THE EFFECTS OF PRESERVATION AND CONSERVATION TREATMENTS ON THE DNA OF MUSEUM INVERTEBRATE FLUID PRESERVED COLLECTIONS.

A THESIS PRESENTED FOR THE
DEGREE OF MASTER OF PHILOSOPHY.
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Contents

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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List of Abbreviations

AFLP	Amplified fragment length polymorphism
CPD	Critical Point Drying
CTAB	Cetyltrimethylammonium bromide
ddH_2O	Double deionised water.
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid.
dNTP's	Deoxynucleoside triphosphates
dsDNA	Double stranded DNA
DTAB	Dodecyltrimethylammonium bromide.
EDTA	Ethylenediaminetetraacetic acid
HMDS	Hexamethylenedisilazane.
IMS	Industrial methylated spirits
Kb	Kilobase
mDNA	Mitochondrial DNA
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nDNA	Nuclear DNA
NMGW	National Museums and Galleries of Wales
PCR	Polymerase chain reaction
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RE	Restriction Endonuclease
RNAase	Ribonuclease

SDS	Sodium dodecyl sulphate
SE	Standard error
TBE	Tris-Borate-EDTA buffer
TE	Tris-HCL EDTA buffer
TIF	Image computer file format
Tris-HCl	Tris hydrochloride
TXT	Text computer file format
UV	Ultraviolet light

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Abstract.

A variety of protocols used to preserve and conserve invertebrate animal museum collections were assessed for their effect on the preservation of DNA. A range of techniques were used to determine both the quantity and quality of extractable DNA from the various protocols assessed. It was shown that, besides cryo-preservation, absolute ethanol preserved the highest quantities and quality of DNA, whilst 100% IMS preserved DNA of a lesser quality. The higher the water content of the preserving solution the greater the damage to the DNA. The presence of formaldehyde or phenoxetol in the solutions proved to be very detrimental to DNA preservation. The use of additives, such as propylene glycol, to ethanol based solutions appears to be of value by enhancing morphological preservation without increasing the degradation of the DNA. Two specialist drying methods, critical point drying and Hexamethylenedisilazane treatment, were also assessed for their affect on DNA. Both preserved good quality DNA.

Chapter 1.

Introduction

1.0 Introduction.

Natural science museum collections have been assembled over the past three centuries at great private and public expense. Whilst these collections were initially put together as 'curiosities', they now form a basis on which evolutionary and systematic studies have developed (Thomas, 1994). Recent years have seen the rapid development and improvement of many techniques in molecular biology, particularly in the study of DNA (figure 1). Of particular note has been the development of the Polymerase Chain Reaction (PCR), (Saiki *et al*., 1988; Arnheim *et al*., 1990), enabling researchers to replicate and amplify very small amounts of DNA for subsequent analysis. The results of this technology have been of considerable importance to the study of many facets of biology, not least of all fields such as evolutionary biology, biosystematics, biodiversity, population biology, conservation biology, and ecology (Baker 1994). As

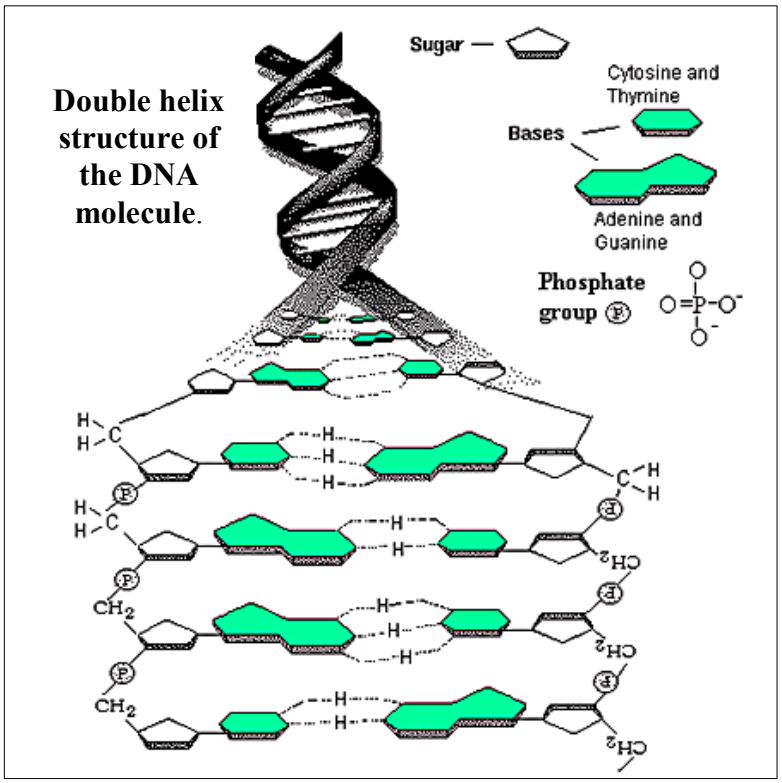


Figure 1: The structure of deoxyribose nucleic acid (DNA), showing the double helix structure of the double stranded molecule. The two strands are linked together by hydrogen bonding between the purine and pyrimidine base units. These are in turn linked together by the phosphodiester backbone of the DNA molecule. (Image source: http://astarte.csustan.edu/~tom/bioinfo-S03/frames/content-bioinfo.html).

the pressure on our natural environment and its biota increases, museum collections are becoming an increasingly important resource for genetic studies (Arnheim *et al.,* 1990). Museum collections are particularly useful in that as well as containing many different species, the specimens also span time and geography (Thomas, 1994). The value of the resource becomes even greater when it is considered that many of the specimens represent species that are now either extinct or so highly endangered, that further collection is not possible or not viable due to financial and / or political reasons. Such material is now regularly used in molecular biological studies (Houde and Braun, 1988; Thomas *et al*., 1989; Ellegren 1991; Leeton *et al*., 1993; Thomas and Pääbo, 1993; Han and McPheron, 1994; France and Kocher, 1996; Roy *et al.,* 1996: Stevens and Wall, 1996; Wirgin *et al*., 1997; Vij *et al*., 1997; Su *et al.,* 1999) and their value is increasingly being recognised by the molecular research community. Some work has been carried out to assess how field collection techniques and preservation practise affects the condition of the specimens DNA (Post *et al*., 1993; Criscuolo, 1994; Reiss *et al*., 1995; Dillon *et al*., 1996; Hammond *et al*., 1996; Vachot and Monnerot, 1996; Austin and Dillon, 1997; Hall *et al*., 1997; Brown, 1999). Research in this field is gradually improving our understanding. However, researchers needing to utilise natural science collections have initiated these studies and not museum workers who specialise in collection care.

During recent years, the concern for the care and conservation of natural science collections has significantly increased. This has been reflected in the formation of specialist groups, such as the Natural Sciences Collections Association in the UK and the Society for the Preservation of Natural History Collections in North America. This concern has also resulted in a growing number of specialist publications covering the

subject (Horie 1989; Stansfield *et al*., 1994; Nudds and Pettitt, 1997; Carter and Walker, 1999). In addition the 'biodiversity crisis' (Barlow, 1995; Reaka-kudla, 1997) and the increased accessibility to the genetic information held in an organism has developed new demands for museum biological collections. Museum conservation is now beginning to seriously tackle the responsibility for the care of this material, and is actively addressing the many issues involved.

1.1 The History of Museum Collections and Preservation Methods

Initially the preservation of Natural Science material was only possible with dry inert materials such as horn, bone, skin, shells, corals, or robust insects (Reid, 1994). It was not until the development of the use of fluid preservation that it became possible to preserve moist, soft biological material. The history of modern fluid preservation effectively dates back to 1644 when Croone presented to the Royal Society two whole puppies preserved in the 'spirit of wine' (Birch, 1756-57). Since this time the general principle of using some form of alcohol solution as a preservative has not fundamentally changed, although at the beginning of the 20th century the use of formaldehyde started to develop and has since become widespread (Down, 1989; Simmons, 1992 *pers. comms.*).

By the end of the seventeenth century, techniques for the preservation of specimens were starting to become established (Reamur, 1748; Wilkinson, 1966). This period also saw the development of natural science collections from eminent collectors such as Tradescent and Sloane, and later from important workers in the history of modern systematics and evolution such as Linnaeus, Banks, Wallace and Darwin. Overall the 18th and 19th Centuries saw many important scientific expeditions, the material from

which now form the core on which modern Natural Science Collections have been built (Davies, 1996). This widespread collecting saw the further development of biological museum methods, many of which persist in current museum practise (Wagstaffe and Fidler, 1955, 1968; Knudson, 1966; Mahoney, 1973; Hangay and Dingley, 1985). Broadly this has led to the preparation of biological museum specimens in two ways;

- Fixation. The killing of the specimen, followed by the treatments to halt decay.
- Preservation: The long-term method used to store and protect the specimen.

Fixation and preservation can be distinctly separate processes e.g. a specimen may be chemically fixed in formaldehyde, and then stored in an ethanol solution for its long-term preservation. Or the two processes can be the same e.g. a specimen may be fixed and preserved in an ethanol solution. The choice of process will depend on the type of specimen and the methods that will be used to study it. Section 1.3 summarises the methods used at NMGW. The long term aim is to preserve, as best we can, the chemical structure and morphology of a specimen. This study will only consider a few of the main processes used on museum specimens, with the aim of providing an overview of the museum methods likely to facilitate DNA preservation.

Whilst some of the specific histochemical changes caused by many of the standard methods of preservation have been actively researched in the last 50 years (Steedman, 1976a; Pearse, 1980), the overall effect of preservation treatments on biological material is still poorly understood. The development of many of the methods currently used for collection preservation has been a result of trial and error, and pure chance. Fixation and preservation technologies have changed little since their discovery (Simmons, 1992 *pers. comms*.; Reid, 1994), and have not been developed by hard scientific research. Many of our existing methods of preservation have significant drawbacks e.g. the use of

ethanol as a preservative causes shrinkage and colour loss. Attempts have been made to develop new techniques. A good example would be the use of the phenol derivative Phenoxetol. This was introduced in the 1970's as an alternative to ethanol preservation as it did not cause shrinkage and retained better colour (Steedman, 1974; Moore, 1999). However there are an increasing number of reports of specimens in Phenoxetol preserved vertebrate collections starting to deteriorate badly (Crimmen, 1989).

It is only in recent years that researchers have been in a position to readily assess the condition of the DNA in a specimen, and indeed analyse the actual genetic information. The use of natural science collections thus enters a new era and the requirements of modern biochemical research needs to be addressed and balanced with the more traditional requirements of whole specimen morphological studies. Many large institutions, especially in the United States (Dessauer *et al*., 1996), have begun to compile tissue banks for the specific preservation of biological samples for biochemical studies. However these compilations require large-scale cryo-preservation, which is expensive, and requires constant monitoring and staff availability in case of power or equipment failure. Such facilities are outside the resources of most institutions holding biological collections. It is thus hoped that future improvements in preservation technology can provided effective solutions for preserving our biological collections for both morphological and molecular use.

1.2 DNA Preservation in Museum Natural History Collections

1.2.1 Current Knowledge.

The ideal storage of specimens for molecular analysis is by using cryo-preservation (Dessauer *et al.*, 1996). However cryo-preservation will not preserve the morphology of a specimen by a means that is easily accessible or visually appealing.

Improvements in molecular techniques, and in particular the advent of PCR (Saiki *et al*., 1988; Arnheim *et al*., 1990) has started to open the vast storehouse of genetic information contained in museum collections (Houde and Braun, 1988; Arnheim *et al*., 1990; Baker, 1994; Thomas, 1994). Some of the first published DNA studies using (non-human) museum specimens examined extinct or rare animals (Higuchi *et al.,* 1984; Higuchi *et al.,* 1987; Houde and Braun, 1988; Thomas *et al.,* 1989). The material used for these studies was dried muscle, skin, feather or bone and tended to recover DNA that was around 200-300bp in length. Whilst this DNA is essentially degraded, it has proved usable for cloning, hybridisation and PCR. During this period, workers were also beginning to look at using the DNA stored in archival histopathological material fixed in formaldehyde, or some other fixative, and then paraffin embedded (Rupp and Locker, 1988; Goelz *et al.,* 1985; Crisan *et al.*, 1990; Greer *et al.*, 1991a; Greer *et al*., 1991b; Malmgren *et al.*, 1992; Chen and Clejan, 1993: Crisan and Mattson, 1993). The use of such archival material demonstrated that biological material that had been exposed to a whole range of chemical treatments could still have a viable use in DNA studies. This view has been further reinforced from studies with ancient DNA, which have obtained DNA from archaeological and subfossil remains (Thomas and Pääbo, 1993; Herrmann and Hummel, 1994; Poinar and Stankiewicz, 1999).

The establishment of usable DNA in museum specimens collected for their gross morphological features, rather than their molecular component, has lead to the consideration of a wide range of museum preserved specimens for molecular work, including fluid-preserved material (examples in photographs 1A-C, *p12*). The use of preserved museum material in DNA studies has continued to increase, with workers beginning to look more closely at the effects of museum preservation treatments on the condition of the DNA, along with the reliability of any analysis carried out to obtain DNA sequences. Findings on fluid preserved specimens (Goebal and Simmons, 1993; Criscuolo, 1994) showed that the DNA extracted from formaldehyde fixed material tended to be of low molecular weight, whereas ethanol-fixed and preserved material gave high molecular weight DNA. Post *et al.* (1993) looked specifically into the preservation of insects of the order Diptera for DNA studies. It was found that liquid nitrogen storage, cold (4°C) ethanol storage and drying over silica gel gave the highest yields of DNA, whilst formaldehyde solutions and dry pinned insects gave very low quantities of DNA. Reiss *et al.* (1995) and Dillon *et al.* (1996) have carried out similar studies using insects from different orders (Coleoptera and Hymenoptera). The results of the two studies broadly correlated. Techniques such as freezing and ethanol preserved good quantities of high molecular weight DNA, whilst treatments such as ethyl acetate and formaldehyde produced degraded low molecular weight DNA. The increased difficulty to extracted intact DNA from specimens treated with fixatives such as formaldehyde has led to the general consideration that the DNA is badly degraded. However, some workers believe the DNA to be difficult to extract rather than highly degraded. Vachot and Monnerot (1996) considered the DNA to be bound to proteins forming DNA-protein complexes (see section 1.2.2; Chaw *et al.,* 1980; Chang and Loew, 1994). This has resulted in attempts to improve extraction techniques in order to

utilise formaldehyde-fixed archival samples more widely (France and Kocher, 1996; Wirgin *et al.*, 1997).

The use of DNA isolation buffers has been considered (Reiss *et al,* 1995; Dawson *et al*., 1998). These were found to work well, but only if the specimen was homogenised. Other workers have advocated the use of buffer solutions for field-work based DNA preservation. Asahida *et al.* (1996) advocated the use of buffers using high concentrations of urea, developed primarily for use with fish tissue samples, whilst Rogstad (1992) and Štorchová *et al.* (2000) have developed the use of NaCl-CTAB (cetyltrimethylammonium bromide) solutions to preserve leaves. Laulier *et al.* (1995) developed buffer solutions using guanidium-isothiocyanate, but again these require homogenisation of the specimen and the solutions can be highly toxic. Similarly, Proebstal *et al.* (1993) developed a preservation method using dimethyl sulfoxide to preserve fish samples that were stored for up to 45 weeks at 5°C. Kuch *et al.*, (1999) demonstrated the use of commercially available laundry detergents for the preservation of DNA in vertebrate blood samples. This study showed that high molecular weight DNA was extractable from the samples after 4 weeks preservation at room temperature, although homogenisation of the tissue samples was again required.

