDE congeners are likely to be adding to the toxicological pressures faced by otters already exposed to relatively high concentrations of other organic pollutants, such as PCBs and DDTs. The question of interaction between low levels of genetic diversity and susceptibility of populations to the challenges faced from the endocrine disrupting and toxicological potential of organic pollutants is still relevant for the continued survival of English and Welsh otter populations.

References

Aasen E. and Medrano J.F. (1990) Amplification of the ZFY and ZFX genes for sex identification in humans, cattle, sheep and goats. *Bio/Technology.* 8: 1279-1281.

Achmann R., Curik I., Dovc P., et al., (2004) Microsatellite diversity, population subdivision and gene flow in the Lipizzan horse. *Animal Genetics.* 35: 285-292.

Adámek Z., Kortan D., Lepič P. and Andreji J. (2003) Impacts of otter (*Lutra lutra* L.) predation on fish ponds: A study of fish remains at ponds in the Czech Republic. *Aquiculture International.* 11: 389-396.

Aguilar A. and Borrell A. (2005) DDT and PCB reduction in the western Mediterranean from 1987 to 2002, as shown by levels in striped dolphins (*Stenella coeruleoalba*). *Marine Environmental Research.* 59: 391-404.

Alcock R.E., Sweetman A.J., Prevedouros K. and Jones K.C. (2003) Understanding levels and trends of BDE-47 in the UK and North America: an assessment of principle reservoirs and source inputs. *Environment International.* 29: 691-698.

Aljanabi S.M. and Martinez I. (1997) Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Research.* 25: 4692-4693.

Allchin C.R., Law R.J. and Morris S. (1999) Polybrominated diphenylethers in sediments and biota downstream of potential sources in the UK. *Environmental Pollution.* 105: 197-207.

Allchin C. and de Boer J. (2001) *Organohalogen Compounds.* 52:30.

Anderson D.M., Gilbert P.M. and Burkholder J.M. (2002) Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. *Estuaries.* 25: 704-726.

Andrén H. and Delin A. (1994) Habitat selection in the Eurasian red squirrel, Sciurus vulgaris, in relation to forest fragmentation. *OIKOS.* 70: 43-48.

Arrendal J., Walker C.W., Sundqvist A.K., Hellborg L. and Vilà C. (2004) Genetic evaluation of an otter translocation program. *Conservation Genetics.* 5: 79-88.

Arrendal J., Vilà C., Björklund M. (2007) Reliability of noninvasive genetic census of otters compared to field censuses. *Conservation Genetics.* 8: 1097-1107.

Ayoub A.T. (1999) Fertilizers and the environment. *Nutrient Cycling in Agroecosystems.* 55: 117-121.

Bachmann B., Lüke W. and Hunsmann G. (1990) Improvement of PCR amplified DNA sequencing with the aid of detergents. *Nucleic Acids Research.* 18: 1309-1309.

Bäcklin B.-M. and Bergman A. (1992) Morphological aspects on the reproductive organs in female mink (*Mustela vison*) exposed to polychlorinated biphenyls and fractions thereof. *AMBIO.* 21: 596-601.

Balch G.C., Vélez-Espino L.A., Sweet C., Alaee M. and Metcalfe C.D. (2006) Inhibition of metamorphosis in tadpoles of *Xenopus laevis* exposed to polybrominated diphenyl ethers (PBDEs). *Chemosphere.* 64: 328-338.

Baldridge M.G., Stahl R.L., Gerstenberger S.L., Tripoli V. and Hutz R.J. (2003) Modulation of ovarian follicular maturation in Long-Evans rate exposed to polychlorinated biphenyls (PCBs) *in-utero* and lactationally. *Reproductive Toxicology.* 17: 567-573.

Baliraine F.N., Bonizzoni M., Guglielmino C.R., *et al.*, (2004) Population genetics of the potentially invasive African fruit fly species, *Ceratitis rosa* and *Ceratitis fasciventris* (Diptera: Tephritidae). *Molecular Ecology.* 13: 683-695.

Barbosa A.M., Real R., Márquez L. and Rendón M.Á. (2001) Spatial, environmental and human influences on the distribution of otter (*Lutra lutra*) in the Spanish provinces. *Diversity and Distribution.* 7: 137-144.

Barbosa A.M., Real R., Olivero J. and Vargas J.M. (2003) Otter (*Lutra lutra*) distribution modeling at two resolution scales suited to conservation planning in the Iberian Peninsula. *Biological Conservation.* 114: 377-387.

Basu N., Scheuhammer A.M. and O'Brien M. (2007) Polychlorinated biphenyls, organochlorinated pesticides, and polybrominated diphenyl ethers in the cerebral cortex of wild river otters (*Lontra canadensis*). *Environmental Pollution.* 149: 25-30.

Beheler A.S., Fike J.A., Dharmarajan G., Rhodes O.E. and Serfass T.L. (2005) Ten new polymorphic microsatellite loci for North American river otters (*Lontra canadensis*) and their utility in related mustelids. *Molecular Ecology Notes.* 5: 602-604.

Bellemain E. and Taberlet P. (2004) Improved noninvasive genotyping method: Application to brown bear (*Ursus arctos*) faeces. *Molecular Ecology.* 4: 519-522.

Bergman A., Bäcklin B.-M., Järplid B., Grimelius L. and Wilander E. (1992) Influence of commercial polychlorinated biphenyls and fractions thereof on liver histology in female mink (*Mustela vison*). *AMBIO.* 21: 591-595.

Bernatchez L. and Duchesne P. (2000) Individual-based genotype analysis in studies of parentage and population assignment: How many loci, how many alleles? *Canadian Journal of Fish and Aquatic Science.* 57: 1-12.

Berteaux D., Duhamel R. and Bergeron J. (1994) Can radio collars affect dominance relationships in Microtus? *Canadian Journal of Zoology.* 72: 785-789.

Beveridge M. and Simmons L.W. (2006) Panmixia: an example from Dawson's burrowing bee (*Amegilla dawsoni*)(Hymenoptera: Anthophorini). *Molecular Ecology.* 15: 951-957.

Bijlsma R., van de Vliet M., Pertoldi C., van Apeldoorn R.C. and van de Zande L. (2000) Microsatellite primers from the Eurasian badger, *Meles meles*. *Molecular Ecology.* 9: 2216.

Blundell G.M., Ben-David M., Groves P., Bowyer R.T. and Geffen E. (2002) Characteristics of sex-biased dispersal and gene flow in coastal river otters: implications for natural recolonization of extirpated populations. *Molecular Ecology.* 11: 289-303.

Bonesi L., Strachan R. and Macdonald D.W. (2006) Why are there fewer signs of mink in England? Considering multiple hypothesis. *Biological Conservation.* 130: 268-277.

Boon J.P., Lewis W.E., Tjoen-A-Choy A.R., *et al.*, (2002) Levels of polybrominated diphenyl ether (PBDE) flame retardants in animals representing different trophic levels of the North Sea food web. *Environmental Science and Technology.* 36: 4025-4032.

Boyle E.E., Zardus J.D., Chase M.R., Etter R.J. and Rex M.A. (2004) Strategies for molecular genetic studies of preserved deep-sea macrofauna. *Deep-Sea Research Part I - Oceanographic Research Papers.* 51: 1319-1336.

Branchi I., Alleva E. and Costa L.G. (2002) Effects of perinatal exposure to a polybrominated diphenyl ether (PBDE 99) on mouse neurobehavioural development. *NeuroToxicology.* 23: 375-384.

Branchi I., Capone F., Vitalone A., *et al.*, (2005) Early developmental exposure to BDE 99 or Aroclor 1254 affects neurobehavioural profile: interference from the administration route. *NeuroToxicity.* 26: 183-192.

Braune B.M., Mallory M.L., Gilchrist H.G., Letcher R.J. and Drouillard K.G. (2007) Levels and trends of organochlorines and brominated flame retardants in Ivory Gull eggs from the Canadian Arctic, 1976 to 2004. *Science of the Total Environment.* 378: 403-417.

Breitburg D. (2002) Effects of hypoxia, and the balance between hypoxia and enrichment, on coastal fishes and fisheries. *Estuaries.* 25: 767-781.

Bromine Science and Environmental Forum (BSEF) (2006) Fact sheet: applications of Deca-BDE. www.bsef.com

Bromine Science and Environmental Forum (BSEF) (2007) Fact sheet: Deca-BDE decabromodiphenyl ether. www.bsef.com

Bromine Science and Environmental Forum (BSEF) (2008) www.bsef.com

Broquet T. and Petit E. (2004) Quantifying genotyping errors in noninvasive population genetics. *Molecular Ecology.* 13: 3601-3608.

Broquet T., Ménard N. and Petit E. (2007) Noninvasive population genetics; a review of sample source, diet, fragment length and microsatellite motif effects on amplification success and genotyping error rates. *Conservation Genetics.* 8: 249-260.