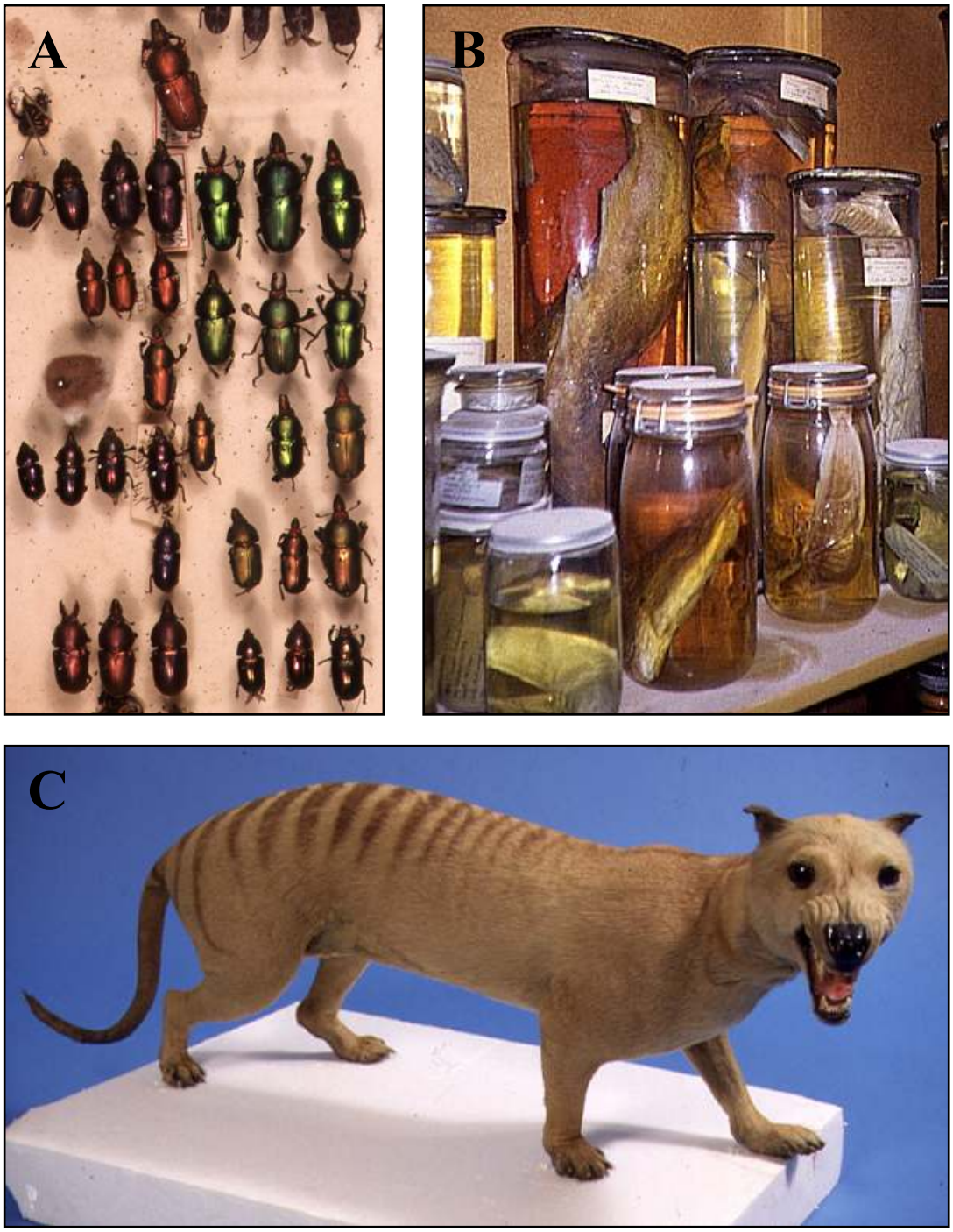
Quicke *et al.,* (1999) provides an overview of the current methods of preservation of hymenopteran specimens for both molecular and morphological study. In this overview they report on the potential uses of specialised drying techniques such as Critical Point Drying (CPD) (Gibson, 1986; Heraty and Hawks*,* 1997 *pers. comms*.), and chemical drying methods such as the use of Hexamethylenedisilazane, (HMDS) (Nation, 1983; Cowan, 1995). Both of these processes potentially give insect specimens that have both

good morphological and DNA preservation (Austin and Dillon, 1997). Drying is a widely used method of preservation in museums that can give good morphological preservation. Many different groups of organisms can be preserved in a dry form. It is a particularly useful method with many groups of insects (Quicke *et al.*, 1999). Harris (1993) reviewed different drying methods for preparing plant material. Overall it was found that no single method stood out, and that the variation in DNA preservation was due to the different plant groups investigated. Dried skin can also be successfully utilised in DNA based studies. For example, Fetzner (1999) demonstrated the use of shed reptile skins as a source of good quality DNA. This method had the additional benefit of being able to sample from rare or highly endangered species without killing the animal. Another interesting protocol, using dried entomological material, has been demonstrated by Phillips and Simon (1995). This protocol extracts DNA from the whole entomological specimen by perforating the exoskeleton with a very fine insect pin, and then soaking the whole specimen in a DTAB (dodecyltrimethylammonium bromide) based extracting solution overnight. After extraction the specimen is washed and returned to the collection morphologically intact.

Table 1 summarises our current knowledge on the effects of various preservation protocols on the gross morphology and molecular preservation of invertebrate material.

Mode of fixation	***Subsequent preservation***	***External morphology***	***Histology***	***Internal anatomy***	***DNA***	***Exhibition display potential***
Cryo preservation	Freezer at –70°C or below.	Can be good.	Poor	Fair to good	Good	None
Absolute ethanol	Absolute ethanol	Poor to good	Poor to fair	Poor to fair	Good	Poor
70-80% IMS	70-80% IMS	Fair to good	Fair to good	Fair to good	Fair - good	Possible
70-80% IMS	CPD or HMDS drying	Good	Poor	Good	Fair - good	Possible
70-80% IMS	Air drying	Good for certain groups.	Poor	Variable	Fair	Possible
4% Formaldehyde	70-80% IMS	Fair to good	Fair	Fair	Poor – fair	Possible
4% Formaldehyde	4% Form-aldehyde	Good	Fair to good	Good	Poor	Possible
Ethyl acetate	Air dried	Fair to good	?	Variable	Very poor	Possible
Formaldehyde based histological.	Same	Fair to good	Good	Good	Very poor to poor	Poor
Mercury based histological.	Same	Fair to good	Good	Good	None or very poor	Poor

Table 1: Summary of the effects of various preservation protocols on invertebrate specimens (after Thomas, 1994; Dillon et al, 1996; Quicke et al, 1999).



Photographs 1A-C: Examples of potential sources of archival DNA held in museum natural science collections. A: Historic dried pinned insect collections. B: Fluid preserved collections. C: Taxidermy mounts (this example is the extinct Thylacine or Tasmanian Wolf).

1.2.2 Factors affecting DNA preservation.

In life an organism has many processes by which to control and repair DNA e.g. figure 2 summarises the formation and control of free radicals in respiratory pathways. Upon death these physiological and cellular control processes are lost and autolytic decay sets in. This causes the rapid degradation of DNA and other macromolecules (Lindahl, 1993; Demple and Harrison, 1994). The key structural feature of DNA influencing its preservation or its destruction is the phosphate diester link (figure 3). It is on this that the macromolecular nature of the DNA chain is dependant (Eglinton and Logan, 1991; Lindahl, 1993). Reactions such as hydrolytic cleavage of this link can result in the loss of the coding base causing changes in the DNA molecule. Effects such as oxidative attack can cause chemical modifications that alter the properties of the DNA molecule.

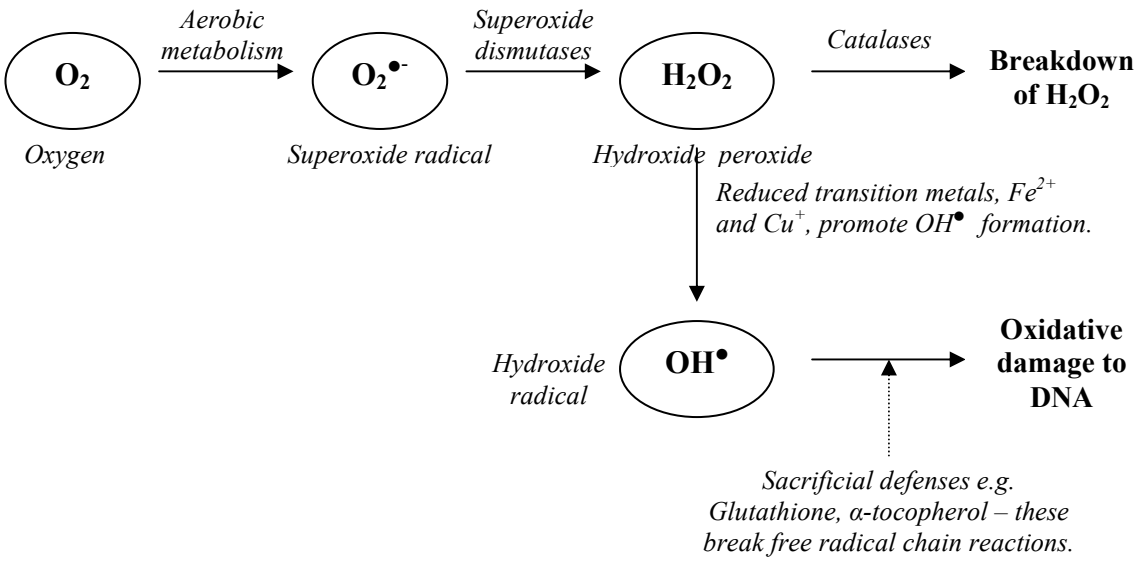


Figure 2: A simple summary of oxygen radical formation and its control in respiratory pathways (Demple and Harrison, 1994).

Museum fixation and preservation treatments are aimed at halting the processes of autolytic decay, allowing the long-term preservation of biological material. Museum specimens are likely to pass through a series of treatments before reaching the final method of preservation (see section 1.3), and many potential factors can affect the integrity of the DNA in a specimen, such as the preservation treatment, age of the specimen or subsequent environmental factors (Lindahl 1993).

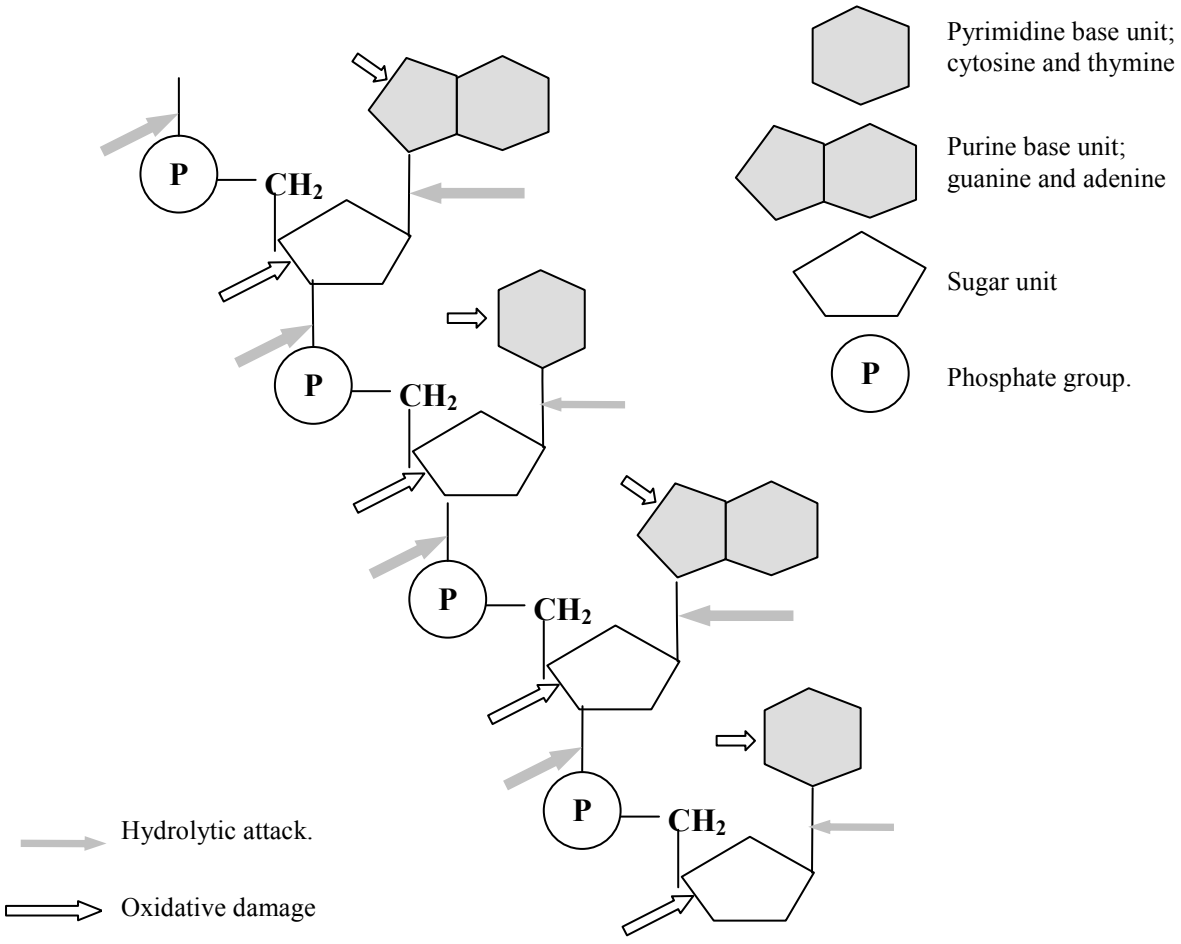


Figure 3: Possible target sites for DNA decay. Arrows indicate key points of attacked on part of a single strand of the dsDNA molecule. Additional base modification and denaturation reactions can also occur (after Lindahl. 1993).

In preserved biological material the DNA is open to a number of key types of degradative process (Brown 1999) such as:

- *Denaturation* – this is where separation of the double stranded DNA occurs. This produces single stranded DNA that is more susceptible to chemical degradation.
- *Cross-linking* - the double stranded DNA becomes chemically cross-linked and bonded to other molecules such as proteins making the DNA less accessible.
- *Strand breakage* – this is where breaks in the sugar-phosphate backbone of the DNA occur causing fragmentation of the DNA.
- *Chemical modification* – chemical changes in the nucleotides can occur through addition, removal or replacement of chemical groups. This can result in alterations to the nucleotide sequence or in how the DNA reacts chemically.

Research on the condition of ancient DNA (Höss *et al.*, 1996; Poinar and Stankiewicz, 1999) suggests that the long term survival of macromolecules such as DNA requires cool and dry conditions. When attempting to preserve the DNA in modern biological materials it is apparent that initial rapid desiccation of tissues limits endogenous hydrolytic damage such as the cleavage of the phosphate links in the sugar–phosphate backbone of dsDNA. The effects of initial oxidative attack on the DNA molecules can be rapid at first but then plateau (Pääbo, 1989). This initial oxidative attack is probably from hydroxyl radicals that are known to attack DNA bases e.g. thymine can be converted to thymine glycols. Deoxyribose is also open to oxidative attack from hydroxide radicals (Muñiz *et al.*, 2001). Oxygen and hydrogen peroxide are not thought to attack DNA, but can be converted to hydroxide radicals by reacting with reduced transition metals such as Fe^{2+} or Cu^{+} (Eglinton and Logan, 1991; Lindahl, 1993; Demple and Harrison, 1994).

In practise it is not possible to adequately preserve all of the components that form an organism. The subsequent decay of inadequately preserved biomolecules such as lipids can lead to the formation of degradative products that can include reactive radicals. Over time these can degrade the DNA through mechanisms such as electrophilic attack. Free radical degradative pathways may be more pronounced in preserved biological material as the normal protective mechanisms, from repair enzymes and sacrificial molecules, are no longer present. As a rule the structure of dsDNA can offer protection to the bases within the double helix structure. However the effects of denaturants, excessive heat or hydrolytic damage can begin to open or disrupt the duplex structure of DNA causing accelerated rates of degradation.

Whether chemical preservation methods potentially cause base changes or chemical modifications, with subsequent changes to the genetic coding, has not been fully explored. Researchers working with ancient DNA templates have tried to consider these factors (e.g. Hansen *et al.*, 2001). There is the suggestion that distinct miscoding lesions in the DNA sequence may occur at different rates over time e.g. the hydrolytic deamination of cytosine occurs at a rate 30-50 times greater than the hydrolytic deamination of adenine in living human cells. The extent such effects occur within preserved material is still under consideration. Douglas and Rogers (1998) appear to have identified increased coding mis-incorporations beyond those normally expected with DNA polymerase mediated replication in sample material exposed to cytological fixatives such as formaldehyde. However, other workers have found no difference in sequence data between formaldehyde fixed and ethanol preserved material (France and Kocher, 1996). The extent of miscoding effects are likely to be very variable, and

dependent on the type of biological material being preserved, the chemical treatments utilised, and the preservation conditions of the material.

Pearse (1980) provides a very good overview of the chemistry and practice of fixation, primarily for use in histochemical studies. Many processes can be considered to be 'fixative', but the most commonly understood process is the use of formaldehyde and other similar aldehydes. Formaldehyde is a mono-functional aldehyde that is extensively used in the fixation of biological tissue for both medical research and museum collections. DNA fragmentation by formaldehyde fixation is a dynamic process (Goebal and Simmons, 1993) that is dependent on the concentration of fixative used, the type of buffer used, the length of exposure and variations in the specimen. The process of formaldehyde fixation has been extensively reviewed for its effects on DNA (Hopwood, 1975; Crisan and Mattson, 1993; France and Kocher, 1996; Vachot and Monnerot, 1996; Shedlock *et al.*, 1997) especially since the establishment of usable DNA within archival histopathological material (Goelz *et al.*, 1985; Crisan *et al.*, 1990; Greer *et al*., 1991a; Malmgren *et al*., 1992; Chen and Clejan, 1993). Some differences exist as to the mechanism by which formaldehyde reacts with the DNA within a specimen. However generally the process is considered to involve DNA-protein and

1. Addition reaction to a compound containing a reactive hydrogen;

$$R,H + CH_2O \leftrightarrow R,CH_2(OH)$$

2. Condensation reaction with a further hydrogen atom to form a methylene bridge (-CH_2-);

$$R,CH_2(OH) + HR' \leftrightarrow R\text{-}CH_2\text{-}R' + H_2O$$

Figure 4: A possible mechanism for methylene bridge formation during formaldehyde fixation.

DNA-interstrand cross-linking (Chang and Loew, 1994). Initially there is considered to be a rapid and reversible hydroxymethylation of the imino and amino groups of the nucleic acid groups, followed by a slower reaction forming methylene bridges between bases (figure 4) (Crisan and Mattson, 1993; France and Kocher, 1996). This can result in the formation of crosslinks with DNA and other cellular components such as proteins. DNA histone complexes are known to form with formaldehyde fixation (Koshiba *et al.*, 1993). Overall a number of distinct interactions can occur between dsDNA and formaldehyde;

(a) Disruption of the purine-pyrimidine hydrogen bonds resulting in rapid and irreversible denaturation of the dsDNA.
(b) Formation of nucleic acid adducts (methylol derivatives).
(c) Methylene bridge crosslinks.
(d) Gradual hydrolysis of the N-glycosidic bonds releasing purine residues.
(e) Scission of the phosphodiester backbone leading to short chains of poly-deoxyribose.

The extent to which formaldehyde degrades DNA in museum specimens can vary significantly from one sample to another and so does not appear to be very predictable (Goebel and Simmons, 1992). Such effects relate to storage temperatures, speed of preservation, pH of the fixative solution and the length of time exposed to formaldehyde (Hopwood, 1975; Jones, 1976; Karlsen et al., 1994).

The fixative effect of formaldehyde is largely due to the formation of the carbonium ion (figure 5). The lower the pH the greater the production of this reactive electrophile, and the greater the effects of fixative based reactions. Formaldehyde naturally attains an acid pH through the production of formic acid through oxidation (Steedman, 1976b).

However the lower the pH of a fixative solution the greater the damage to dsDNA. This has been shown by studies such as Douglas and Rogers (1998) where greater damage to dsDNA occurred with un-buffered formaldehyde solutions than with buffered formaldehyde solutions.

Previously, it was considered that the DNA extracted from formaldehyde fixed material was significantly degraded. However, it is now considered that the DNA is more intact

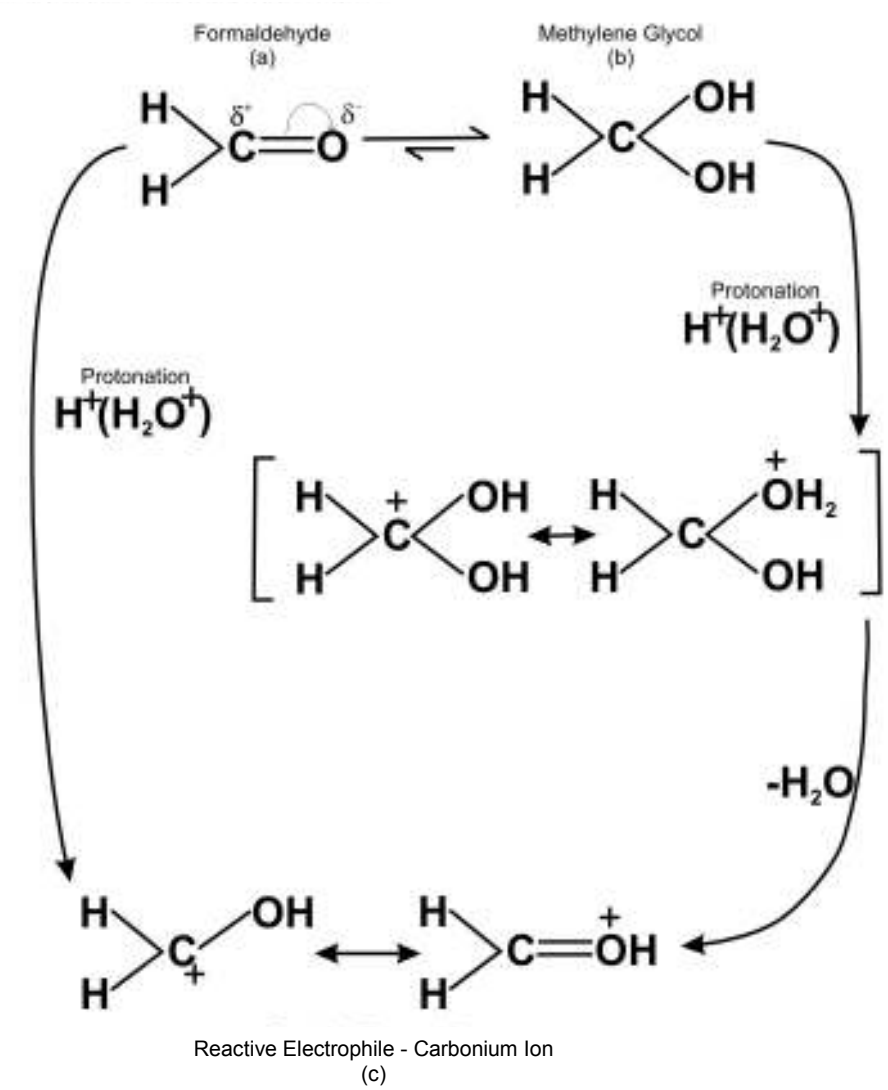


Figure 5: Possible mechanisms for the production of the carbonium ion responsible for the main fixation reactions of formaldehyde. (Adapted from Jones, 1976)

than originally thought, but through the formation of the formaldehyde-induced protein complexes the DNA is harder to extract (Crisan and Mattson, 1993; Vachot and Monnerot, 1996; Shedlock *et al.*, 1997). There is also some evidence to suggest the DNA is unfragmented, but altered in structure (Karlsen *et al.*, 1994).

Other fixatives have also been explored for their effects on DNA in archival or museum preserved specimens (Greer *et al.*, 1991a; Crisan and Mattson, 1993; Post *et al.,* 1993) and it is generally found that most histological style fixatives appear to be poor for retaining the integrity of DNA. Whether this is due to the actual degradation of the DNA, a result of fixation reactions making the DNA difficult to extract, or secondary products inhibiting reaction enzymes is unknown. Certainly acidic fixatives such as Bouin's or Carnoy's reagents significantly degrade DNA, probably through acid depurination (Crisan and Mattson, 1993; Quicke *et al*., 1999).

Formaldehyde, along with propylene glycol, are also used as components in a preserving solution known as Steedman's solution (Steedman, 1974). In addition this solution uses a phenol, propylene phenoxetol ($C_6H_{12}O_2$), which has bactericidal, fungicidal and anti-oxidant properties. The use of this solution has become widespread in some areas of museum work, most notably with herpetological and ichthyological collections. In spite of this, questions have begun to be raised as to the long-term stability of material preserved in Steedman's solution (Crimmen, 1989). No retrospective DNA studies have appeared to have been carried out using Steedman's preserved material. However the components of the preservative suggest that DNA preservation would be very poor. There are potential degradative effects from the formaldehyde in the solution, and additional hydrolytic reactions due to the water in the

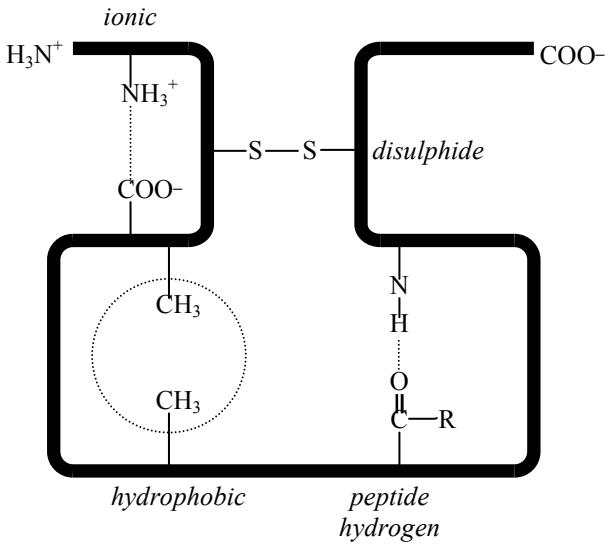


Figure 6: Diagrammatic representation of the tertiary structure of protein. Three types of noncovalent bond are responsible for the tertiary structure: hydrogen bonds; ionic bonds; and hydrophobic bonds. A strong covalent bond, the disulphide bond, also helps maintain the tertiary structure. (After Pearse 1980).

solvent mixture. Phenols are more strongly acidic than alcohol's (Simmons, 1995), and this could be a strong factor causing further degradation of dsDNA. Another effect is possibly through the absorption of phenoxetol into lipids (*pers com* Van Dam, 2001) reducing the solutions long-term effectiveness as a preservative.

Ethanol can be considered to have both a denaturant fixative effect (with proteins) and a preservation action. Ethanol replaces the water molecules in biological tissues that can cause morphological disturbance through shrinkage, hardening and distortion. Ethanol also has the effect of bringing about the aggregation or precipitation of proteins (Pearse 1980; Stoddart, 1989), hence its denaturant fixative action. This alteration of the structure of proteins is brought about by disruption to the hydrophobic bonding within the tertiary structure of proteins by ethanol (figure 6). The result is the loss of the tertiary structure, but retention of the secondary structure of proteins, which is determined by peptide hydrogen bonding within the protein structure. The result is a

change in the steric arrangement. This causes the resulting precipitation and aggregation of the proteins and aids in the entrapment of other cellular components. It is possible that ethanol may induce similar conformation changes within DNA molecules, although this is probably more of an effect with the associated nucleoproteins. Overall, the duplex structure of DNA should be essentially unaffected by the action of ethanol, a factor regularly utilised by standard molecular laboratory techniques which use ethanol to precipitate and store extracted DNA. The double helices of the DNA molecule are bound by hydrogen bonding, which is relatively unaffected by the action of ethanol. DNA is probably preserved by ethanol through a combination of conformational change of the duplex molecule, entrapment by the precipitated protein macromolecular mass and by the elimination of free water reducing hydrolytic damage. The resulting inactivation of enzymes through steric changes and the prevention of biological decay due to the antiseptic properties of ethanol further enhances the preservation of the dsDNA molecules. The result is that ethanol has the potential to be a good preservative for DNA and other macromolecules. In practise the results can be more erratic, and this can be due to a combination of factors:

- Dilution of the ethanol from water within the specimen allowing hydrolytic degradation to occur.
- Not all biological materials will be adequately preserved e.g. lipids which will dissolve into the ethanol solution and degrade into glycols and fatty acids (Moore, 1999). This can have possible degradative effects on the preservation of DNA.
- Contaminating materials in the specimen causing DNA degradation or preventing subsequent DNA analysis e.g. tannins and resins in plant material (Štorchová *et al*, 2000).

In theory industrial grade methylated spirit (IMS) should have a similar effect on DNA preservation as ethanol. IMS is approximately 95% ethanol with the addition of a denaturant, usually around 4% methanol. The addition of the denaturant is necessary to make IMS exempt from customs duty, hence reducing its purchase cost. Thus IMS represents a much more affordable supply of ethanol resulting in its wide scale use as a biological preservative. However, is the use of IMS as effective as absolute ethanol for the preservation of DNA? At the time of the compilation of this study no direct comparison between the use of IMS and absolute ethanol could be found, and this was one of the driving factors for this study. One of the problems of using methylated spirits as a preservative is the range of denaturants that can be used to make it duty exempt (Stecher, 1968). However IMS is far less variable in its composition. Methanol and ethanol are closely related primary alcohols (table 2). The action of ethanol on proteins and nucleic acids has previously been discussed, however methanol is closely related in structure to water and thus can effectively compete with water for hydrogen bonds (Pearse, 1980). It is possible that methanol's smaller molecular size and greater affinity for hydrogen bonding may lead to localised points of weakness in the dsDNA molecule. Due to the low concentrations of methanol this is a slow and gradual effect over time. Methanol may also be potentially causing other problems. Methanol itself is not considered to be a good preservative (Simmons, 1995) since it is a powerful solvent that oxidises to formaldehyde and then formic acid. These additional oxidation products could be further enhancing the gradual degradation of the dsDNA.

Formula	Name	Type	Molecular weight
CH_3OH	Methanol	Primary	32.04
CH_3CH_2OH	Ethanol	Primary	46.07
$CH_3CHOHCH_3$	Isopropanol	Secondary	60.10
$CH_3CHOHCH_2OH$	Propylene glycol	Primary diol and secondary	76.09
$CH_2OHCHOCH_2OH$	Glycerol	Primary and secondary triol	92.09
$C_8H_{10}O_2$	Phenoxetol	Phenol	138.16

Table 2: The basic properties and formulae of alcohols commonly used in the preservation of zoological specimens (after Simmons, 1995).

When IMS is diluted with water, to improve its general morphological preservation characteristics, a considerable drop off in both the amount and the quality of the DNA can occur (Quicke *et al*., 1999). This is almost certainly due to hydrolytic damage from the action of water to which the labile base-sugar glycosyl bond in deoxyribose is susceptible (Lindahl, 1993). This can result in a greater loss of the purine bases that are not greatly inhibited by the double helical structure of dsDNA. The DNA bases, particularly cytosine, are also open to hydrolytic deamination although the double helical structure does offer greater protection from this form of degradation. Additionally hydrolytic damage causing nicking and fragmentation of the dsDNA can affect the sugar-phosphate backbone of the DNA molecule. Water is an aggressive polar solvent, the action of which will possibly also affect the hydrogen bonding between the base pairs in the dsDNA causing a weakening in the dsDNA structure and reducing its structural resilience. Unless biological material can be protected from the action of water in some way, its presence in the preserving solution will almost certainly be detrimental to the long term preservation of the DNA, and other biomolecules, in the preserved material.

The use of pre-treatments or additional additives to improve preservation has seen some study especially with the use of ethyl acetate (Reiss *et al.*, 1995; Dillon *et al.*, 1996). Other chemicals are in common usage with preserved material such as propylene glycol and 2-ethoxy ethanol, neither of which appear to have been previously considered for their effect on DNA preservation. In summary;

- Ethyl acetate; $CH_3OOC_2H_5$. Clear volatile flammable liquid that is miscible with ethanol. Although generally considered stable, it is slowly decomposed by moisture, acquiring an acid reaction.
- Propylene Glycol; $CH_3CHOHCH_2OH$. Hygroscopic viscous liquid, miscible with water and ethanol. Generally stable but can oxidise at high temperatures to aldehydes and organic acids. Considered harmless.
- 2-ethoxy ethanol; $HOCH_2CH_2OC_2H_5$. Colourless liquid that is miscible with water and ethanol. It dissolves many fats and oils and can de-fat the skin. It can also form explosive peroxides on storage and is significantly toxic.

Ethyl acetate appears to be highly damaging to DNA and this has been noted in other studies (Reiss *et al.*, 1995; Dillon *et al.*, 1996), although one recent study (Fukatsu, 1999) has considered ethyl acetate to have beneficial effects on the preservation of dsDNA. However the use of ethyl acetate in the preparation of entomological collections (Reiss *et al.*, 1995) has also been considered to be detrimental when using such collections in retrospective DNA studies. The evidence suggests that if ethyl acetate is present when biological material is fixed or preserved then significant change to the dsDNA can occur. Whether this change is from extensive fragmentation or from chemical alteration of the dsDNA is uncertain.

Propylene glycol is used extensively as an additive in alcohol-based fluid preservatives (Boase and Waller, 1994). It appears to enhance morphological preservation by reducing the shrinkage effects induced by alcohol based preservation, and imparting greater flexibility in preserved proteins. In addition propylene glycol is non-toxic and acts as an inhibitor of mould growth. The use of 2-ethoxy ethanol is somewhat more problematic. Its ability to dissolve fats and oils is probably the factor that makes its use attractive to some entomologists. Fats and oils are difficult to adequately preserve (Jones, 1976) and their breakdown products can cause subsequent degradation problems. However this must be balanced out with the excessive removal of these components which are all part of the bio-molecular makeup of an organism. Its potential toxicity and possible storage by products require this chemical to be used with care.