Brownstein M.J., Carpten J.D. and Smith J.R. (1996) Modulation of non-templated nucleotide addition by *Taq* DNA polymerase: primer modifications that facilitate genotyping. *BioTechniques*. 20: 1004-1010.

Bruford M.W., Hanotte O., Brookfield J.F.Y. and Burke T. (1998) Multilocus and single-locus DNA fingerprinting. In: *Molecular Genetic Analysis of Populations: A Pracical Approach, 2nd edition.* Ed Hoelzel A. R. IRL Press, Oxford. pp. 287-336.

Buskirk S.W. and Lindstedt S.L. (1989) Sex biases in trapped samples of mustelidae. *Journal of Mammology.* 70: 88-97.

Camargo J.A. and Alonso Á. (2006) Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. *Environment International.* 32: 831-849.

Carr S.M. (2002) www.mun.ca/biology/scarr/DNA_Forensics_PopGen.htm

Cassens I., Tiedemann R., Suchentrunk F. and Hartl G.B. (2000) Mitochondrial DNA variation in the European otter (*Lutra lutra*) and the use of special autocorrelation analysis in conservation. *Journal of Heredity.* 91:31-35.

Chadwick E.A. (2007) *Post mortem* study of otters in England and Wales 1992-2003. *Environment Agency, Science Report SC010065/SR.*

Chanin P.R.F. and Jefferies D.J.J. (1978) The decline of the otter *Lutra lutra* L. in Britain: an analysis of hunting records and discussion of causes. *Biological Journal of the Linnean Society.* 10: 305-328.

Chanin P. (1981) The diet of the otter and its relations with the feral mink in two areas of Southwest England. *Acta Theriologica.* 26: 83-95.

Chanin (1985) *The Natural History of Otters.* Croom Helm, London.

Chanin P. (2004) River Itchen sustainability study: DNA fingerprinting of otters 2003. *Report.*

Chen J.-P., Sun D.-J., Dong C.-Z., *et al.*, (2005) Genetic analysis of four wild chum salmon *Oncorhynchus keta* populations in China based on microsatellite markers. *Environmental Biology of Fishes.* 73: 181-188.

Christensen J.R., MacDuffee M., MacDonald R.W., Whiticar M. and Ross P. (2005) Persistent organic pollutants in British Columbia grizzly bears: consequence of divergent diets. *Environmental Science and Technology.* 39: 6952-6960.

Clarke G.P., White P.C.L. and Harris S. (1998) Effects of roads on badger *Meles meles* populations in south-west England. *Biological Conservation.* 86: 117-124.

Clavero M., Prenda J. and Delibes M. (2003) Trophic diversity of the otter (*Lutra lutra* L.) in temperate and Mediterranean freshwater habitats. *Journal of Biogeography.* 30: 761-769.

Clutton-Brock T.H. (1989) Female transfer and inbreeding avoidance in social mammals. *Nature.* 337: 70-71.

Coltman D.W., Pilkington J.G. and Pemberton J.M. (2003) Fine-scale genetic structure in a free-living ungulate population. *Molecular Ecology.* 12: 733-742.

Copp G. H. and Roche K. (2003) Range and diet of Eurasian otters *Lutra lutra* (L.) in the catchment of the River Lee (south-east England) since re-introduction. *Aquatic Conservation - Marine and Freshwater Ecosystems.* 13: 65-76.

Couceiro L., Míguez A., Ruiz J.M. and Barreiro R. (2008) Introduced status of *Cyclope neritea* (Gastropoda, Nassariidae) in the NW Iberian Peninsula confirmed by mitochondrial sequencing data. *Marine Ecology Progress Series.* 354: 141-146.

Covaci A., Bervoets L., Hoff., *et al.*, (2004) PBDEs in freshwater mussels and fish from Flanders, Belgium. *Organohalogen Compounds.* 66: 3848-3855.

Coxon K., Chanin P., Dallas J. and Sykes T. (1999) The use of DNA fingerprinting to study the population dynamics of otters (*Lutra lutra*) in southern Britain – a feasibility study. *Environment Agency Report. TR W202.*

Coxon K., Chanin P. (2003) DNA extraction from otter spraint and tissue samples. *Environment Agency Report. W1-025.*

Coyer J.A., Peters A.F., Stam W.T. and Olsen J.L. (2003) Post-ice age recolonization and differentiation of *Fucus serratus* L. (Phaeophyceae; Fucaceae) populations in Northern Europe. *Molecular Ecology.* 12: 1817-1829.

Crawford A. (2003) Fourth otter survey of England 2000-2002. *Environment Agency Report W1-061/TR*

Creel S., Spong G., Sands J.L., *et al.*, (2003) Population size estimation in Yellowstone wolves with error-prone noninvasive microsatellite genotypes. *Molecular Ecology.* 12: 2003-2009.

Dallas J.F. and Piertney S.B. (1998) Microsatellite primers for the Eurasian otter. *Molecular Ecology.* 7: 1248-1251.

Dallas J.F., Bacon P.J., Carss D.N., *et al.* (1999) Genetic diversity in the Eurasian otter, *Lutra lutra*, in Scotland. Evidence from microsatellite polymorphism. *Biological Journal of the Linnean Society.* 68: 73-86.

Dallas J.F., Carss D.N., Marshall F., *et al.* (2000) Sex identification of the Eurasian otter *Lutra lutra* by PCR typing of spraints. *Conservation Genetics.* 1: 181-183.

Dallas J.F., Marshall F., Piertney S.B., Bacon P.J. and Racey P.A. (2002) Spacially restricted gene flow and reduced microsatellite polymorphism in the Eurasian otter *Lutra lutra* in Britain. *Conservation Genetics.* 3: 15-29.

Dallas J.F., Coxon K.E., Sykes T., *et al.* (2003) Similar estimates of population genetic composition and sex ratio derived from carcasses and faeces of Eurasian otter *Lutra lutra*. *Molecular Ecology.* 12: 275-282.

Darnerud P.O., Eriksen G.S., Jóhannesson T., Larsen P.B. and Viluksela M. (2001) Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. *Environmental Health Perspectives.* 109: 49-68.

Davis C.S. and Strobeck C. (1998) Isolation, variability and, cross-species amplification of polymorphic microsatellite loci in the family Mustelidae. *Molecular Ecology.* 7: 1776-1778.

de Boer J. and Allchin C. (2001) *Organohalogen Compounds.* 52:13.

de Boer J., Wester P.G., van der Horst A. and Leonards P.E.G.(2003) Polybrominated diphenyl ethers in influents, suspended particulate matter, sediments, sewage treatment plant and effluents and biota from the Netherlands. *Environmental Pollution.* 122: 63-74.

de Boer J., Leslie H.A., Leonards P.E.G., *et al.*, (2007) Screening and time trend study of decabromodiphenylether and hexabromodiphenylether in birds. *Proceedings Forth International Workshop on Brominated Flame Retardants.*

de Wit C.A., Alaee M. and Muir D.C.G. (2006) Levels and trends of brominated flame retardants in the Arctic. *Chemosphere.* 64: 209-233.

de Wit C.A., Nylund K., Eriksson U., *et al.*, (2007) Brominated flame retardants in sludge from 50 sewage treatment plants: evidence of anaerobic degradation of HBCD and TBBPA. *Proceedings Forth International Workshop on Brominated Flame Retardants.*

Deuter R., Pietsch S., Hertel S. and Müller O. (1995) A method for preparation of fecal DNA suitable for PCR. *Nucleic Acids Research.* 23: 3800-3801.

Drake J. W., Charlesworth B., Charlesworth D. and Crow J. F. (1998) Rates of spontaneous mutation. *Genetics.* 148: 1667-1686.

Durbin L.S. (1998) Habitat selection by five otters *Lutra lutra* in rivers of northern Scotland. *Journal of Zoology, London.* 245: 85-92.

Edwards C.J., Dolf G., Looft C., Loftus R.T. and Bradley D.G. (2000) Relationships between the endangered Pusteraler-Sprinzen and three related European cattle breeds as analysed with 20 microsatellite loci. *Animal Genetics.* 31: 329-332.

Effenberger S. and Suchentrunk F. (1999) RFLP analysis of the mitochondrial DNA of otters (*Lutra lutra*) from Europe – implications for conservation of a flagship species. *Biological Conservation.* 90: 229-234.

Efford M. (1998) Demographic consequences of sex-biased dispersal in a population of brushtail possums. *Journal of Animal Ecology.* 67: 503-517.

Ehrich D. and Stenseth N.C. (2001) Genetic structure of Siberian lemmings (*Lemmus sibiricus*) in a continuous habitat: large patches rather than isolation by distance. *Heredity.* 86: 716-730.

Eriksson J., Green N., Marsh G. and Bergman A. (2004) Photochemical decomposition of 15 polybrominated diphenyl ether congeners in methanol/water. *Environmental Science and Technology.* 38: 3119-3125.

Erlinge S. (1967) Home range of the otter *Lutra lutra* L. in Southern Sweden. *OIKOS.* 18: 186-209.

Ernest H.B., Penedo M.C.T., May B.P., Syvanen M. and Boyce W.M. (2000) Molecular tracking of mountain lions in the Yosemite Valley region in California: genetic analysis using microsatellites and faecal DNA. *Molecular Ecology.* 9: 433-441.

Esposito D.L., Palmirotta R., Verì M.C., *et al.* (1998) Optimized PCR labeling in mutational and microsatellite analysis. *Clinical Chemistry.* 44: 1381-1387.

Estoup A., Garnery L., Solignac M. and Cornuet J.–M. (1995) Microsatellite variation in honey bee (*Apis mellifera* L.) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models. *Genetics.* 140: 679-695.

Fabrellas B., Larrazabal D., Martinez M.A., Eljarrat E. and Barcelo D. (2004) Presence of polybrominated diphenyl ethers in Spanish sewage sludges: important contribution of Deca-BDE. *Organohalogen Compounds.* 66: 3706-3711.

Fang L., Huang J., Yu G. and Wang L. (2008) Photochemical degredation of six polybrominated diphenyl ether congeners under ultraviolet irradiation in hexane. *Chemosphere.* 71: 258-267.

Felsenstein F. (1995) PHYLIP (Phylogeny Inference Package; version 3.6). http://evolution.genetics.washington.edu/phylip.html

Fernando P. and Melnick D.J. (2001) Molecular sexing eutherian mammals. *Molecular Ecology Notes.* 1: 350-353.

Fernie K.J., Shutt L., Mayne G., *et al.*, (2005) Exposure to polybrominated diphenyl ethers (PBDEs): changes in thyroid, vitamin A, glutathione homeostasis, and oxidative stress in American kestrels (*Falco sparverius*). *Toxicological Sciences.* 88: 375-383.

Ferrando A., Pons M., Marmi J. and Domingo-Roura X. (2004) Eurasian otters, *Lutra lutra*, have a dominant mtDNA haplotype from the Iberian peninsula to Scandinavia. *Journal of Heredity.* 95: 430-435.

Field A. (2005) *Discovering statistics using SPSS. 2nd Edition.* Saga, London.

Fleming M.A., Ostrander E.A. and Cook J.A. (1999) Microsatellite markers for American mink (*Mustela vison*) and ermine (*Mustela erminea*). *Molecular Ecology.* 8: 1351-1362.

Fortin C., Beauchamp G., Dansereau M., Larivière N. and Bélanger D. (2001) Spatial variation in mercury concentrations in wild mink and river otter carcasses from the James Bay Territory, Québec, Canada. *Archives of Environmental Contamination and Toxicology.* 40: 121-127.

Frantzen M.A.J., Silk J.B., Ferguson J.W.H., Wayne R.K. and Kohn M.H. (1998) Empirical evaluation of preservation methods for faecal DNA. *Molecular Ecology.* 7: 1423-1428.

Frantz A.C., Pope L.C., Carpenter P.J., *et al.*, (2003) Reliable microsatellite genotyping of the Eurasian badger (*Meles meles*) using faecal DNA. *Molecular Ecology.* 12: 1649-1661.

Frantzen M.A.J., Ferguson J.W.H. and de Villiers M.S. (2001) The conservation role of captive African wild dogs (*Lycaon pictus*). *Biological Conservation.* 100: 253-260.

Fuller S.J., Bull C.M., Murray K. and Spencer R.J. (2005) Clustering of related individuals in a population of Australian lizard, *Egernia frerei*. *Molecular Ecology.* 14: 1207-1213.

Galan M., Cosson J.F., Aulagnier S., *et al.* (2003) Cross-amplification tests of ungulate primers in roe deer (*Capreolus capreolus*) to develop a multiplex panel of 12 microsatellite loci. *Molecular Ecology Notes* 3: 142-146.

Gama A.C., Sanatcumar P., Viana P., Barceló D. and Bordado J.C. (2006) The occurrence of polybrominated diphenyl ethers in river and coastal biota from Portugal. *Chemosphere.* 64: 306-310.

Garg S.K. and Bhatnagar A. (1999) Effect of different doses of organic fertilizer (cow dung) on pond productivity and fish biomass in stillwater ponds. *Journal of Applied Ichthyology.* 15: 10-18

Gauthier L.T., Hebert C.E., Weseloh C.D.V. and Letcher R.J. (2007) Decabromodiphenyl ether (BDE-209) and degredation products in the eggs of herring gulls (*Larus argentatus*) from the Laurentian Great Lakes of North America. *Proceedings Forth International Workshop on Brominated Flame Retardants.*

Goodman S.J., Tamate H.B., Wilson R., *et al.* (2001) Bottlenecks, drift and differentiation: The population structure and demographic history of sika deer (*Cervus nippon*) in the Japanese archipelago. *Molecular Ecology.* 10: 1357-1370.

Griffiths R. and Tiwari B. (1993) Primers for the differential amplification of the sex-determining region Y gene in a range of mammal species. *Molecular Ecology.* 2: 405-406.

Guo S. W. and Thompson E. A. (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics.* 48: 361-372.

Gutiérrez-Espeleta G.A., Kalinowski S.T., Boyce W.M. and Hendrick P.W. (2000) Genetic variation and population structure in desert bighorn sheep: implications for conservation. *Conservation Genetics.* 1: 3-15.

Hájková P., Zemanová B., Bryja J., *et al.*, (2006) Factors affecting success of PCR amplification of microsatellite loci from otter faeces. *Molecular Ecology Notes.* 6: 559-562.

Hájková P., Pertoldi C., Zemanová B., *et al.* (2007) Genetic structure and evidence for recent population decline in Eurasian otter populations in the Czech and Slovak Republics: implications for conservation. *Journal of Zoology.* 272: 1-9.

Håkansson H., Manzoor E. and Ahiborg U.G. (1992) Effects of technical PCB preparations and fractions thereof on vitamin A levels in the mink (*Mustela vison*). *AMBIO.* 21: 588-590.

Hale M.L., Lurz P.W.W., Shirley M.D.F., *et al.* (2001) Impact of landscape management on the genetic structure of red squirrel populations. *Science.* 293: 2246-2248.

Hall A.J., Kalantzi O.I. and Thomas G.O. (2003) Polybrominated diphenyl ethers (PBDEs) in grey seals during their first year of life – are they thyroid hormone endocrine disrupters? *Environmental Pollution.* 126: 29-37.

Hallgren S., Sinjari T., Håkansson H. and Darnerud P.O. (2001) Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. *Archives of Toxicology.* 75: 200-208.

Hallgren S. and Darnerud P.O. (2002) Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats – testing interactions and mechanisms for thyroid hormone effects. *Toxicology.* 177: 227-243.

Handt O., Höss M., Krings M. and Pääbo S. (1994) Ancient DNA: Methodological challenges. *Experientia.* 50: 524-529.

Hansen M.M. and Jacobsen L. (1999) Identification of mustelid species: otter (*Lutra lutra*), American mink (*Mustela vison*) and polecat (*Mustela putorius*), by analysis of DNA from faecal samples. *Journal of Zoology, London.* 247: 177-181.

Hansen H., Ben-David M. and McDonald D.B. (2008) Effects of genotyping protocols on success and errors in identifying individual river otters (*Lontra canadensis*) from their faeces. *Molecular Ecology Resources.* 8: 282-289.

Harrad S., Hazrati S., Ibarra C. (2006) Concentrations of polychlorinated biphenyls in indoor air and polybrominated diphenyl ethers in indoor air and dust in Birmingham, United Kingdom: Implications for human exposure. *Environmental Science and Technology.* 40: 4633-4638.

Harrad S., Ibarra C., Diamond M., *et al.*, (2008) Polybrominated diphenyl ethers in domestic indoor dust from Canada, New Zealand, United Kingdom and United States. *Environment International.* 34: 232-238.

Hartmann P.C., Burkhardt P. and Giger W. (2007) Occurrence of polybrominated diphenyl ethers (PBDEs) in brown trout bile and liver from Swiss rivers. *Environmental Pollution.* 146: 107-113.

Hauer S., Ansorge H. and Zinke O. (2002a) Mortality patterns of otters (*Lutra lutra*) from eastern Germany. *Journal of Zoology, London.* 256: 361-368.

Hauer S., Ansorge H. and Zinke O. (2002b) Reproductive performance of otters *Lutra lutra* (Linnaeus, 1758) in Eastern Germany: low reproductive in a long-term strategy. *Biological Journal of the Linnean Society.* 77: 329-340.

Heggberget T.M. and Moseid K.-E. (1994) Prey selection in coastal Eurasian otters *Lutra lutra*. *Ecography.* 17: 331-338.