Subsequent drying of material is considered to be a practical long-term method of preservation where the morphological structure of a specimen allows (Goebel and Simmons, 1992). However Lindahl (1993) discusses potential problems with simply air drying methods, where the material remains partially hydrated and open to decay. Similarly if the DNA is completely 'dry' then it will loose the double helical conformation, making the bases more vulnerable to damage (Lindahl, 1993). This is because some water molecules are required in the grooves of the double helix in order to maintain the structure. Two specialist drying methods are increasingly commonly used to prepare specimens that have originally been fluid preserved. Both CPD and HMDS have given good dsDNA preservation results in past studies (Austin and Dillon, 1997). The preservation action of CPD techniques is understood. The method replaces the free water in a specimen with a dehydrating solvent such as ethanol, and then uses pressure and temperature to dry the specimen using carbon dioxide without exposure to

a liquid-gas boundary. This method of drying greatly reduces the structural distortion on a specimen normally caused by the surface tension effect of water. This removal of free water also removes the solvent environment in which DNA degrading reactions can occur, and prevents microbial decomposition activity (Hayat, 1978). HMDS treatment also involves replacing the water in a specimen with a dehydrating solvent, and then soaking the specimen in HMDS. The HMDS is then simply allowed to evaporate off, greatly reducing morphological distortion, yet the mechanism by which HMDS works on biological tissue does not appear to have been investigated in any detail. HMDS is used in gas chromatography to prepare silyl ethers of compounds with one or more reactive hydrogen atoms, such as sugars, amino acids and alcohols (Nation, 1983). However it is not known whether HMDS reacts with some of these compounds within the biological tissues, or reacts with the ethanol used in the dehydration treatment step of the process. It is probable that HMDS reacts with some biological tissues to form silyl ethers that may cross-link and subsequently strengthen proteins allowing the subsequent retention of morphological features on drying. The resulting preservation of dsDNA may be attributable to removal of free water, but not structural water, preventing hydrolytic damage and autolytic decay.

One of the biggest risks facing the long-term preservation of fluid preserved material is curatorial neglect (Waller, 1999). This results in collections receiving inadequate monitoring for preservation problems. One of the key problems with fluid preserved collections is fluid loss causing the drying out of the preserved material. The extent this can effect the gross morphology of the preserved material depends on the animal type being preserved (Carter, 1998). Animal groups with hard exoskeletons, such as insects and crustaceans, will be relatively unchanged over time. Soft bodied animal groups,

such as worms and anemones, lack this robust supporting structure and will become significantly shrivelled and deformed. Another key problem is that structural proteins will become shrunken and brittle making the preserved material more difficult to handle and manipulate. Many animal groups with long appendages can become extremely difficult to handle without significant breakage to limbs and antennae. Such issues can make the rehydration of biological material desirable, especially with neglected collections requiring extensive conservation and curation. However, what is the overall effect on the preservation of dsDNA in the preserved material? This is likely to be due to a number of factors. The history of preservation treatments used on the biological material will have the greatest effect on the condition of the dsDNA. Subsequently the storage environment will also have an effect on long-term molecular preservation. High temperature and humidity have the greatest degradative influence on the integrity of biological collections. Any subsequent conservation or preservation treatment is also likely to affect the condition of the preserved dsDNA. A common method to rehydrate dried biological material is to use a common laboratory detergent, Decon90. This is an unknown mix of anionic and non-ionic detergents, and has a strongly alkaline pH. Its rehydration action is believed to be through the partial maceration of biological tissues by detergent action. This alters the permeability of cellular membranes allowing the ingress of water into the biological tissue. Once the process is considered to be complete, the specimen can be transferred to a suitable preservative. Decon90 is just one of many chemicals suggested for the rehydration of biological tissue (Marhue, 1983; Jeppesen, 1988; Harris, 1990). The effect of the action of Decon90 on DNA preservation has not been investigated, although the use of laundry detergents has been previously suggested for the preservation of DNA in reptile blood samples (Kuch *et al.*, 1999).

1.3 The Invertebrate Collections of the National Museum and Gallery of Wales

The invertebrate collections at the NMGW number approximately 3 million specimens, and are particularly well developed in the areas of Mollusca, Annelida (marine), Crustacea and Insecta. These animal groups are preserved in a variety of ways, depending on the animal type, history and main uses of the collection. For example it is traditional to prepare most insect collections as dry specimens on pin mounts, although the specimens may originally have be collected by trapping using a fluid fixative or preservative. The more delicate soft bodied specimens often remain fluid-preserved or prepared as a slide mount, depending on the means used to identify the animal originally. Within the mollusc collections it is common to separate the shell and preserve this as a dry specimen, whilst the body is removed and fluid-preserved. The result is that the collections contain specimens that may well have been through a variety of preservation procedures, and are ultimately preserved as fluid specimens, dry preparations, slide mounts or SEM preparations.

1.3.1 Standard preservation protocols as used at the NMGW.

The methods of preserving an animal specimen can be diverse and even specific to a type of animal group (Wagstaffe and Fidler, 1955 and 1968; Hangey and Dingley, 1985; Harris, 1990). However, a large percentage of the NMGW collections have been put together from whole habitat sampling through trapping for terrestrial invertebrates (Southwood, 1978), or through marine sampling using benthic grabs or dredges (Mackie *et al.*, 1995). The result is that most of the recent collections, i.e. the bulk of the collections put together in the last 30 years, have been preserved along reasonably standard protocols and these have been summarised in Figure 7.

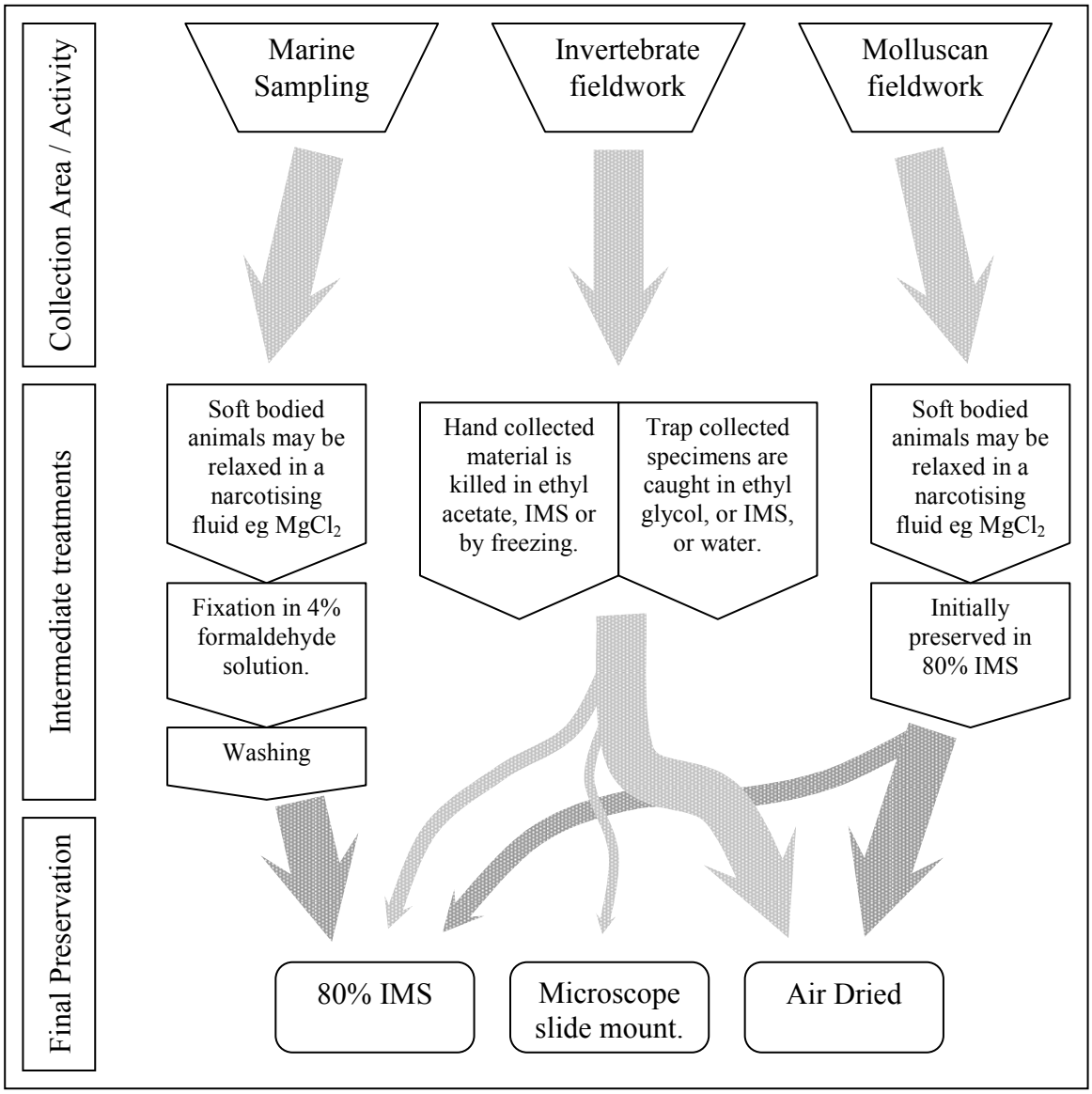


Figure 7: A summary of the main collection and preservation treatments used at the NMGW for the treatment and preservation of the invertebrate collections. The typical field work methods used are as outlined in Southwood (1978).

1.3.2 Conservation Treatments used at the NMGW

Conservation methods are considered to be protocols carried out on a specimen, once it has been collected and preserved, in order to prevent specimen deterioration or even destruction. The ultimate aim of any conservation action is to ensure that any treatment alters the specimen as little as possible, and is fully reversible. However, in practise any treatment will cause change and is never technically reversible (Horie, 1987). When working with invertebrate animal specimens the practical aim is to stop deterioration and enhance the stability of the specimen. The usual aim has been to attempt to restore the specimen to as correct a morphological state as possible, but now the uses of these collections are changing and the effect of these treatments on the DNA now needs to be considered. Figure 8 summarises some of the key conservation actions used with invertebrate collection conservation at NMGW (Carter, 1998).

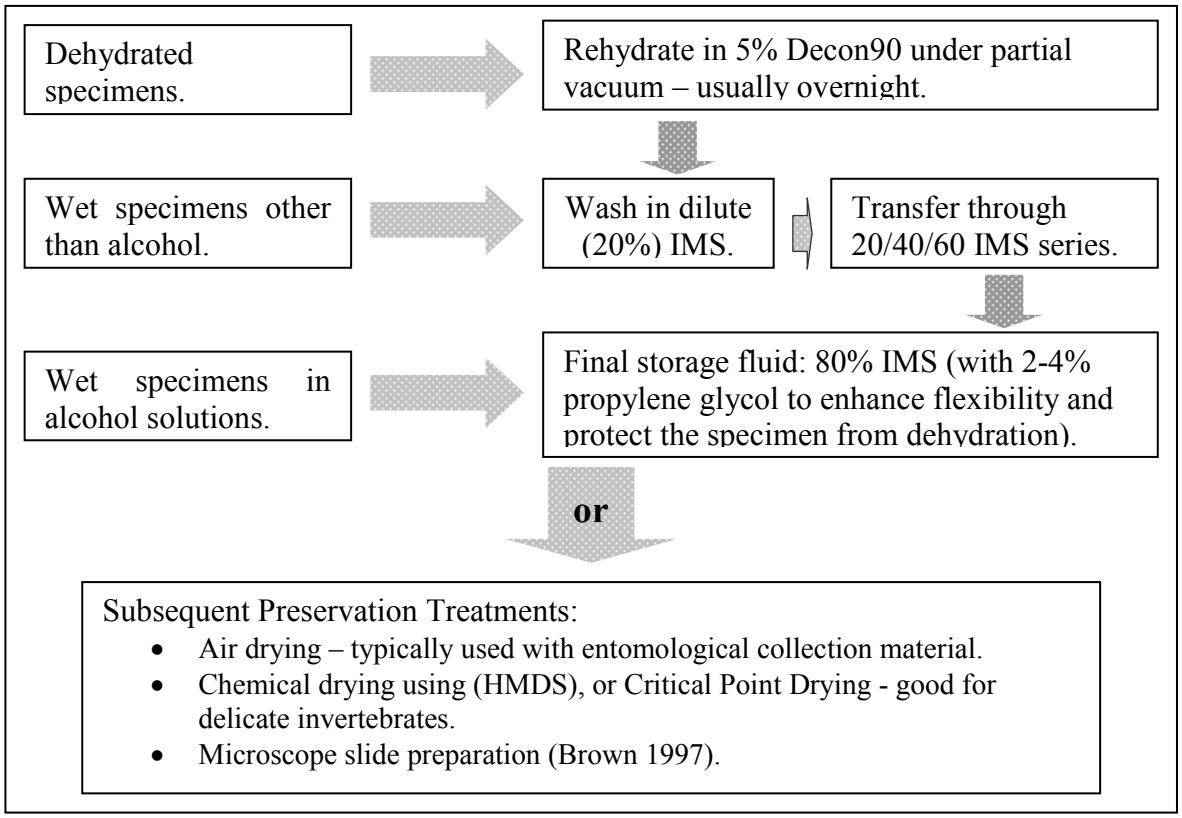


Figure 8: A summary of the main conservation actions used to conserve invertebrate collection material at the NMGW.

1.4 Project Rationale and Aims

The aim of this project is to further improve our understanding of how collection, preservation and conservation processes affect the DNA of specimens in museum invertebrate collections, particularly fluid preserved collections. It is now important to review the status of museum methods of specimen preservation and storage in order that our understanding of these processes can be improved, with the aim of balancing whole specimen morphology with biochemical analysis. The main aim of this project is to review the existing literature in order to compile the known data, and to then carry out a study to further explore the effects of the standard methods of preservation and conservation as used at the National Museum of Wales from a genetic perspective. An assessment of the effects of the various preservation and conservation treatments on the DNA of a selected invertebrate species will be investigated in order to deduce their effects.

The study focused on considering the methods of fixation and preservation utilised around the fluid preserved collections of the NMGW's zoological based collections. These have been summarised in section 1.3. In principle a number of areas desired investigation;

- A review of ethanol-based preservation, including the effects of using IMS, the effects of additives to the alcohol-based preservative, and the effects of storage time.
- The effects on the integrity of the DNA of subsequent preservation and conservation methods such as specialist drying, CPD and HMDS, fluid changes, and rehydration of dried out material.

As the aim of the study was to monitor the quantity and quality of the DNA that could be extracted from an invertebrate species that has undergone a series of preservation and

conservation treatments, a single species needed to be chosen as a standard to work upon. The species chosen for the study was the common woodlouse, *Porcellio scaber*, (figure 9) which is very easy to collect and identify. It is also very numerous and is present all year round. All the specimens were collected from NGR ST126818.

To assess how the preservation and conservation treatments affected the integrity of the DNA, numerous methods needed to be considered on how best to achieve this. The study needed to consider how to:

- reliably extract DNA from the woodlouse, *Porcellio scaber*.
- quantify the extracted DNA
- assess the quality of the extracted DNA.

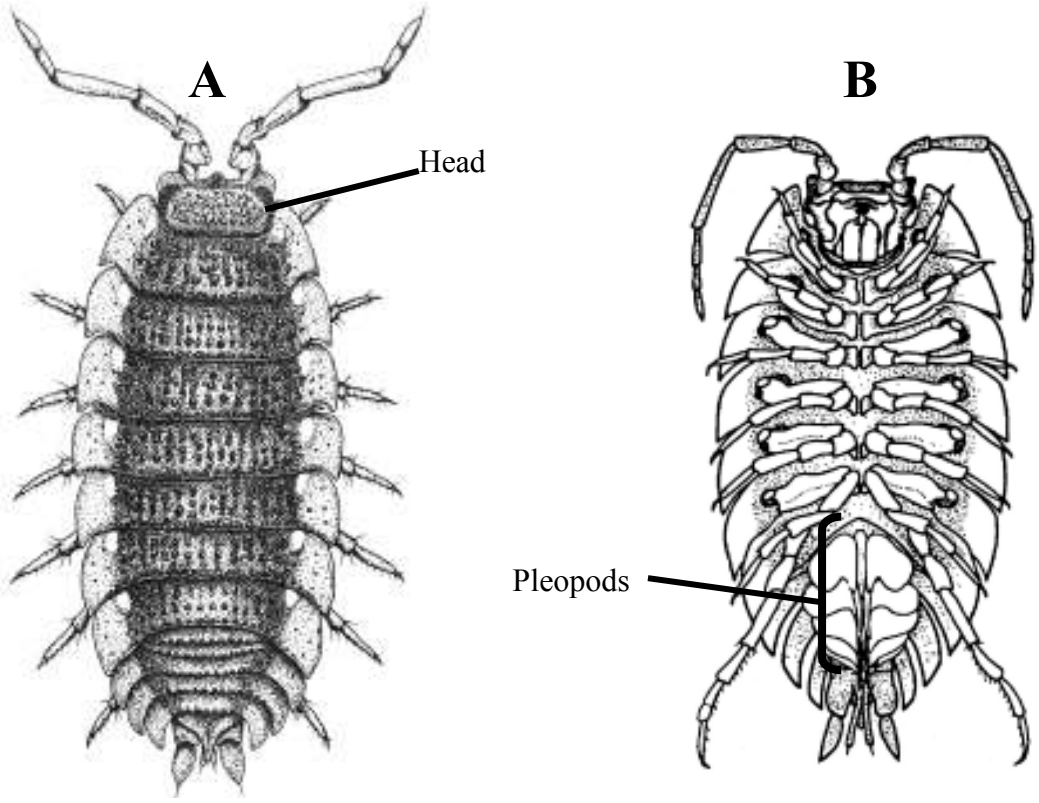


Figure 9: The woodlouse, *Porcellio scaber*. A: shows the dorsal surface. B: shows the ventral surface and the position of the pleopods. (Illustration by Chris Meecham, NMGW).