Herzke D., Berger U., Nygård T., Vetter W. (2003) Organochlorines, organobromines and their metabolites in eggs of Norwegian birds of prey. *Organohalogen Compounds.* 61: 466-469.

Hewitt G. (1999) Post glacial re-colonisation of European biota. *Biological Journal of the Linnean Society.* 68: 87-112.

Hewitt G. (2000) The genetic legacy of the Quaternary ice ages. *Nature.* 405: 907-913.

Hoff-Olsen P., Mevåg B., Staalstrøm E., *et al.* (1999) Extraction of DNA from decomposed human tissue. An evaluation of five extraction methods for short tandem repeat typing. *Forensic Science International.* 105: 171-183.

Hooper K., Holden A. and She J. (2004) Deca-BDE: old myths, new realities. *Proceedings Third International Workshop on Brominated Flame Retardants.*

Höss M. and Pääbo S. (1993) DNA extraction from Pleistocene bones by a silica-based purification method. *Nucleic Acids Research.* 21: 3913-3914.

Howarth R.W., Billen G., Swaney D., *et al.*, (1996) Regional nitrogen budgets and riverine N & P fluxes for the drainages to the North Atlantic Ocean: natural and human influences. *Biogeochemistry.* 35: 75-139.

Huang C.-C., Hsu Y.-C., Lee L.-L. and Li S.-H. (2005) Isolation and characterisation of tetramicrosatellite DNA markers in the Eurasian otter (*Lutra lutra*). *Molecular Ecology Notes.* 5: 314-316.

Hung C.-M., Li S.-H. and Lee L.-L. (2004) Faecal DNA typing to determine the abundance and special organisation of otters (*Lutra lutra*) along two stream systems in Kinmen. *Animal Conservation.* 7: 301-311.

Hyvärinen H., Tyni P. and Nieminen P. (2003) Effects of moult, age, and sex on the accumulation of heavy metals on the otter (*Lutra lutra*) in Finland. *Bulletin of Environmental Contamination and Toxicology.* 70: 278-284.

Idaghdour Y., Broderick D. and Korrida A. (2003) Faeces as a source of DNA for molecular studies in a threatened population of great bustards. *Conservation Genetics.* 4: 789-792.

Ikonomou M.G., Rayne S., Fischer M., Fernandez M.P. and Cretney W. (2002) Occurrence and congener profiles of polybrominated diphenyl ethers (PBDEs) in environmental samples from coastal British Columbia, Canada. *Chemosphere.* 46: 649-663.

Ikonomou M.G., Fernandez M.P., Addison R.F. and Stobo W.T. (2004) PBDE profiles in aquatic species at different levels of the food chain. *Proceedings Forth International Workshop on Brominated Flame Retardants.*

International Program on Chemical Safety. (1994) Polybrominated diphenyl ethers. *Environmental Health Criteria 162, Geneva World Health Organisation.*

Jamshidi A., Hunter S., Hazrati S. and Harrad S. (2007) Concentrations and chiral signatures of polychlorinated biphenyls in outdoor and indoor air and soil in a major UK conurbation. *Environmental Science and Technology.* 41: 2153-2158.

Jarne P. and Lagoda P.J.L. (1996) Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution.* 11: 424-429.

Jaspers V., Covaci A., Maervoet J., *et al.*, (2004) Brominated flame retardants in Belgian little owl (*Athene noctua*) eggs. *Organohalogen Compounds.* 66: 3856-3860.

Jaspers V., Covaci A., Voorspoels S., *et al.*, (2006) Brominated flame retardants and organochlorine pollutants in aquatic and terrestrial predatory birds of Belgium: levels, patterns, tissue distribution and condition factors. *Environmental Pollution.* 139: 340-352.

Jędrzejewska B., Sidorovich V.E., Pikulik M.M. and Jędrzejewski W. (2001) Feeding habits of the otter and the American mink in Białowieża Primeval Forest (Poland) compared to other Eurasian populations. *Ecography.* 24: 165-180.

Jefferies D.J. and Walker C.H. (1966) Uptake of *p,p´*-DDT and its post-mortem breakdown in the avian liver. *Nature.* 5061: 533-534.

Jefferies R.L and Maron J.L. (1997) The embarrassment of riches: atmospheric deposition of nitrogen and community and ecosystem processes. *Trends in Ecology and Evolution.* 12: 74-78.

Jeffreys A.J., Royle N.J., Wilson V. and Wong Z. (1988) Spontaneous mutation rates to new length alleles at tandem-repetitive hypervariable loci in human DNA. *Nature.* 332: 278-281.

Jensen S., Kihlström J.E., Olsson M., Lundberg C. and Örberg J. (1977) Effects of PCB and DDT on mink (*Mustela vision*) during the reproductive season. *AMBIO.* 6:239-239.

Jenssen B.M., Sørmo E.G., Salmer M.P., Baek K. and Skaare J.U. (2004) Brominated flame retardants (BFRs) in the Arctic marine food chain. *Proceedings Forth International Workshop on Brominated Flame Retardants.*

Johansson I., Héas-Moisan K., Guiot N., Munschy C. and Tronczyński J. (2006) Polybrominated diphenyl ethers (PBDEs) in mussels from selected French coastal sites: 1981-2003. *Chemosphere.* 64: 296-305.

Kajiwawa N., Kamikawa S., Ramu K., *et al.*, (2006) Geographical distribution of polybrominated diphenyl ethers (PBDEs) and organochlorines in small cetaceans from asian waters. *Chemosphere.* 64: 287-295.

Kameyama Y., Isagi Y. and Nakagoshi N. (2002) Relatednes structure in *Rhododendron metternichii* var. *hondoense* revealed by microsatellite analysis. *Molecular Ecology.* 11: 519-527.

Kannan K., Perrotta E., Thomas N.J. and Aldous K.M. (2007) A comparative analysis of polybrominated diphenyl ethers and polychlorinated biphenyls in southern sea otters that died of infectious diseases and non-infectious causes. *Archives of Environmental Contamination and Toxicology.* 53: 293-302.

Keeney D.R. (2002) Reducing nonpoint nitrogen to acceptable levels with emphasis on the upper Mississippi river basin. *Estuaries.* 25: 862-868.

Keller L.F. and Waller D.M. (2002) Inbreeding effects in wild populations. *Trends in Ecology and Evolution.* 17: 230-241.

Kelly R., Gibbs M., Collick A. and Jeffreys A.J. (1991) Spontaneous mutation at the hypervariable mouse minisatellite locus Ms6-hm: Flanking DNA sequence and analysis of germline and early somatic mutation events. *Proceedings of the Royal Society of London, Series B.* 245: 235-245.

Ketmaier V. and Bernardini C. (2005) Structure of the mitochondrial control region of the Eurasian otter (*Lutra lutra*; Carnivora, Mustelidae): patterns of genetic

heterogeneity and implications for conservation of the species in Italy. *Journal of Heredity.* 96: 318-328.

Kihlström J.E., Olasson M., Jensen S., *et al*. (1992) Effects of PCB and different fractions of PCB on the reproduction of the mink (*Mustela vison*). *AMBIO.* 21: 563-569.

Klosterhaus S.L., Dreis E. and Baker J.E. (2007) Bioavailability of brominated diphenyl ethers to the marine polychaete worm, *Nereis virens*. *Proceedings Forth International Workshop on Brominated Flame Retardants.*

Knoth W., Mann W., Meyer R. and Nebhuth J. (2007) Polybrominated biphenyl ether in sewage sludge in Germany. *Chemosphere.* 67: 1831-1837.

Kohn M., Knauer F., Schröder W. and Pääbo S. (1995) Conservation genetics of the European brown bear - a study using excremental PCR of nuclear and mitochondrial sequences. *Conservation Genetics.* 4: 95-103.

Kohn M.H. and Wayne R.K. (1997) Facts from feces revisited. *Trends in Ecology and Evolution* 12: 223-227.

Kohn M.H., York E.C., Kamradt D.A., *et al.* (1999) Estimating population size by genotyping faeces. *Proceedings of the Royal Society of London, Series B.* 266: 657-663.

Kruuk H. and Conroy W.H. (1991) Mortality of otters (*Lutra lutra*) in Scotland. *Journal of Applied Ecology.* 28: 83-94.

Kruuk H. (1992) Scent marking by otters (*Lutra lutra*): signaling the use of resources. *Behavioural Ecology.* 3: 133-140.

Kruuk H.and Conroy W.H. (1996) Concentrations of some organochlorines in otters (*Lutra lutra*) in Scotland: implications for populations. *Environmental Pollution.* 92: 165-171.