Chapter 2.

DNA Extraction: Quantity and Quality.

2.0 Introduction

Table 3 summarises the treatments that were considered in this study, while table 9 details the samples analysed, including the number of samples and any replications. Figure 10 summarises the experimental procedure developed for use in this study. Prior to finalising the methods described in 'Materials and Methods', an extensive review into possible protocols was carried out. This considered DNA extraction protocols, quantifying the extracted DNA and assessing the quality of the extracted DNA.

Treatment	Summary of processes considered in the study
Cryopreservation	A standard method by which the other methods will be assessed for their affects on the extractable DNA.
Absolute Ethanol	The effects of ethanol and DNA preservation were revisited, taking into account storage temperature, storage time; ethanol change and additives.
Industrial Methylated Spirits	The main preservative used at NMGW. The study considered the effects of undiluted IMS, 80% IMS, storage time and additives.
Formaldehyde	A 4% solution was used to compare the effects of formaldehyde treatment with the other methods considered.
Steedmans Solution	Fixative / preservative solution that uses formaldehyde, phenoxetol and propylene glycol.
Additives / Pretreatments	The whole scale preservation effects of propylene glycol, ethyl acetate, and 2 ethoxy ethanol on DNA were considered.
CPD / HMDS drying methods	Specialist drying methods. Their effect on DNA preservation was considered.
Rehydration in Decon90	Dried out specimens, initially ethanol preserved, were rehydrated in Decon90 to assess the effects on DNA.

Table 3: A summary of the treatments considered in this study.

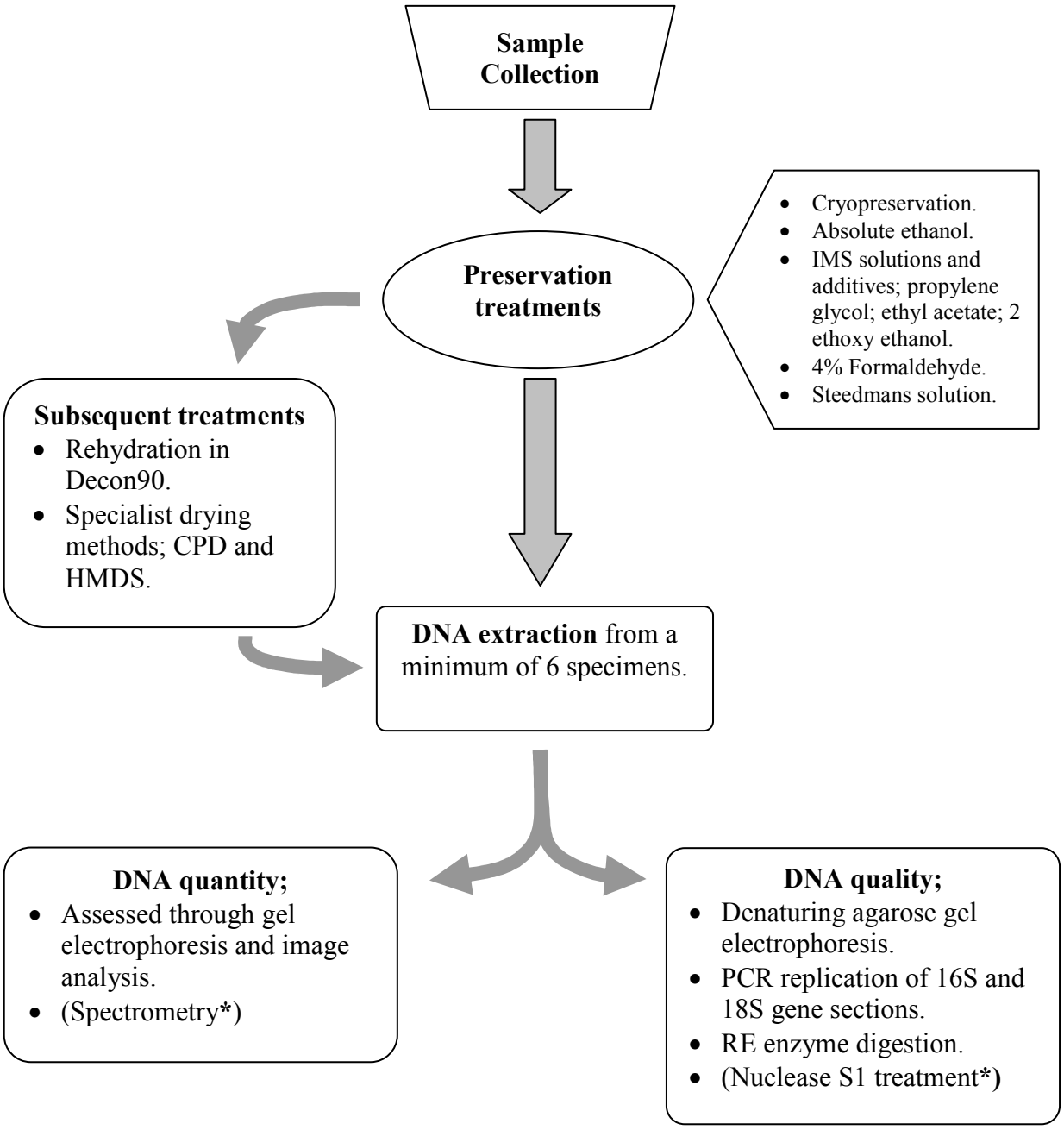


Figure 10: Flow diagram summarising the main steps in the experimental protocol used for this study. (* not used in the full study).

2.1 DNA Extraction

The study required a standard method of DNA extraction to be developed. The overall quality of the DNA depends on the extraction protocol used (Reineke *et al.*, 1998). This will also depend on the DNA source because of differences in interfering substances

present in biological tissue. Prior to the method used in this study a search of the literature revealed a wide range of DNA extraction methods and adaptations to techniques (Sambrook *et al.*, 1989; Heller *et al.,* 1991; Towner, 1991; Walsh *et al.,* 1991; Mőller *et al.,* 1992; Cano and Poinar, 1993: Cushwa and Medrano, 1993; DeSalle *et al.,* 1993; Rowland and Nguyen, 1993; Stewart and Via, 1993; Gibb and Padovan, 1994; Laulier *et al.,* 1995; Reiss *et al.,* 1995; Austin and Dillon, 1997; D'Amato and Coracti, 1996; Dillon *et al.,* 1996; Hammond *et al.,* 1996; Taylor *et al.,* 1996; Vachot and Monnerot, 1996; Hunt, 1997; Reineke *et al.,* 1998). A standard DNA extraction technique would allow a comparison of the ease of DNA extraction from the differently-treated specimens. However it must be stressed that any one method may not be the optimal technique for extraction of DNA for that particular treatment e.g. formaldehyde preserved material would require extended digestion times (Vachot and Monnerot, 1996).

A variety of DNA methods were considered, with the aim of using a method that gave DNA of high molecular weight and good quality from fresh material, but with as simple a protocol as possible to reduce the chance of errors and the need to use hazardous chemicals. Crustacea are considered to be a difficult animal group to work upon with DNA studies. The potential supply of Ca^{2+} ions from the calcareous integument can promote DNAase activity (Adams *et al.*, 1999) and degrade the DNA, thus methods that focused on working with difficult animal groups were reviewed. This led to the use of the method described in 'Materials and methods'.

2.2 DNA Quantity

It was found that the use of spectrometry readings to quantify the extracted DNA obtained in this study was not a reliable measure of the DNA content. The spectrophotometer and the Genequant instrument readings could not be correlated to each other (table 4). Other workers have found similar problems when trying to quantify dsDNA extracted from invertebrates (Reiss *et al*., 1995), and it is felt that problems may be associated with pigments or other cellular components co-purifying with the DNA during the extraction process causing inaccurate spectrometric readings. It was considered that the use of image analysis of the extracted DNA on agarose gels was a more robust method of quantifying the DNA. Table 4 illustrates examples of the DNA quantities obtained on the same samples with the three methods assessed in this study to quantify the DNA.

Sample (from ethanol preserved material)	Spectrophotometer readings (260nm) ($\mu g\ ml^{-1}$)	Pharmacia Genequant ($\mu g\ ml^{-1}$)	Image analysis of agarose gel ($\mu g\ ml^{-1}$)
1	110	319	58
2	300	170	38
3	100	162	34
4	180	207	53
5	200	140	38

Table 4: Comparison of the DNA quantities using the three methods assessed in this study: 260/280nm spectrophotometer readings; Genequant dedicated instrument; image analysis of whole genomic extract on agarose gel.

2.3 DNA Quality

A review was made of the main methods utilising DNA analysis with invertebrate animals. Hillis *et al.* (1996a) provides a valuable overview of the methodologies utilised in molecular systematic research, and short papers such as Cook (1996) help guide invertebrate workers into the field of molecular study. The majority of these techniques require the DNA to be able to act as a substrate for key enzymes such as polymerases and restriction endonucleases. Many of these methods utilise the PCR reaction in some way to replicate the DNA. PCR is normally carried out on extracted DNA, but methods of direct PCR can also be used (Burns *et al.*, 1997). The PCR reaction itself can be the provider of information through the use of quantitative PCR (Zimmermann and Mannhalter, 1996; Cayouette *et al.*, 1999). It is more usual for the amplified DNA product to be further analysed through methods such as DNA base sequencing (Thomas and Kocher, 1992; Hillis *et al.*, 1996b), Random Amplified Polymorphic DNA, RAPD (Hadrys *et al.*, 1992; Haymer, 1994; Pascual *et al.*, 1997; Wilson *et al.*, 1997; Cobb, 1997); Amplified Fragment Length Polymorphism, AFLP (Vos *et al.*, 1995; Semblat *et al.*, 1998); microsatellite markers (Hughes and Queller, 1993; Shaw, 1997).

DNA quality in preserved specimens has also been assessed using agarose gel electrophoresis (Post *et al*. 1993). The profile of the DNA on the gel can indicate the integrity of the extracted DNA. Other studies have initially assessed the extracted DNA on a gel, and have then used the DNA in a variety of molecular techniques utilised in molecular systematic research e.g. Greer *et al.* (1991a and 1994) measured the effect of fixation of biological tissue by the ability of the DNA in a treated tissue to act as a template for the amplification of DNA fragments of increasing length. By sequencing the PCR product other workers (France and Kocher, 1996; Vachot and Monnerot, 1996)

have assessed whether preservation treatments alter the DNA sequence itself. RAPD has been use by a number of workers (Stewart and Via, 1993; Hammond *et al.*, 1996; Štorchová *et al.*, 2000), with the quality of the resulting RAPD pattern giving a measure of quality of the DNA. RFLP has also been used (Rogstad, 1992; Harris, 1993; Rowland and Nguyen, 1993; Asahiba *et al*,. 1996), with the effectiveness of Restriction Endonucleases to act on the DNA being a measure of quality. Other studies have assessed the usability of the DNA in hybridisation studies (Houde and Braun, 1988; Vij *et al.*, 1997). The whole variety of the methods that have been used to assess the quality of the extracted DNA from preserved specimens indicates the wide range of techniques now open to the molecular biologist for studying the information in DNA.

From the review a number of methods of analysis were considered for assessing DNA quality;

- Gel electrophoresis.
- Restriction Endonuclease enzyme activity.
- Polymerase enzyme activity.
- Nuclease S1 enzyme activity.

2.3.1 Gel Electrophoresis.

The use of agarose gel electrophoresis techniques forms an important part of many of the methods used in this study. Using agarose gels, combined with ethidium bromide staining and subsequent UV visualisation, is an important method of viewing and analysing the products of DNA extraction and analysis. However, the use of electrophoresis in the analysis of nucleic acids is not limited to the use of standard agarose gels (Andrews, 1991; Lessa and Applebaum, 1993). Methods of denaturing gel

electrophoresis were also assessed (Myersa *et al.*, 1987; Lessa, 1993; Dowling *et al.*, 1996). The use of alkaline gel electrophoresis has been found to be a simple method of estimating single strand breaks in DNA (Freeman *et al.*, 1986; Shin and Day, 1995; Zirkle and Krieg, 1996). Under alkaline conditions the hydrogen bonding between the base pairs is disrupted and the DNA is denatured. The result would be that dsDNA that has been badly nicked would fragment into smaller pieces when denatured. This could then be assessed from the gel image.

2.3.2 Restriction Endonucleases screening.

Restriction endonucleases (RE) are enzymes that cut dsDNA at a constant position within a specific recognition sequence, typically 4-6bp long. One way to compare the quality of the extracted DNA is to compare the effectiveness of RE enzymes on the extracted dsDNA. The assumption is that the better the preservation condition of the DNA the more effectively it will be cut by RE enzymes. The effectiveness of the RE enzymes will be dependent on the overall structural condition of the DNA, and the extent of site specific modifications such as methylation (Yosef *et al.*, 1981; Kupper *et al.*, 1997; Nelson *et al.*, 1993). The effect of RE enzyme action on the extracted DNA can then be assessed through agarose gel electrophoresis and image analysis.

Table 5 shows the range of RE enzymes screened and summarises their effectiveness on the extracted DNA from ethanol killed specimens. RE's with adenine and thymine in their recognition sequences tended to give a greater 'cutting' effect than GC rich recognition sequences. The 'rare cutter' RE's appeared to have little effect on the extracted DNA. The result of the screening was that the RE *Hinf*1, and the RE *Dra*1

were used for this study for the RE analysis of the extracted DNA as these RE's demonstrated an effective action on the extracted DNA template.

2.3.3 PCR amplification of specific genes

The successful amplification of extracted DNA by the PCR cycle would indicate the presence of good quality DNA that would denature and re-anneal during the PCR cycle, allowing polymerase action to occur. In considering the use of PCR, thought was given as to what DNA to try and replicate. Invertebrate cells have two key forms of DNA, multicopy mitochondrial DNA (mDNA) and single/low copy nuclear DNA (nDNA). The nDNA is associated with complex protein structures, nucleosomes, and other non-

Enzyme	Recognition Sequence	'Cutting' effect on extracted DNA
Dra I	TTTAAA	Good, some banding on gels
Eco RI	GAATTC	Partial
Hin fI	GANTC	Good, some banding on gels
Hind III	AAGCTT	Partial to Good, faint banding on gel
Hpa I	GTTAAC	Partial
Hpa II	GCGG	Good, faint banding on gels
Msp I	CCGG	Good
Nci I	CC(G,C)GG	None
Pst I	CTGCAG	None
Sal I	GTCGAC	None
Sau 3A	GATC	Good, no banding pattern
Sfi I	GGCCNNGGCC	None
Sma I	CCCGGG	None
Swa I	ATTTAAAT	None
Xba I	TCTAGA	Partial

N – any sequence

Table 5: List of RE enzymes that have been screened for this study.

histone proteins to make chromatin. If the nDNA cannot be dissociated from these associations then it is unavailable for PCR (Simpson *et al*, 1999). Alternatively the low copy number of nDNA in a cell can make less DNA available for PCR based studies, especially if the DNA becomes degraded. The mDNA is relatively easily available since it occurs in an organelle other than the nucleus, it does not associate with nucleosomes, and it is available in a multicopy number. The mDNA is therefore available in higher concentrations than the nDNA. For this study both mDNA and nDNA genes were used as targets for PCR amplification when comparing the quality of the DNA extracted from the various treatments.

For the PCR it was necessary to find suitable primers for the amplification of the DNA targets. Palumbi (1996) provides a good review of both PCR techniques and useful primers. Commonly used animal mitochondrial gene primers are the small subunit ribosomal RNA gene 16S and the subunit of the cytochrome oxidase complex, COI. Both of these genes have been widely used in invertebrate animal molecular investigations, including crustacean groups (Bucklin *et al.*, 1992; DeSalle *et al.*, 1993; Folmer *et al.*, 1994; Kann and Wishner, 1996; Geller *et al.*, 1997; Meyran *et al.*, 1997; Baldwin *et al.*, 1998; Harrison and Crespi, 1999). For the nuclear DNA, the 18S subunit of the nuclear ribosomal gene has been commonly used with invertebrate animal groups (Brower and DeSalle, 1994; Han and McPheron, 1994; Spears *et al.*, 1994; Barques and Mas-Coma, 1997; Black *et al.*, 1997; Mizrahi *et al.,* 1998; Maddison *et al.*, 1999).

2.3.4 Nuclease S1 treatment.

In moderate quantities Nuclease S1 will cleave dsDNA at nick points (Sambrook *et al.,* 1989; Jaraczewski and Jahn, 1993). If the dsDNA has been nicked by a particular

treatment, then Nuclease S1 treatment can be used to cleave the dsDNA at that position. DNA that has been nicked will thus be fragmented when compared to good quality DNA. Other nucleases such as Mung Bean Nuclease (Kroeker and Kowalski, 1978) or Bal31 can also be used to break apart large DNA fragments.

A series of experiments were run comparing the action of nuclease S1 on aliquots of high molecular weight DNA extracted from ethanol killed specimens. The nuclease S1 was used at a variety of concentrations on non-UV exposed and UV exposed DNA (figure 11). However it was difficult to decide what concentration was a 'moderate' quantity of Nuclease S1. This is the point where the S1 enzyme action was causing strand breakage at nick points, rather than the enzymes 'normal' action of removing end

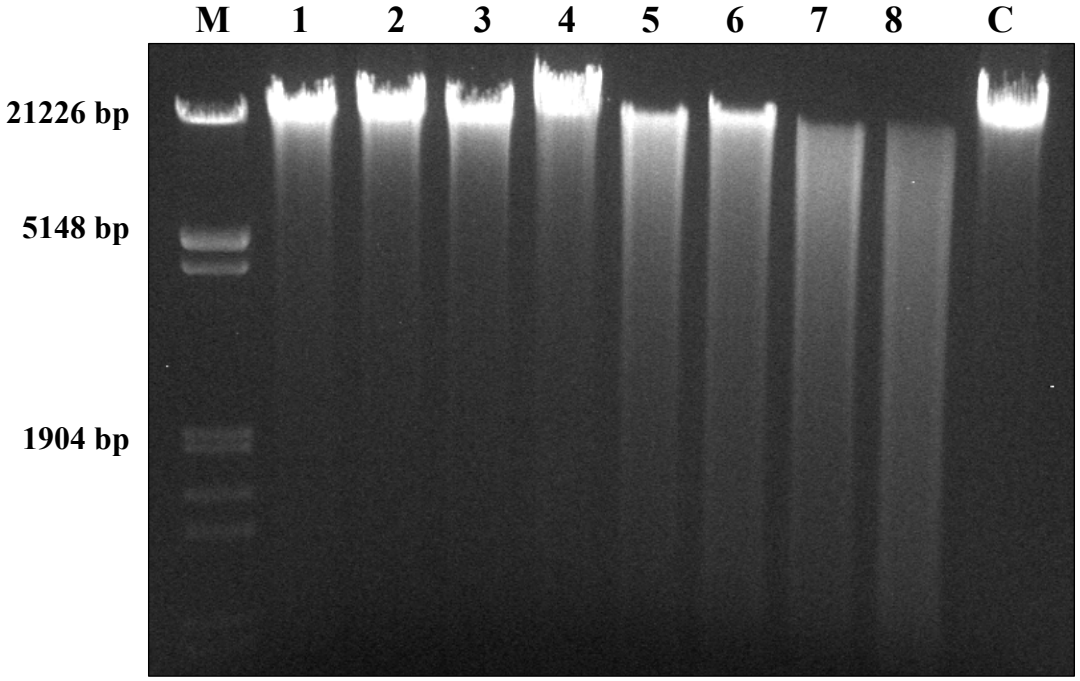


Figure 11: An example of the effects of nuclease S1 activity on high molecular weight dsDNA extracted from ethanol killed specimens. M; 10 µl Boehringer Mannheim DNA molecular weight marker III. Lanes 1 and 2; effect of 10 U of nuclease S1 on dsDNA. Lanes 3 and 4; effect of 10 U of nuclease S1 on dsDNA exposed to UV light for 10 minutes. Lanes 5 and 6; 50 U of nuclease S1 on dsDNA. Lanes 7 and 8; 50 U of nuclease S1 on dsDNA exposed to UV light for 10 minutes. C; control dsDNA sample (no nuclease S1 treatment).

bases (Sambrook *et al*., 1989). Thus, it was difficult to reliably define how the nuclease S1 action was affecting the sample DNA and the method was not further used in this study.

Chapter 3.

Materials and Methods

3.0 Introduction

General laboratory practise and gel electrophoresis protocols were taken from Sambrook *et al.* (1989); Andrews (1991); Hillis *et al*. (1996a).

3.1 Preparation of agarose gels

Agarose gel electrophoresis of DNA samples was carried out using 1.0% (wv^{-1}) agarose (for whole genomic DNA) or 1.5% (wv^{-1}) agarose (for RE digested DNA) in 1 × Tris-Borate-EDTA buffer (TBE, 10.8 g l^{-1} tris base, 5.5 g l^{-1} boric acid, 5ml 0.5 M EDTA pH 8.0, containing 5 µg ml^{-1} of ethidium bromide in both gel and running buffer. DNA samples to be electrophoresed were mixed with 10% loading dye (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol, 50 mM EDTA pH 8.0) prior to loading. Electrophoresis was carried out at 60 V for 2 hours for whole genomic DNA samples, or at 20 V overnight for RE digested DNA samples. DNA was visualised by illumination with UV light (310 nm), and images recorded as TIF files using a digital gel documentation system.

3.2 DNA Extraction

A CTAB extraction method, based on Möller *et al.* (1992) and Reinke *et al.* (1998), was adapted. The rear part of the woodlouse, containing the pleopods (figure 9), was removed. The gut (a source of microbial DNA) and excess cuticle was then also removed with fine tweezers and discarded. The woodlouse tissue was then placed in a 1.5 ml microcentrifuge tube containing 200 µl TE (0.05 M EDTA; 0.1 M Tris HCl pH 8.0) and homogenised using a micro-pestle. After homogenisation a further 200 µl TE; 50 µl 20% wv^{-1} SDS; 15µl Proteinase K (2 mg ml^{-1}); was added to each tube. The tubes were then incubated for 2 hours at 60°C, after which 200 µl 5 M NaCl and $1/10^{th}$

volume of 10% wv^{-1} CTAB was added, briefly mixed by inversion of the tubes several times, and then allowed to incubate for 15 minutes at 60°C. 20 µl of RNAse A (10 mg ml^{-1}) was added to each tube, and incubated at 37°C for 30 minutes. The tubes were then removed from incubation and an equal volume of cold 24:1 chloroform:isoamyl alcohol was added. The tubes were then gently, but thoroughly mixed by inversion, and incubated at room temperature for 5 minutes. The samples were then microcentrifuged for 5 minutes, and the upper, aqueous, layer removed to a new tube using a wide bore pipette tip. Promega Protein Precipitation solution (1/3rd volume) was added to the aqueous phase and vortexed prior to microcentrifuging for 5 minutes. The supernatant was then removed and the DNA was precipitated with 1 volume of cold isopropanol. The tubes were then gently inverted to mix the isopropanol and precipitate out the DNA. They were then placed in the freezer overnight to maximise the DNA yield, after which they were microcentrifuged for 10 minutes. The isopropanol was then removed and the tubes allowed to air dry inverted for 10 minutes, before the addition of 200 µl of TE buffer to re-dissolve the DNA pellet. Once dissolved the extracted DNA was microcentrifuged for 2 minutes before removing 10 µl for analysis by agarose gel electrophoresis. This was to remove any remaining precipitated material.

3.3 Quantifying the DNA

Two key methods were initially assessed for this study: spectrometry and image analysis. In practice it was felt that the image analysis method was the more reliable for use in this study (see section 3.1).

2.3.1 Spectrometry

Two types of spectrometer were assessed, a Cecil Series 6000 spectrophotometer and a dedicated Pharmacia Genequant instrument. The Cecil Series 6000 spectrophotometer can be used to measure both the quantity and quality of the DNA. This is based on the reasoning that 50 μg ml^{-1} of dsDNA will have an O.D. of 1 at 260nm, and that a 260nm/280nm ratio of around 1.8 would indicate a pure sample (although this ratio is variable depending on the base pair ratio in the DNA). One problem with using this instrumentation was the quantity of sample that was required, the smallest practical volume that could be used proved to be about 500 μl. This involved taking 50 μl of extracted DNA and diluting with TE buffer to 500 μl. Readings were then taken at 260nm and 280nm, and the ratio calculated.

The Pharmacia Genequant is a dedicated instrument that uses very low volumes of extract by using microcapillary tubes. The extract is drawn up into the tube through capillary action. The top of the tube is sealed with Clingfilm. The tube is then placed in the light cell, which is in turn placed in the machine. The Genequant automatically gives dsDNA quantity in μg ml^{-1}, purity and 260/280nm ratio.

3.3.2 Quantifying the DNA through image analysis of agarose gels

Agarose gel computer TIF images were analysed using the freeware image analysis programme Scion Image (www.scioncorp.com). The images were inverted and a 2D rolling ball background subtraction carried out over the area of interest. A broad band density plot was then carried out on each gel lane (Freeman *et al.,* 1986; Hadrys *et al.,* 1992), followed by an Integrated Density reading. The readings were then saved as

TXT files. The TXT files were then compiled and imported into Microsoft Excel. Each lane could then be compared to the known DNA in the DNA marker ladder.

The Image Analysis gel readings could then be used in two ways;

- Broad band density plot: The readings from this can be plotted graphically (Excel). By plotting the density data readings from the sample lanes with the DNA marker then the quantity, distribution and size of the DNA in the sample can be assessed (figure 12).
- Integrated density reading: The integrated density feature of Scion Image measures the sum of the grey values in the selection, with the background value subtracted. This can be used to effectively measure the volume of DNA in a sample band;

  Integrated Density = N × (Mean gray value – Background gray value)

  where N is the number of pixels in the selection.

Comparing the integrated density reading for a known quantity of DNA to the unknown integrated density measurements of the extracted DNA samples can then be used to assess the quantity of DNA in a sample. These readings were averaged from a number of separate sample extractions (usually six). From these results an error reading was calculated. This sampling error was assessed from the standard error (S.E.) of the mean where;

$$\text{S.E.} = \frac{\text{sample standard deviation}}{\sqrt{\text{number of sampling units}}} = \frac{s}{\sqrt{n}}$$

$$s = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$$

The sample standard deviation, s, is calculated from;

where x is the sample value, $\bar{x}$ is the sample mean and n is the number of samples.

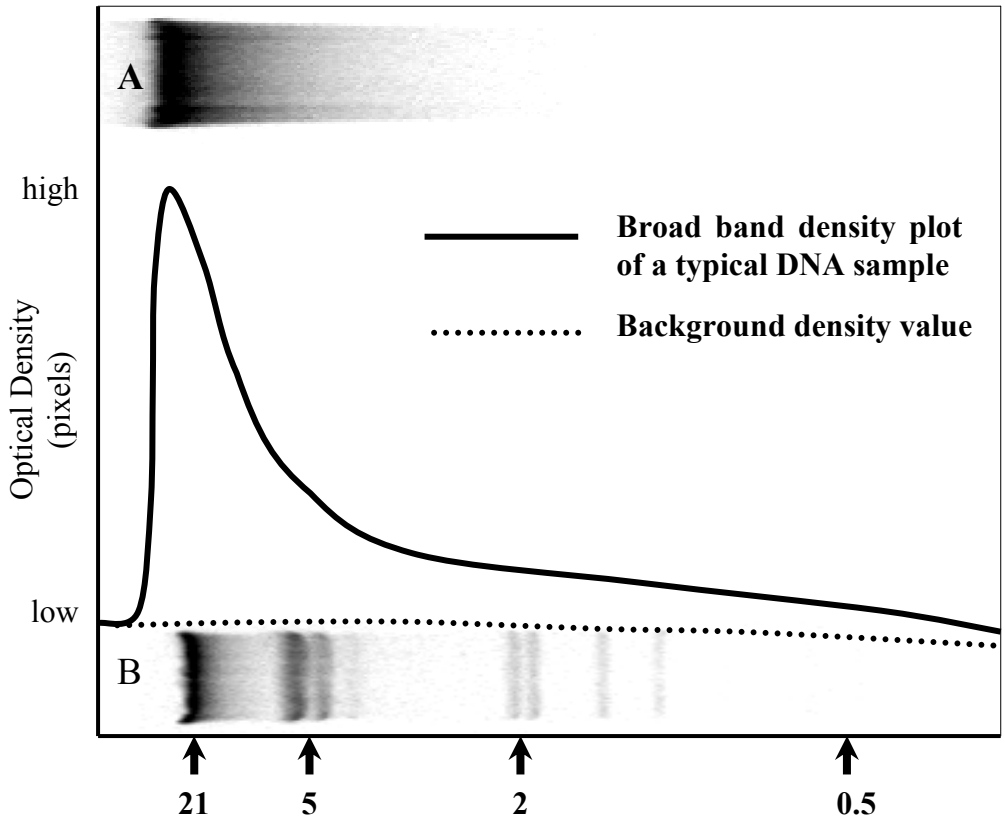


Figure 12: Graphical representation of broad band density plot data. The y axis is the optical density, calculated from the pixel values of the broad band density plot of the sample lanes on the agarose gel. The x axis represents the distribution of the molecular size of the DNA fragments of the extracted DNA. **A** shows a typical agarose gel image of an aliquot of extracted DNA, whilst the solid graph line represents the density reading along the band from the gel image. The higher the optical density reading, the higher the DNA concentration at that point on the gel band. The total DNA concentration in the aliquot is in effect the area under the curve in the graph, which can also be estimated through the integrated density reading. **B** represents the DNA marker band, which is used to estimate the size distribution of the DNA in the sample aliquot.

3.4 Restriction Endonuclease digestion of the extracted DNA

A process of screening for suitable RE enzymes was carried out, using information provided by manufacturers, REBASE (Roberts and Macelis, 1993) and research (Hughes and Queller, 1993; Vogler *et al.*, 1993; Zeh *et al.*, 1993; Taylor et al, 1996; Pascual *et al.*, 1997; Semblat *et al.*, 1998). Consideration was given to a range of RE enzymes starting with the common 'frequent' cutters such as the tetranucleotide to hexanucleotide recognising RE's, to some of the 'rare cutter' enzymes. These have octanucleotide recognition sequences and are used to generate large fragments (McClelland, 1987; Qiang and Schildkraut, 1987). The procedure used for the RE digestion of the extracted DNA was as follows;

A 20 µl aliquot of the extracted DNA was mixed with 2 µl RE enzyme; 5 µl ddH_2O and 3 µl RE 10× buffer. The tubes were then briefly 'flick' mixed and incubated at 37°C for 3 hours. The digest was then stopped with 10% of load dye and examined by agarose gel electrophoresis.

3.5 Nuclease S1 treatment.

The methods of Jaraczewski and Jahn (1993) and Kroeker and Kowalski (1978) were adapted. Stock Nuclease S1 (400 U μl^{-1}) was diluted to a working concentration of 10 U μl^{-1} using ddH_2O. A solution of 10× incubation buffer was prepared (330 mM l^{-1} sodium acetate; 500 mM l^{-1} sodium chloride; 10 mM l^{-1} zinc sulphate). A 10 µl sample of extracted DNA (of known concentration) was added to the side of a 1.5 ml microcentrifuge tube, along with 2 µl 10× buffer, 30 U μl^{-1} nuclease S1 per 1.0 µg DNA, and sufficient ddH_2O to bring the total reaction volume to 20 µl. The tube was then very briefly centrifuged to start the reaction, which was run at 37°C for 30 minutes. The reaction was then stopped by the addition of 3 µl of loading dye. The results were

analysed by running the samples on a 0.5% agarose gel at 40 V until the dye band was approximately 2/3rds along the length of the gel. The gel was visualised under UV light and a digital TIF image captured.

3.6 Alkaline denaturing agarose gel electrophoresis.

The methods of Zirkle and Krieg (1996) were adapted. A 0.8% agarose gel was prepared in 50 mM NaCl and 4 mM EDTA. The gel was then allowed to pre-soak in the alkaline running buffer, consisting of 30 mM NaOH and 2 mM EDTA, for 30 minutes. The gel was then pre-run for 10 minutes. 20 µl aliquots of extracted DNA were denatured in $1/5^{th}$ volume of alkaline stop buffer; consisting of 25% (v/v) glycerol; 0.125% (w/v) bromocresol green; 0.5 M NaOH; 5 mM EDTA. The samples were then incubated at room temperature for 40 minutes prior to loading onto the gel. The loaded gel was then run at 40V for approximately 3 hours, after which time the gel was neutralised in 0.1 M Tris-HCl pH 8.0 for 30 minutes, stained in 1 µg ml^{-1} ethidium bromide for 30 minutes and then washed in ddH_2O for 30 minutes. The gel was then visualised under UV and the image recorded as a TIF file.