Kuch B., Hagenmaier H., Krner W. (2001) Poster presentation: determination of brominated flame retardants in sewage sludges and sediments in South-West Germany. In: *11th Annual Meeting of SETAC Europe, Madrid, Spain.*

Kunieda T., Xian M., Kobayashi E., *et al.* (1992) Sexing of mouse preimplantation embryos by detection of Y chromosome-specific sequences using polymerase chain reaction. *Biology of Reproduction.* 46: 692-697.

Kunisue T., Takayanagi N., Isobe T., *et al.*, (2008) Regional ternd and tissue distribution of brominated flame retardants and persistent organochlorines in racoon dogs (*Nyctereutes procyonoides*) from Japan. *Environmental Science and Technology.* 42: 685-691.

Kyle C.J. and Strobeck C. (2001) Genetic structure of North American wolverine (*Gulo gulo*) populations. *Molecular Ecology.* 10: 337-347.

Kwok S. and Higuchi R. (1989) Avoiding false positives with PCR. *Nature.* 339: 237-238.

Lacy R.C. (1987) Loss of genetic diversity from managed populations: interacting effects of drift, mutation, immigration, selection, and population subdivision. *Conservation Biology.* 1: 143-158.

Lam J.C.W., Kajiwara N., Ramu K., Tanabe S. and Lam P.K.S. (2007) Assessment of polybrominated diphenyl ethers in eggs of waterbirds from South China. *Environmental Pollution.* 148: 258-267.

Lampa S., Gruber B., Henle K. and Hoehn M. (2008) An optimisation approach to increase DNA amplification success of otter faeces. *Conservation Genetics.* 9: 129-139.

Lampert K.P., Rand S.A., Mueller U.G. and Ryan M.J. (2003) Fine-scale genetic pattern and evidence for sex-biased dispersal in the túngara frog, *Physalaemus pustulosus*. *Molecular Ecology.* 12: 3325-3334.

Langford K., Scrimshaw M. and Lester J. (2007) The impact of process variables on the removal of PBDEs and NPEOs during simulated active sludge treatment. *Archives of Environmental Contamination and Toxicology.* 53: 1-7.

Lanszki J., Pallos Z.S., Nagy D. and Yoxon G. (2007) Diet and fish choice of Eurasian otters (*Lutra lutra* L.) in fish wintering pools in Hungary. *Aquiculture International.* 15: 393-402.

Law R.J., Allchin C.R., Bennett M.E., Morris S. and Rogan E. (2002) Polybrominated diphenyl ethers in two species of marine top predators from England and Wales. *Chemosphere.* 46: 673-681.

Law R.J., Alaee M., Allchin C.R., *et al.*, (2003) Levels and trends of polybrominated diphenylethers and other brominated flame retardants in wildlife. *Environment International.* 29: 757-770.

Law R.J., Allchin C.R., de Boer J., *et al.*, (2006) Levels and trends of brominated flame retardants in the European environment. *Chemosphere.* 64: 187-208.

Legler J. and Brouwer A. (2003) Are brominated flame retardants endocrine disruptors? *Environment International.* 29: 879-885.

Lema S.C., Schultz I.R., Scholz N.L., Incardona J.P. and Swanson P. (2007) Neural defects and cardiac arrhythmia in fish larvae following embryonic exposure to 2,2′,4,4′-tetrabromodiphenyl ether (PBDE 47). *Aquatic Toxicology.* 82: 296-307.

Lenton E.J., Chanin P.R.F. and Jefferies D.J. (1980) Otter survey of England 1977-1979. Nature Conservancy Council, London.

Levinson G. and Gutman G.A. (1987) Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Molecular Biology and Evolution.* 4: 203-221.

Li A., Rockne K.J., Sturchio N., *et al.*, (2006) Polybrominated diphenyl ethers in the sediments of the Great Lakes. 4. Influencing factors, trends, and implications. *Environmental Science and Technology.* 40: 7528-7534.

Lindahl T. (1993) Instability and decay of the primary structure of DNA. *Nature.* 362: 709-715.

Loew S.S. (1999) Sex-biased dispersal in eastern chipmunks, *Tamias striatus*. *Evolutionary Ecology.* 13: 557-577.

Loughlin T.R., Castellini M.A. and Ylitalo G. (2002) Spatial aspects of organochlorine contamination in northern fur seal tissues. *Marine Pollution Bulletin.* 44: 1024-1034.

Lucchini V., Fabbri E., Marucco F., *et al.* (2002) Noninvasive molecular tracking of colonizing wolf (*Canis lupus*) packs in the western Italian Alps. *Molecular Ecology.* 11: 857-868.

Lunn N.L., Paetkau D., Calvert W., *et al.* (2000) Cub adoption by polar bears (*Ursus maritimus*): determining relatedness with microsatellite markers. *Journal of Zoology.* 251: 23-30.

Lynch M. (1991) The genetic interpretation of inbreeding and outbreeding depression. *Evolution.* 45: 622-629.

Macdonald D. (1984) *The encyclopedia of mammals.* Andromeda Oxford

Madsen A.B., Dietz H.H., Henriksen P. and Clausen B. (1999) Survey of Danish free living otters *Lutral lutra* - a consecutive collection and necropsy of dead bodies. *IUCN Otter Specialist Group Bulletin.* 16: 65-76.

Manchester-Neesvig J.B., Valters K. and Sonzogni W.C. (2001) Comparison of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenlys (PCBs) in Lake Michigan salmonids. *Environmental Science and Technology.* 35:

Mariussen E., Steinnes E., Breivik K., *et al.*, (2008) Spatial patterns of polybrominated dyphenyl ethers (PBDEs) in mosses, herbivores and a carnivore from the Norwegian terrestrial biota. *Science of the Total Environment.* 404: 162-170.

Marsh G., Athanasiadou M., Athanassiadis I., *et al.*, (2005) Identification, quantification, and synthesis of a novel dimethoxylated polybrominated biphenyl in marine mammals caught off the coast of Japan. *Environmental Science and Technilogy.* 39: 8684-8690.

Martin M., Lam P.K.S. and Richardson B.J. (2004) an Asian quandary: where have all the PBDes gone? *Marine Pollution Bulletin.* 49: 375-382.

Mason and Macdonald (1986) *Otters: Ecology and Conservation.* Cambridge University Press.

Mason C.F. and Stephenson A. (2001) Metals in tissues of European otters (*Lutra lutra*) from Denmark, Great Britain and Ireland. *Chemosphere.* 44: 351-353.

Maudet C., Miller C., Bassano B., *et al.* (2002) Microsatellite DNA and recent statistical methods in wildlife conservation management: applications in Alpine ibex [*Capra ibex* (*ibex*)]. *Molecular Ecology.* 11: 421-436.

Mayne G.J., Bishop C.A., Martin P.A., Boermans H.J. and Hunter B. (2005) Thyroid function in nesting tree swallows and eastern bluebirds exposed to non-persistent pesticides and *p,p´*-DDE in apple orchards of Southern Ontario, Canada. *Ecotoxicology.* 14:381-396.

McDonald R.A., O'Hara K. and Morrish D.J. (2007) Decline of invasive alien mink (*Mustela vison*) is concurrent with recovery of native otters (*Lutra lutra*). *Diversity and Distribution.* 13: 92-98.

Marshall T.C., Slate J., Kruuk L.E.B. and Pemberton J.M. (1998) Statistical confidence for likelihood-based paternity inferences in natural populations. *Molecular Ecology.* 7, 639-655.

Meerts I.A.T.M., van Zanden J.J., Luijks E.A.C., *et al.*, (2000) Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin *in Vitro*. *Toxicological Sciences.* 56: 95-104.

Meerts I.A.T.M., Letcher R.J., Hoving S., *et al.*, (2001) *In vitro* estrogenicity of polybrominated diphenyl ethers, hydroxylated PBDEs, and polybrominated bisphenol A compounds. *Environmental Health Perspectives.* 109: 399-407.

Michaux J.R., Libois R., Davison A., Chevret P. and Rosoux R. (2004) Is the western population of the European mink, (*Mustela vison*), a distinct management unit for conservation? *Biological Conservation.* 115: 357-367.

Mierle G., Addison E.M., MacDonald K. and Joachim D.G. (2000) Mercury levels on tissue of otters from Ontario, Canada: variation with age, sex, and location. *Environmental Toxicology and Chemistry.* 19: 3044-3051.

Minch E., Ruiz-Linares A., Goldstein D., Feldman M. and Cavalli-Sforza L.L. (1996) Microsat (version 1.5): A computer program for calculating various statistics on microsatellite allele data. http://hpgl.stanford.edu/projects/microsat/

Moore W.S., (1995) Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear gene trees. *Evolution.* 49: 718-726.

Morell V. (1995) Dogfight erupts over animal studies in the Serengeti. *Science.* 270: 1302-1303.

Morin P.A., Wallis J., Moore J.J., Chakraborty R. and Woodruff D.S. (1993) Non-invasive sampling and DNA amplification for paternity exclusion, community structure, and phylogeography in wild chimpanzees. *Primates.* 34: 347-356.

Mucci N., Pertoldi C., Madsen A.B., Loeschcke V. and Randi E. (1999) Extremely low mitochondrial DNA control-region sequence variation in the otter *Lutra lutra* population of Denmark. *Hereditas.* 130: 331-336.

Mucci N and Randi E. (2007) Sex identification of Eurasian otter (*Lutra lutra*) non-invasive DNA samples using ZFX/ZFY sequences. *Conservation Genetics.*

Murk A.J., Leonards P.E.G., van Hattum B., *et al.* (1998) Application of biomarkers for the exposure and effect of polyhalogenated aromatic hydrocarbons in naturally exposed European otters (*Lutra lutra*). *Environmental Toxicology and Pharmacology.* 6: 91-102.

Murphy M.A., Waits L., Kendall K.C., *et al.*, (2002) An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples. *Conservation Genetics.* 3: 435-440.

Murphy M.A., Waits L.P. and Kendall K.C. (2003) The influence of diet on faecal DNA amplification and sex identification in brown bears (*Ursus arctos*). *Molecular Ecology.* 12: 2261-2265.

Murvoll K.M., Skaare J.U., Anderssen E. and Jenssen B.M. (2006) Exposure and effects of persistent organic pollutants in European shag (*Phalacrocorax aristotelis*) hatchlings from the coast of Norway. *Environmental Toxicology and Chemistry.* 25: 190-198.

Nachman M. W. and Crowell S. L. (2000) Estimate of the mutation rate per nucleotide in humans. *Genetics.* 156: 297-304.

Naert C., van Peteghem C., Kupper J., Jenni L. and Naegeli H. (2007) Distribution of polychlorinated biphenyls and polybrominated diphenyl ethers in birds of prey from Switzerland. *Chemosphere.* 68: 977-987.

Nei M. (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Science, USA.* 70: 3321-3323.

Nei M. (1977) F-statistics and analysis of gene diversity in subdivided populations. *Annual of Human Genetics.* 41: 225-233.

Nicholls C.N. and Pullin A.S. (2000) A comparison of larval survivorship in wild and introduced populations of the large copper butterfly (*Lycaena dispar batavus*). *Biological Conservation.* 93: 349-358.

Nyakaana S. and Arctander P. (1999) Population genetic structure of the African elephant in Uganda based on variation at mitochondrial and nuclear loci: evidence for male-biased gene flow. *Molecular Ecology.* 8: 1105-1115.

O'Brien S.J., Wildt D.E., Bush M., *et al.* (1987) East African cheetahs: Evidence for two population bottlenecks? *Proceedings of the National Academy of Science USA.* 84: 508-511.

O'Connell M., Wright J.M. and Farid A. (1996) Development of PCR primers for nine polymorphic American mink *Mustela vison* microsatellite loci. *Molecular Ecology.* 5: 311-312.

Ormiston B.G. (1985) Effects of a subminiature radio collar on activity of free-living white-foot mice. *Canadian Journal of Zoology.* 63: 733-735.

Paetkau D. and Strobeck C. (1995) The molecular basis and evolutionary history of a microsatellite null allele in bears. *Molecular Ecology.* 4: 519-520.

Page D.C., Mosher R., Simpson E.M., *et al.* (1987) The sex-determining region of the human Y chromosome encodes a finger protein. *Cell.* 51: 1091-1104.

Palomares F., Godoy J.A., Piriz A., O'Brien S.J. and Johnson W.E. (2002) Faecal genetic analysis to determine the presence and distibution of elusive carnivores: design and feasibility for the Iberian lynx. *Molecular Ecology.* 11: 2171-2182.

Palsbøll P.J., Vader A., Bakke I. and El-Gewely R. (1992) Determination of gender in cetaceans by the polymerase chain reaction. *Canadian Journal of Zoology.* 70: 2166-2170.

Payne E.J. and Cotinot C.Y. (1993) Comparative HMG-box sequences of the SRY gene between sheep, cattle and goats. *Nucleic Acids Research.* 21: 2772.

Pérez-Haro M., Viñas J., Mañas F., *et al.* (2005) Genetic variability in the complete mitochondrial control region of the Eurasian otter (*Lutra lutra*) in the Iberian Peninsular. *Biological Journal of the Linnean Society.* 86: 397-403.

Perrin N. and Mazalov V. (1999) Dispersal and inbreeding avoidance. *The Americal Naturalist.* 154: 282-292.

Pertoldi C., Hansen M.M., Loeschcke V., *et al.* (2001) Genetic consequences of population decline in the European otter (*Lutra lutra*): An assessment of microsatellite DNA variation in Danish otters from 1883 to 1993. *Proceedings of the Royal Society of London, Series B.* 268: 1775-1781.

Philcox C.K., Grogan A.L. and MacDonald D.W. (1999) Patterns of otter *Lutra lutra* road mortality in Britain. *Journal of Applied Ecology.* 36: 748-762.

Piggott M.P., Banks S.C., Stone N., Banffy C. and Taylor A.C. (2006) Estimating population size of endangered brush-tailed rock-wallaby (*Petrogale penicillata*) colonies using faecal DNA. *Molecular Ecology.* 15: 81-91.

Prichard J.K., Stephens M. and Donnelly P. (2000) Inference of population structure using multilocus genotype data. *Genetics.* 155: 945-959.

Prigioni C., Balestrieri A. and Remonti L. (2007) Decline and recovery in otter *Lutra lutra* populations in Italy. *Mammal Review.* 37: 71-79.

Prugh L.R., Ritland C.E., Arthur S.M. and Krebs C.J. (2005) Monitoring coyote population dynamics by genotyping faeces. *Molecular Ecology.* 14: 1585-1596.

Queller D.C. and Goodnight K.F. (1989) Estimating relatedness using genetic markers. *Evolution.* 43: 258-275.

Rabalais N.N., Turner R.E., Dortch Q., *et al.*, (2002) Nutrient-enhanced productivity in the northern Gulf of Mexico: past present and future. *Hydrobiologia.* 475/476: 39-63.

Radespiel U., Lutermann H., Schmelting B., Bruford M.W. and Zimmermann E. (2003) Patterns and dynamics of sex-biased dispersal in a nocturnal primate, the grey mouse lemur, *Microcebus murinus*. *Animal Behaviour.* 65: 709-719.

Raff J.D. and Hites R.A. (2007) Deposition versus photochemical removal of PBDEs from Lake Superior air. *Environmental Science and Technology.* 41: 6725-6731.

Raldúa D., Padrós F., Solé M., *et al.*, (2008) First evidence of polybrominated diphenyl ether (flame retardants) effects in feral barbell from the Ebro River basin (NE, Spain). *Chemosphere.* 73: 56-64.

Randi E., Davoli F., Pierpaoli M., *et al.* (2003) Genetic structure in otter (*Lutra lutra*) populations in Europe: Implications for conservation. *Animal Conservation.* 6: 93-100.

Raymond M. and Rousset F. (1995a) An exact test for population differentiation. *Evolution.* 49: 1280-1283.

Raymond M. and Rousset F. (1995b) Genepop (version 1.2): Population genetics software for exact tests and ecumenicism. *Journal of Heredity.* 86: 248-249.

Reed J.Z., Tollit D.J., Thompson P.M. and Amos W. (1997) Molecular scatology: The use of molecular genetic analysis to assign species, sex and individual identity to seal faeces. *Molecular Ecology.* 6: 225-234.

Rengmark A.H., Slettan A., Skaala Ø., Lie Ø. and Lingaas F. (2006) Genetic variability in wild and farmed Atlantic salmon (*Salmo salar*) strains estimated by SNP and microsatellites. *Aquaculture.* 253: 229-237.

Richard K.R., McCarrey S.W. and Wright J.M. (1994) DNA sequence from the *SRY* gene of the sperm whale (*Physeter macrocephalus*) for use in molecular sexing. *Canadian Journal of Zoology.* 72: 873-877.

Robertson A. and Hill W. G. (1984) Deviations from Hardy-Weinberg proportions: Sampling variances and use in estimation of inbreeding coefficients. *Genetics.* 107: 703-718.

Roos A., Greyerz E., Olsson M. and Sandegren F. (2001) The otter (*Lutra lutra*) in Sweden – population trends in relation to ∑DDT and total PCB concentrations during 1968-99. *Environmental Pollution.* 111: 457-469.

Rousset F. and Raymond M. (1995) Testing heterozygote excess and deficiency. *Genetics.* 140: 1413-1419.

Rousset F. (2007) Genepop 4.0 for Windows and Linux. http://genepop.curtin.edu.au/

Rowe G., Beebee T.J.C. and Burke T. (1998) Phylogeography of the natterjack toad *Bufo calamita* in Britain: genetic differentiation of native and translocated populations. *Molecular Ecology.* 7: 751-760.