3.7 PCR amplification of extracted DNA

The PCR reaction followed the general guidelines of Palumbi (1996). The following primer options gave good amplification products with the extracted DNA;

16S (Bucklin *et al.*, 1992; Palumbi, 1996; Geller *et al.*, 1997). Expected product approximately 500 bp.

16SAR (forward primer) 5'-CGCCTGTTTAACAAAAACAT-3'

16SBR (reverse primer) 5'-CCGGTTTGAACTCAGATCACGT-3'

18S (Black *et al.*, 1997). Expected product approximately 1400 bp with NS3 and NS4, and approximately 800 bp with NS3 and NS34-.

NS3 (forward primer) 5'-GCAAGTCTGGTGCCAGCAGCC-3'

NS4 (reverse primer) 5'-CTTCCGTCAATTCCTTTAAG-3'

NS34- (reverse primer) 5'-CGRTCCAAGAATTTCACCTCT-3'

The reaction volume used was 50 µl, but the reagents were prepared in larger 'master mix' to reduce pipetting errors. The 50 µl reaction volume consisted of 36.5 µl ddH_2O; 5 µl 10× reaction buffer; 1 µl 10 mM dNTP's; 2 µl of each primer; 1.5 µl $MgCl_2$; 1 µl template DNA and 0.5 U Bio-X-act DNA polymerase (Bioline). Negative controls, where the DNA had been replaced by water, were used to monitor for contamination. The DNA was amplified in a PTC-100 Thermocycler (MJ Research) with a heated lid, using the following amplification cycle; an initial denaturation of 4 minutes at 94°C, followed by 40 cycles of: 1 minute denaturation at 96°C, 1 minute annealing at 51°C for the 16SAR/SBR primers, or 1 minute annealing at 64°C for the NS primers, and 1 min extension at 72°C. The programme ended with 5 minutes at 72°C to complete extension of the DNA. The PCR amplification products were then examined using 10 µl of each PCR product, plus 2 µl of load dye and running on a 1% Agarose gel, run for approximately 1 hour at 100 V. The DNA was visualised by ethidium bromide staining and fluorescence with UV. A computer TIF image was captured for later examination.

Chapter 4.

Results.

4.0 Results

The results of the samples analysed in this study are summarised in Table 9 (section 4.8). Due to time restraints and problems with equipment availability the full range of treatments planned could not be assessed. However it was considered that a sufficient range of results had been obtained, and a number of interesting observations noted. The results from the study will be considered in treatment groups;

- The results from standard treatments cryo-preservation and absolute ethanol. This allows comparison of the effects of the various treatments on the condition of the DNA.
- The effects of ethanol preservation.
- The effects of IMS preservation.
- The effects of other chemicals used as additives in preservation fluids.
- The effects of other fluid preservatives, notably formaldehyde.
- The rehydration of dried material using Decon90.
- Subsequent treatments; the effects of specialist drying methods.
- Summary table of results.

4.1 The integrity of the DNA from cryo-preserved and absolute ethanol preserved material.

The use of absolute ethanol and cryo-preservation are considered standard methods for the preservation of material for DNA studies. The results obtained with samples preserved by these methods were used to allow comparison with the other treatments used in this study.

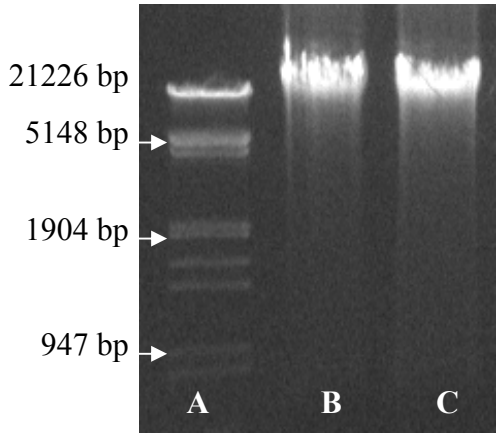


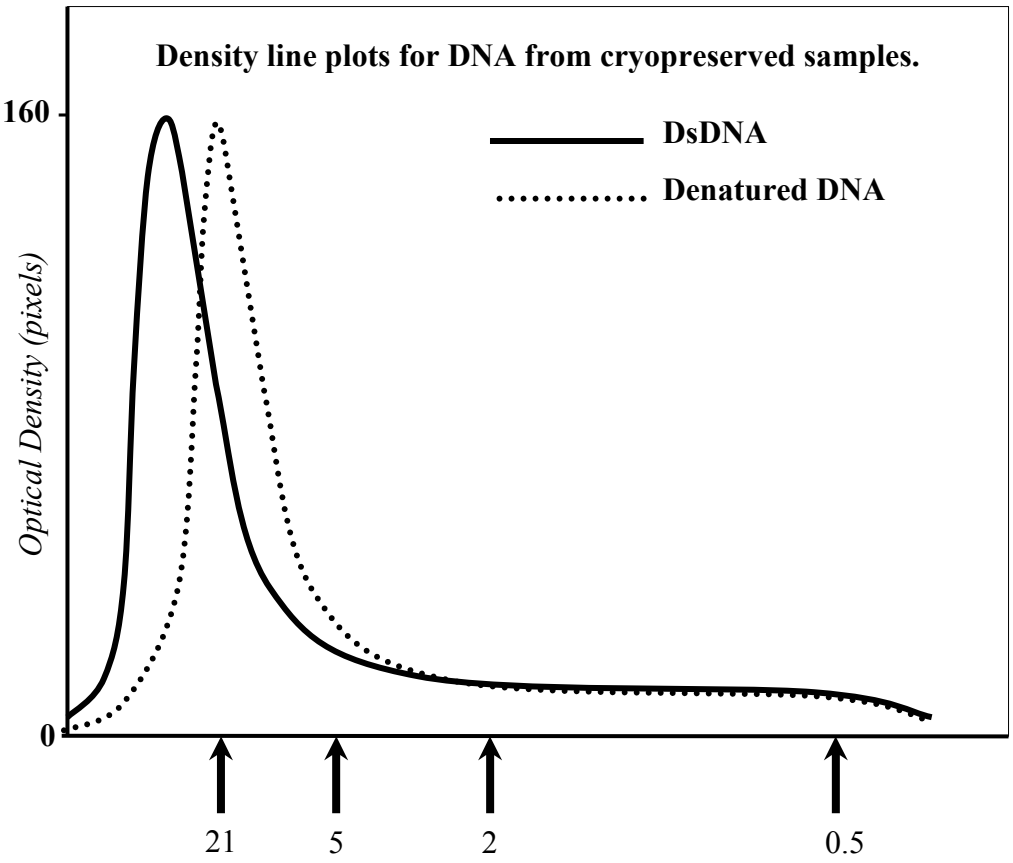
Figure 13: Typical agarose gel image. Lane A: 10 µl of Boehringer Mannheim DNA molecular weight marker III. Lanes B and C are replicates of 10 µl of high molecular weight dsDNA from cryo-preserved samples preserved for 20 months, and extracted using the method as described in section 3.2.

4.1.1 Cryo-preserved.

The gel image, figure 13, of the cryo-preserved sample shows that DNA of a high molecular weight has been extracted from the sample (78.7 µg ml^{-1}±40.5). Figure 14 shows in diagrammatic form the densiometry trace of the ethidium bromide stained agarose gel. The single strong peak at the start of the graph confirms that the extracted dsDNA is of high molecular weight, with very little fragmentation. Figure 14 also shows the same extracted DNA samples that have been denatured into ssDNA. The graph again shows a strong peak, with a reduction in overall molecular weight (which is expected as the molecular weight has effectively been halved by the denaturation

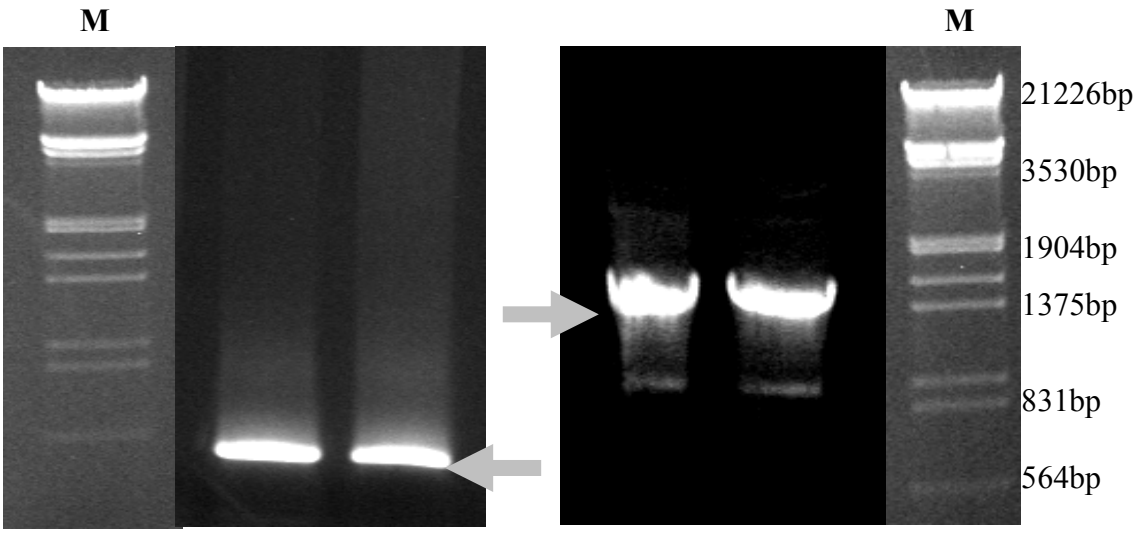
process). The results of the denaturation DNA profile suggest that the preservation treatment, and the DNA extraction process, have not extensively damaged the DNA.

PCR amplification was successfully achieved from both the 16S and 18S primers (figure 15). In addition the RE *Hin*f1 enzyme digestion suggested successful RE cutting of the extracted DNA (Figure 16).



Approximate position of DNA marker points on the agarose gel.
DNA size in kilobase pairs (kbp).

Figure 14: Graphical representation of an broad band density plot along a gel band (see section 3.3.2) where the optical density is a measure of the DNA concentration, and the area under the curve represents the total quantity of DNA in the sample. The graph shows a density line plot for DNA extracted from the cryopreserved specimens. The solid line represents the extracted dsDNA, and the dashed lined represents the alkaline denatured DNA samples.



M: Molecular Weight marker III (0.12-21.2kbp) from Boehringer Mannheim

Figure 15: 16S and 18S (using NS3 / NS4 primer pair) PCR products observed by agarose gel electrophoresis with DNA extracted from cryo-preserved samples.

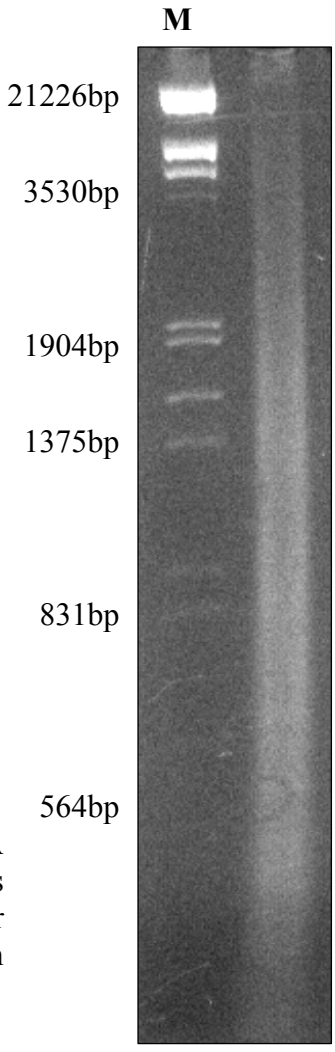


Figure 16: Agarose gel image of *Hin*f1 digested DNA extracted from cryo-preserved samples preserved for 20 months. M: Molecular Weight marker III (0.12-21.2kbp) from Boehringer Mannheim.

4.1.2 Absolute Ethanol preserved.

The gel image (figure 17) of the absolute ethanol preserved sample suggests that DNA of a high molecular weight has been extracted from the sample (54.2 μg ml^{-1}±14.7). The densiometry trace (figure 18) confirms the presence of high molecular weight dsDNA from the sample, with a single strong peak before the 22kb marker. The denatured DNA graph profile also shows a strong peak.

The PCR analysis gave successful amplification products with the 16S and 18S primers, whilst *Hin*f1 digestion suggested successful RE enzyme activity on the extracted DNA template (data not shown).

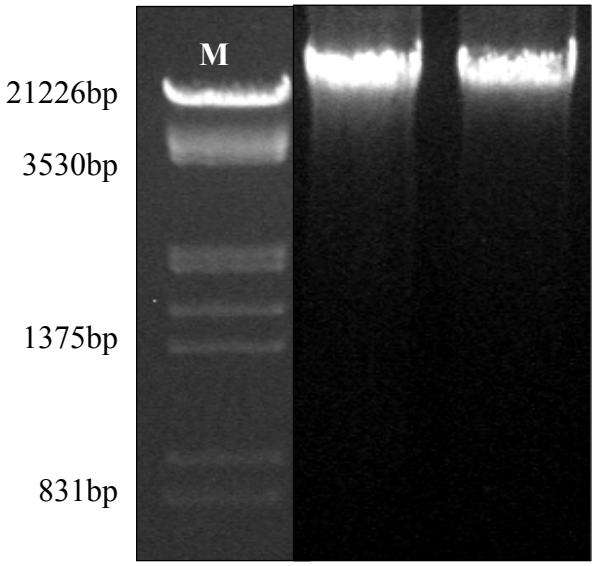
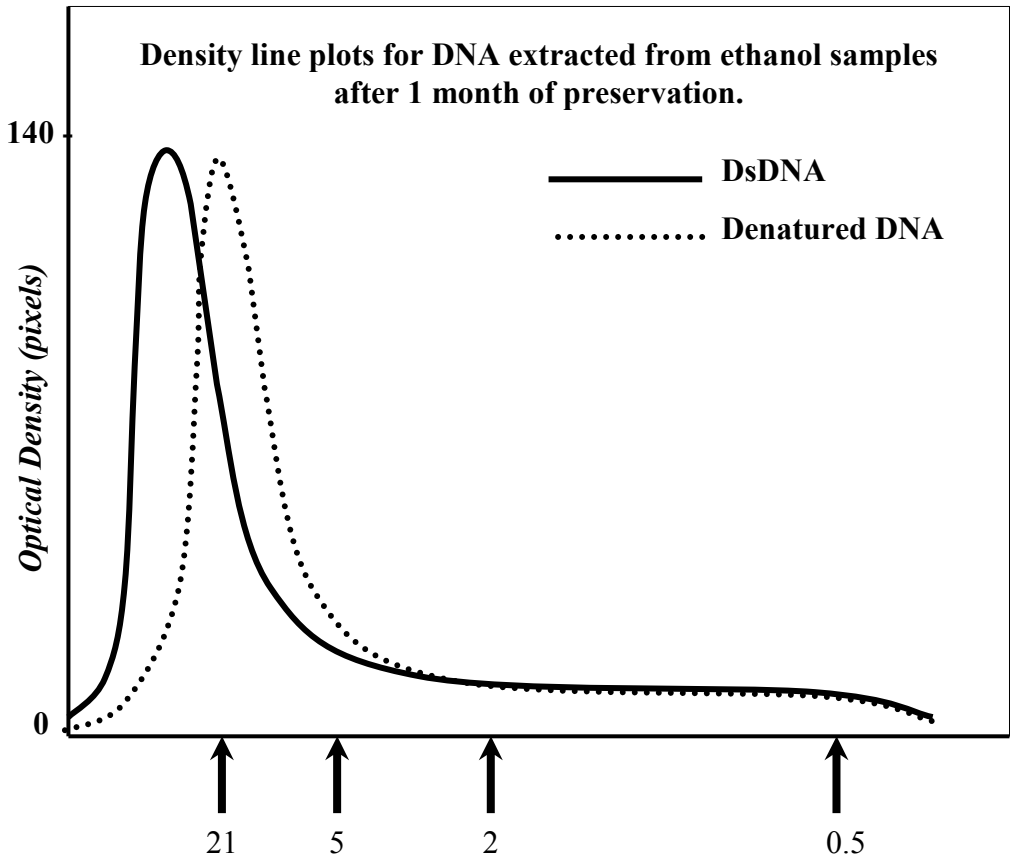


Figure 17: Agarose gel image of ethanol preserved dsDNA extracted after 1 month of preservation. **M:** Molecular Weight marker III (0.12-21.2kbp) from Boehringer Mannheim



Approximate position of DNA marker points on the agarose gel.
DNA size in kilobase pairs (kbp).

Figure 18: Density line plot for DNA extracted from ethanol preserved specimens after 1 month of preservation. The solid line represents the extracted dsDNA, and the dashed lined represents the alkaline denatured DNA samples.