Ruano G., Fenton W. and Kidd K.K. (1989) Biphasic amplification of very dilute DNA samples via 'booster' PCR. *Nucleic Acids Research.* 17: 5407.

Ruiz-Olmo J., Lafontaine L., Prignioni C., López-Martín J.M. and Santos-Reis M. (2002) Pollution and its effects on otter populations in south-western Europe. *Journal of the International Otter Survival Fund.* 1: 63-82.

Ruiz-Olmo J., Margalida A. and Batet A. (2005) Use of small rich patches by Eurasian otter (*Lutra lutra* L.) females and cubs during the pre-dispersal period. *Journal of Zoology, London.* 265: 339-346.

Sacks B.N., Brown S.K. and Ernest H.B. (2004) Population structure of California coyotes corresponds to habitat-specific breaks and illuminates species history. *Molecular Ecology.* 13: 1265-1275.

Saitou N. and Nei M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution.* 4: 406-425.

Sales-Luís T., Pedroso N.M. and Santos-Reis M. (2007) Prey availability and diet of the Eurasian otter (*Lutra lutra*) on a large reservoir and associated tributaries. *Canadian Journal of Zoology.* 85: 1125-1135.

Salonen A. and Peuhkuri N. (2004) A short hatchery: does it make a difference to aggressiveness in European grayling? *Journal of Fish Biology.* 65: 231-239.

Sambrook J., Fritsch E.F. and Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual. 2nd Edition.* Cold Spring Harbour Laboratory Press, New York.

Sawal G., Stachel B. and Lepom P. (2004) Polybrominated diphenyl ethers in sediments from the River Elbe, Germany. *Proceedings Fourth International Workshop on Brominated Flame Retardants.*

Schlabach M., Fjeld E. and Borgen A.R. (2004) Brominated flame retardants in Drammens river and the Drammensfjord, Norway. *Proceedings Fourth International Workshop on Brominated Flame Retardants.*

Schwenk S. (1986) The otter (*Lutra lutra*) as seen in German and Austrian hunting statistics between 1830 and 1936. *International Journal of Mammalian Biology.* 32: 239-248.

Sellström U. (1999) Determination of some polybrominated flame retardants in biota, sediment and sewage sludge. *Dortoral Dissertation, University of Stockholm, Sweden.*

Sellström U., Lindberg P., Häggberg L., de Wit C. (2001) Higher brominated PBDEs found in eggs of peregrine falcons (*Falco peregrinus*) breeding in Sweden. *Proceedings Second International Workshop on Brominated Flame Retardants.*

Seppä P. and Laurila A. (1999) Genetic structure of island populations of the anurans *Rana temporaria* and *Bufo bufo*. *Heredity.* 82: 309-317.

Seutin G., White B.N. and Boag P.T. (1991) Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology.* 69: 82-90.

She J., Petreas M., Winkler J., *et al.*, (2002) PBDEs in the San Fransisco Bay Area: measurements in harbour seal blubber and human breast adipose tissue. *Chemosphere.* 46: 697-707.

Simpson V.R. (1998) A post mortem study of otter *(Lutra lutra*) found dead in South West England. *Environment Agency, Technical Report W148.*

Simpson V.R., Bain M.S., Brown R., Brown B.F. and Lacey R.F. (2000) A long-term study of vitamin A and polychlorinated hydrocarbon levels in otters (*Lutra lutra*) in south west England. *Environmental Pollution.* 110: 267-275.

Simpson V.R. (2007) Health status of otters in southern and south west England 1996-2003. *Environment Agency, Science Report SC010064/SR1.*

Sjodin A., Hagmar L., Klasson-Wehler E., *et al.*, (1999) Flame retardant exposure: Polybrominated diphenyl ethers in blood from Swedish workers. *Environmental Health Perspectives.* 107: 643-648.

Slate J., Coltman D.W., Goodman S.J., *et al.* (1998) Bovine microsatellite loci are highly conserved in red deer (*Cervus elaphus*), sika deer (*Cervus nippon*) and Soay sheep (*Ovis aries*). *Animal Genetics.* 29: 307-315.

Slatkin M. (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics.* 139: 457-462.

Smith V.H., Tilman G.D., Nekola J.C. (1999) Eutrophication: impacts of excess nutrient input on freshwater, marine, and terrestrial ecosystems. *Environmental Pollution.* 100: 179-196.

Sommer R. and Benecke N. (2004) Late- and post-glacial history of the Mustelidae in Europe. *Mammal Review.* 34: 249-284.

Sommer R. and Nadachowski A. (2006) Glacial refugia of mammals in Europe: evidence from fossil records. *Mammal Review.* 36: 251-265.

Stapleton H.M., Dodder N.G., Kucklick J.R., *et al.*, (2006) Determination of HBCD, PBDEs and MeO-BDEs in Californian sea lion (*Zalophus californianus*) stranded between 1993 and 2003. *Marine Pollution Bulletin.* 52: 522-531.

Stephan W. (1989) Tandem-repetitive non-coding DNA: Forms and forces. *Molecular Biology and Evolution.* 6: 198-212.

Storelli M.M., Storelli A. and Marcotrigiano G.O. (2005) Concentrations and hazard assessment of polychlorinated biphenyls and organochlorine pesticides in shark liver from the Mediterranean Sea. *Marine Pollution Bulletin.* 50: 850-855.

Strachan R., Birks J.D.S., Chanin P.R.F. and Jefferies D.J. (1990) Otter survey of England 1984-1986. *Nature Conservancy Council, Peterborough.*

Strachan R and Jefferies D.J. (1996) Otter survey of England 1991-1994. *The Vincent Wildlife Trust, London.*

Strandberg B., Dodder N.G., Basu I. and Hites R.A. (2001) Concentrations and spatial variations of polybrominated diphenyl ethers and other organohalogen compounds in Great Lakes air. *Environmental Science and Technology.* 35: 1078-1083.

Taastrøm H.-M. and Jacobsen L. (1999) The diet of otters (*Lutra lutra* L.) in Danish freshwater habitats: comparisons of prey fish populations. *Journal of Zoology, London.* 248: 1-13.

Taberlet P., Mattock H., Dubois-Paganon C. and Bouvet J. (1993) Sexing free-ranging brown bears *Ursus arctos* using hairs found in the field. *Molecular Ecology.* 2: 399-403.

Taberlet P., Griffin S., Goosens B., *et al.* (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research.* 24: 3189-3194.

Taberlet P., Camarra J.J., Griffin S., *et al.* (1997) Noninvasive genetic tracking of the endangered Pyrenean brown bear population. *Molecular Ecology.* 6: 869-876.

Taberlet P., Fumagalli L., Wust-Saucy A.-G. and Cosson J.-F. (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology.* 7: 453-464.

Taggart J.B., Hymes R.A., Prodöuhl P.A. and Ferguson A. (1992) A simplified protocol for routine total DNA isolation from salmonid fishes. *Journal of Fish Biology.* 40: 963-965.

Tapio I., Tapio M., Grislis Z., *et al.* (2005) Unfolding a population structure in Baltic sheep breedis using microsatellite analysis. *Heredity.* 94: 448-456.

Taylor A.C., Horsup A., Johnson C.N., Sunnucks P. and Sherwin B. (1997) Relatedness structure detected by microsatellite analysis and attempted pedigree reconstruction in an endangered marsupial, the northern hairy-nosed wombat *Lasiorhinus krefftii*. *Molecular Ecology.* 6: 9-19.

Thomas V. (2000) Impact of exposure to maternal PCBs and dioxins on the neonate's thyroid hormone status. *Epidemiology.* 11: 239.

Thomas G.O., Moss S.E.W., Asplund L. and Hall A.J. (2005) Absorption of decabromodiphenyl ether and other organohalogen chemicals by grey seals (*Halichoerus grypus*). *Environmental Pollution.* 133: 581-586.

The European Parliament and the Council of the European Union (2003) Directive 2003/11/EC of the European Parliament and of the Council of 6 February 2003 amending for the 24th time Council Directive 76/769/EEC relating to restrictions on the marketing and use of certain dangerous substances and preparations (pentabromodiphenyl ether, octabromodyphenyl ether). *Official Journal of the European Union.* 15.2.2003: L42-45.

The Otter Trust (2006) The Otter Trust Website. http://www.ottertrust.org.uk (accessed 25/03/2008)

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., Higgins D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nuclear Acid Research.* 25: 4876-4882.

Thuillet A.-C., Bataillon T., Poirier S., Santoni S. and David J.L. (2005) Estimation of long-term effective population sizes through the history of durum wheat using microsatellite data. *Genetics.* 169: 1589-1599.

Trujillo R.G., Loughlin T.R., Gemmell N.J., Patton J.C. and Bickham J.W. (2004) Variation in microsatellites and mtDNA across the range of the Steller sea lion, *Eumetopias jubatus*. *Journal of Mammology.* 85: 338-346.

Tseng L.-H., Lee, C.-W., Pan M.-H., *et al.*, (2006) Postnatal exposure of the male mouse to 2,2′,3,3′,4,4′,5,5′,6,6′,-decabrominated diphenyl ether: decreased epididymal sperm functions without alterations in DNA content and histology in testis. *Toxicology.* 224: 33-43.

Tsutsui N.D., Suarez A.V., Holway D.A. and Case T.J. (2001) Relationships among native and introduced populations of the Argentine ant (*Linepithema humile*) and the source of introduced populations. *Molecular Ecology.* 10: 2151-2161.

Utami S.S., Goossens B., Bruford M., de Ruiter J.R. and van Hooff J.A.R.A.M. (2002) Male bimaturism and reproductive success in Sumatran orang-utans. *Behavioural Ecology.* 13: 643-652.

Valdiosera C.E., García N., Anderung C., *et al.*, (2007) Staying out in the cold: glacial refugia and mitochondrial DNA phylogeography in ancient European brown bears. *Molecular Ecology.* 16: 5140-5148.

Valiela I., Collins G., Kremer J., *et al.*, (1997) Nitrogen loading from coastal watersheds to receiving estuaries: new method application. *Ecological Applications.* 7: 358-380.

Valière N. (2002) GIMLET: a computer program for analysing genetic individual identification data. *Molecular Ecology Notes.* 2: 377-379.

Van Apeldoorn R.C., Oostenbrink W.T., van Winden A. and van der Zee F.F. (1992) Effects of habitat fragmentation on the bank vole, *Clethrionomys glareolus* , in an agricultural landscape. *OIKOS* 65: 265-274.

Van der Ven L.T.M., van de Kuil T., Leonards P.E.G., *et al.*, (2008) A 28-day oral dose toxicity study in Winster rats enhanced to detect endocrine effects of decabromodiphenyl ether (decaBDE). *Toxicology Letters.* 179: 6-14.

Van de Zande L., van Apeldoorn R.C., Blijdenstein A.F., *et al.* (2000) Microsatellite analysis of population structure and genetic differentiation within and between populations of the root vole, *Microtus oeconomus* in the Netherlands. *Molecular Ecology.* 9: 1651-1656.

Vernesi C., Pecchioli E., Caramelli D., *et al.*, (2002) The genetic structure of natural and reintroduced roe deer (*Capreolus capreolus*) populations in the Alps and central Italy, with reference to the mitochondrial DNA phylogeography of Europe. *Molecular Ecology.* 11: 1285-1297.

Verreault J., Gebbink W.A., Gauthier L.T., Gabrielsen G.W. and Letcher R.J. (2007) Brominated flame retardants in glaucous gulls from the Norwegian Arctic: more than just an issue of polybrominated diphenyl ethers. *Environmental science and Technology.* 41: 4925-4931.

Vethaak A.D., Rijs G.B.J., Schrap S.M., *et al.*, (2002) Polybrominated diphenyl ethers, polybrominated biphenyls. In: *Estrogens and Xeno-Estrogens in the Aquatic Environment of the Netherlands – Occurrence, Potency and Biological Effects.* Pg136-153.

Viberg H., Fredriksson A., Jakobsson E., Örn U. and Eriksson P. (2003) Neurobehavioural derangements in adult mice receiving decabrominated diphenyl ether (PBDE 209) during a defined period of neonatal brain development. *Toxicological Sciences.* 76: 112-120.

Viberg H., Fredriksson A. and Eriksson P. (2004) Investigations of strain and/or gender differences in developmental neurotoxic effects of polybrominated diphenyl ether in mice. *Toxicological Sciences.* 81: 344-353.

Vitousek P.M., Aber J.D., Howarth R.W., *et al.*, (1997) Human alteration of the global nitrogen cycle: sources and consequences. *Ecological Applications.* 7: 737-750.

Von Segesser F., Menard N., Gaci B. and Martin R.D. (1999) Genetic differentiation within and between isolated Algerian subpopulations of Barbary macaques (*Macaca sylvanus*): Evidence from microsatellites. *Molecular Ecology.* 8: 433-442.

Voorspoels S., Covaci A., Lepom P., Escutenaire S. and Schepens P. (2006) Remarkable findings concerning PBDEs in the terrestrial top-predator red fox (*Vulpes vulpes*). *Environmental Science and Technology.* 40: 2937-2943.

Vorkamp K., Christensen J.H. and Ridet F. (2004) Polybrominated diphenyl ethers and organochlorine compounds in biota from the marine environment of East Greenland. *Science of the Total Environment.* 331: 143-155.

Waits L.P., Luikart G. and Taberlet P. (2001) Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. *Molecular Ecology.* 10: 249-256.

Walker C.H. (2001) *Organic Pollutants: An Ecotoxicological Perspective.* Taylor and Francis, London.

Walker C.W., Vilà C., Landa A., Lindén M. and Ellegren H. (2001) Genetic variation and population structure in Scandinavian wolverine (*Gulo gulo*) populations. *Molecular Ecology.* 10: 53-63.

Wan Q.-H., Fang S.G., Chen G.-F., *et al.*, (2003) Use of oligonucleotide fingerprinting and faecal DNA in identifying the distribution of the Chinese tiger (*Panthera tigris amoyensis* Hilzheimer). *Biodiversity and Conservation.* 12: 1641-1648.

Wang M. and Schreiber A. (2001) The impact of habitat fragmentation and social structure on the population genetics of roe deer (*Capreolus capreolus* L.) in Central Europe. *Heredity.* 86: 703-715.

Wang M., Lang G. and Schreiber A. (2002) Temporal shifts of DNA-microsatellite allele profiles in roe deer (*Capreolus capreolus* L.) within three decades. *Journal of Zoological Systems ad Evolutionary Research.* 40: 232-236.

Weber J.L. and Wong C. (1993) Mutation of human short tandem repeats *Human Molecular Genetics.* 2: 1123-1128.

Webster M.T., Smith N.G.C., Lercher M.J. and Ellegren H. (2004) Gene expression, synteny, and local similarity in human noncoding mutation rates. *Molecular Biology and Evolution.* 21: 1820-1830.

Weir B.S. and Cockerham C.C. (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution.* 38: 1358-1370.

Whittier C.A., Dhar A.K., Stem C., *et al.*, (1999) Comparison of DNA extraction methods for PCR amplification of mitochondrial cytochrome c oxidase subunit II (COII) DNA from primate fecal samples. *Biotechnology Techniques.* 13: 771-779.

Whittle D.M., MacEachen D.C., Sergeant D.B. and Keir M.J. (2004) Food web biomagnification of brominated diphenyl ethers in two Great Lakes fish communities. *Proceedings Fourth International Workshop on Brominated Flame Retardants.*

Williamson J.E., Huebinger R.M., Sommer J.A., Louis E.E. and Barber R.C. (2002) Development and cross-amplification of 18 microsatellite markers in the Sumatran tiger (*Panthera tigris sumatrae*). *Molecular Ecology Notes.* 2: 110-112.

Wolkers H., Lydersen C., Kovacs K.M., Burkow I. and van Bavel B. (2006a) Accumulation, metabolism and food chain transfer of chlorinated and brominated contaminants in subadult white whales (*Delphinapterus leucas*) and narwhals (*Monodon monoceros*) from Svalbard, Norway. *Archives of Environmental Contamination and Toxicology.* 50: 69-78.

Wolkers H., van Bavel B., Ericson I., Skoglund E., Kovacs K.M. and Lydersen C. (2006b) Congener-specific accumulation and patterns of chlorinated and brominated contaminants in adult male walruses from Svalbard, Norway: indications from individual-specific prey selection. *Science of the Total Environment.* 370: 70-79.

Woxvold I.A., Adcock G.J. and Mulder R.A. (2006) Fine-scale genetic structure and dispersal in cooperative breeding apostlebirds. *Molecular Ecology.* 15: 3139-3146.

Wright S. (1951) The genetical structure of populations. *Annual of Eugenics* 15: 323-354.

Yamada N.A., Smith G.A., Castro A., *et al.*, (2002) Relative rates of insertion and deletion mutations in dinucleartide repeats of various lengths in mismatch repair proficient mouse and mismatch repair deficient human cells. *Mutation Research – Fundamental and Molecular Mechanisms of Mutagenesis.* 499: 213-225.

Yamaguchi N., Gazzard D., Scholey G. and Macdonald D.W. (2003) Concentrations and hazard assessment of PCBs, organochlorine pesticides and mercury in fish species from the upper Thames: river pollution and its potential effects on top predators. *Chemosphere.* 50: 265-273.

Zhou T., Ross D.G., DeVito M.J. and Crofton K.M. (2001) Effects of short-term *in vivo* exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicological Science.* 61: 76-82.