4.2 The effects of ethanol preservation on the integrity of the DNA.

The previous section (4.1.2) already discusses the results of short-term ethanol preservation on the integrity of the DNA. The results in this section will consider the effects of storage conditions and time on the integrity of the DNA in absolute ethanol preserved samples.

4.2.1 Sample age and the integrity of the DNA.

The DNA from specimens that had been recently killed and preserved in absolute ethanol was compared with the DNA extracted from specimens killed and preserved in absolute ethanol for 3 months and 24 months respectively. All samples were stored at room temperature prior to DNA extraction. Figure 19 shows the dsDNA profiles from the various sample sets. There appears to be little overall change in the DNA over the first 3 months, but the 24 month specimens are beginning to show extensive fragmentation of the dsDNA. This change is also shown in the gel images (figure 20). The denatured DNA profiles also reflect this pattern, but with even greater fragmentation (Figures 21 and 22).

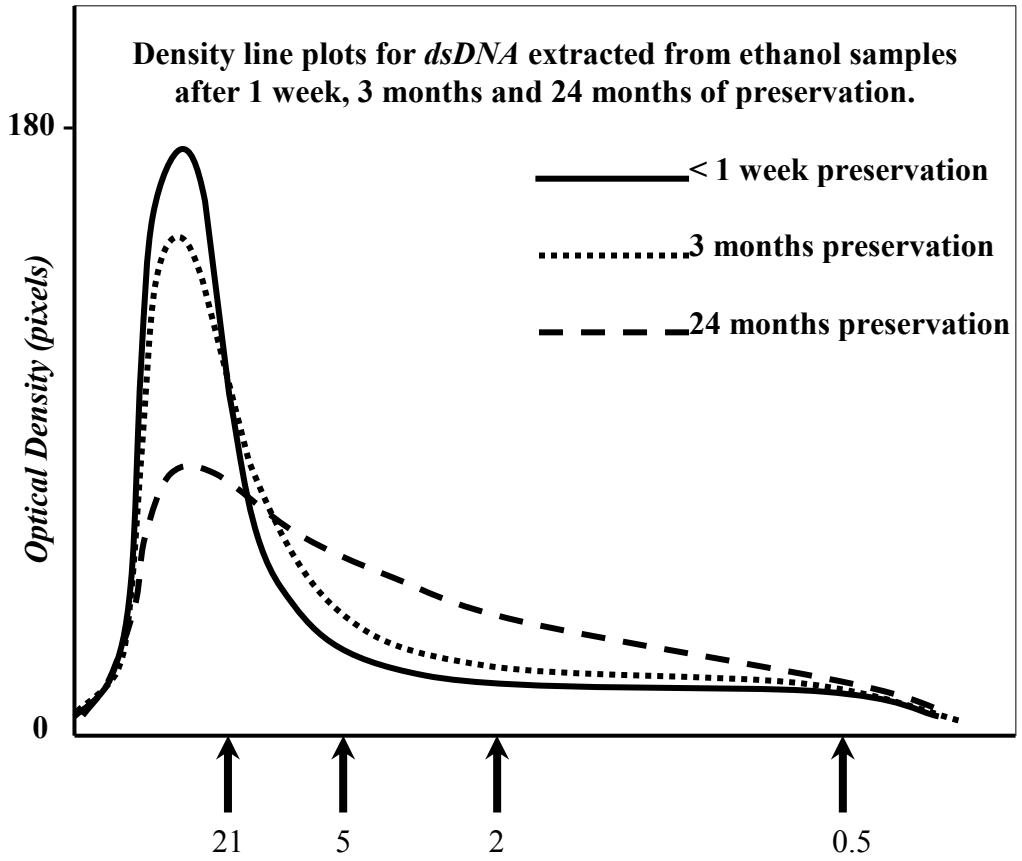


Figure 19: Density line plot for DNA extracted from ethanol preserved specimens after 1 week; 3 months; and 24 months preservation. All samples represent non-denatured dsDNA. Note the fragmentation of the dsDNA over time.

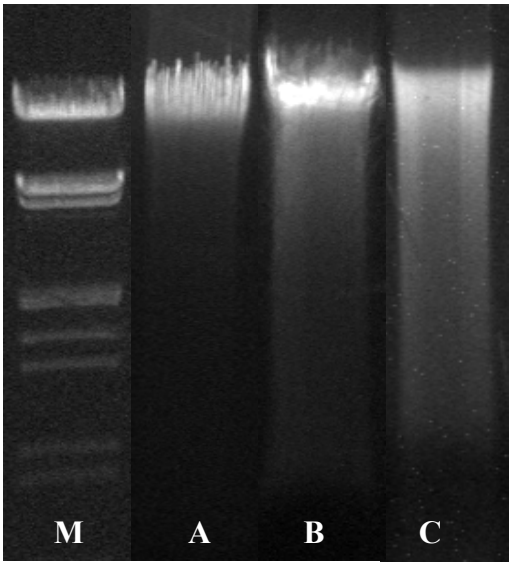


Figure 20: Examples of agarose gel lane images for whole genomic DNA extracted from ethanol preserved samples after: A - 1 week; B – 3 months; C – 24 months. M – molecular weight marker III from Boehringer Mannheim.

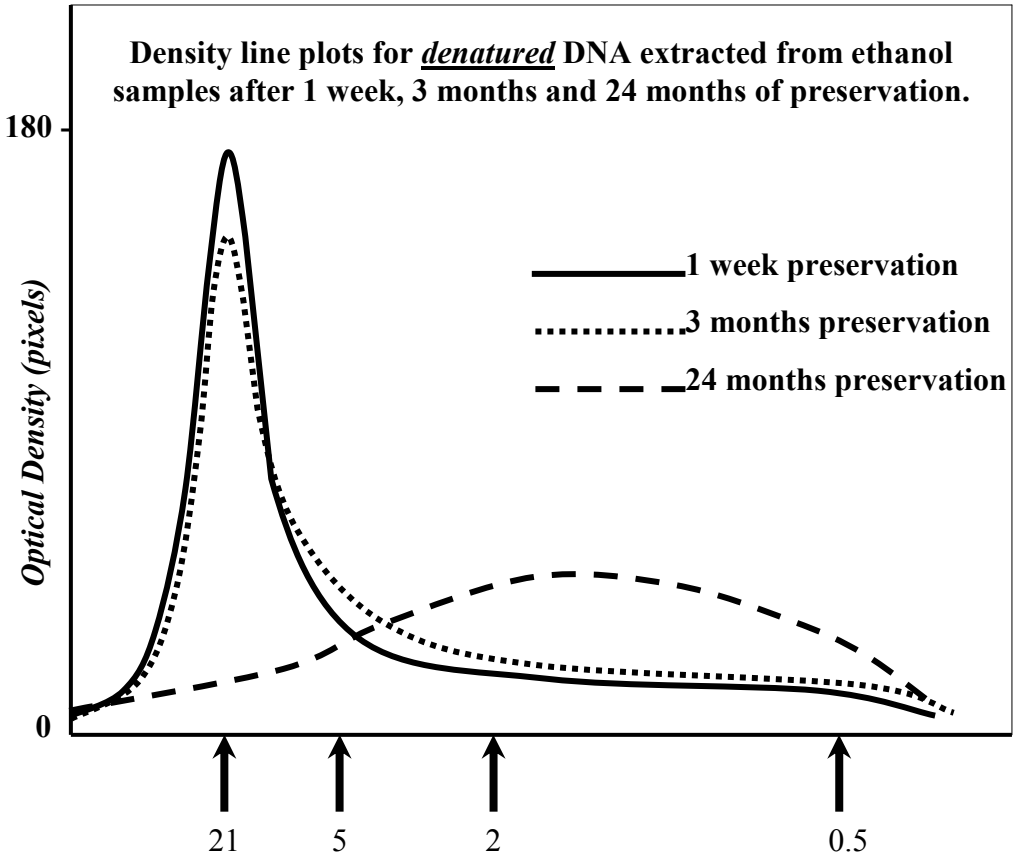


Figure 21: Density line plot for DNA extracted from ethanol preserved specimens after 1 week; 3 months; and 24 months preservation. All samples represent denatured DNA. Note that the denatured DNA has become significantly fragmented in the 24 month sample.

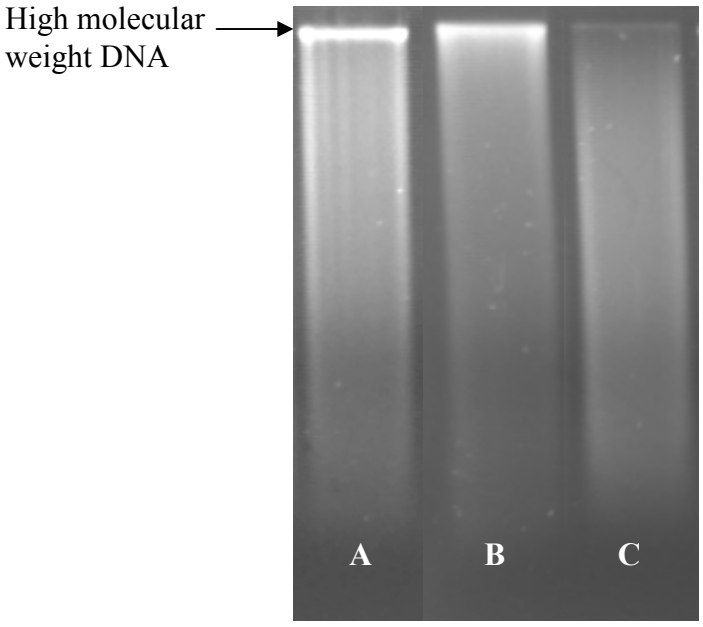


Figure 22: Denaturing agarose gel images of ethanol preserved DNA after; A: 1 week; B: 3 months; and C: 24 months preservation.

Despite the evidence for increased fragmentation of the DNA over time, the overall quantities of DNA extracted, as calculated in 'Materials and Methods', did not appear to significantly decrease over the study period. The quantities extracted between age samples remained broadly comparable (table 6).

All the samples gave successful 16S and 18S amplifications (Figure 23), and all samples demonstrated a similar degree of RE enzyme cutting with *Hin*f1 (figure 24).

	<1 week	3 month	24 month
Quantity ±95% C.L. ($\mu g\ ml^{-1}$)	54.2 ±14.7	49.4 ±6.2	54.9 ±8.0

Table 6: The quantity of dsDNA extracted from samples over time.

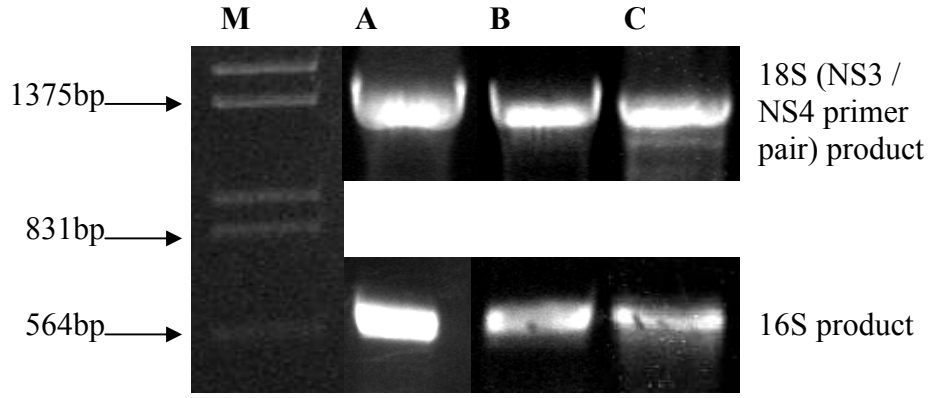


Figure 23: Examples of 16S and 18S amplification products from DNA extracted from ethanol preserved samples after: A - 1 week; B – 3 months; C – 24 months. M – molecular weight marker III from Boehringer Mannheim.

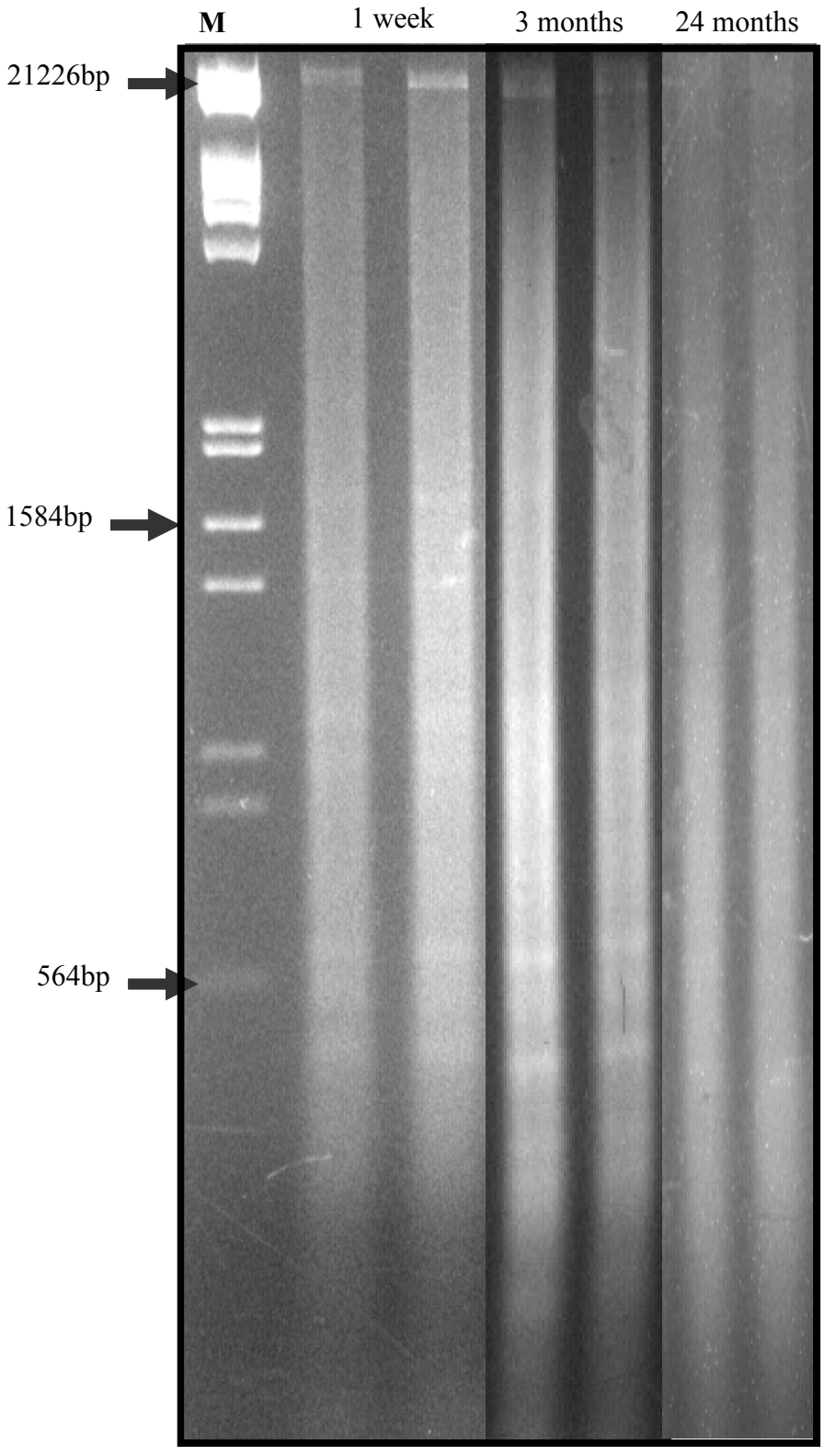


Figure 24: Agarose gel images of Hinf1 RE enzyme digested dsDNA extracted from 1 week, 3 month and 24 month ethanol preserved samples. Some high molecular weight DNA remains undigested in the 1 week samples, and faint bands are visible either side of the 564bp marker in the 1 week and 3 month samples. **M**: Molecular Weight marker III (0.12-21.2kbp) from Boehringer Mannheim.

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4.2.2 Low temperature storage and DNA integrity

The use of cryo-preservation has been previously discussed, along with the results of the cryo-preserved samples used in this study. The aim of this section was to consider the possible benefit of storing ethanol preserved sample at about 4°C in a domestic refrigerator. Figure 25 shows the dsDNA and denatured DNA profiles typical for 12 month old samples, while figure 26 shows examples of agarose gel images of uncut and denatured DNA from the same samples. These profiles appear to be similar to those obtained from ethanol preserved samples stored at ambient conditions. However the quantity of DNA extracted was possibly more significant at an average value of 67.2 μg ml^{-1}±19.0, when compared to samples stored at ambient temperatures, which gave DNA quantities of about 50 μg ml^{-1}.

With the PCR analysis, the extracted DNA gave successful amplification products with both the 16S and 18S primers, and was successfully digested by the *Hin*f1 RE enzyme (figure 27).

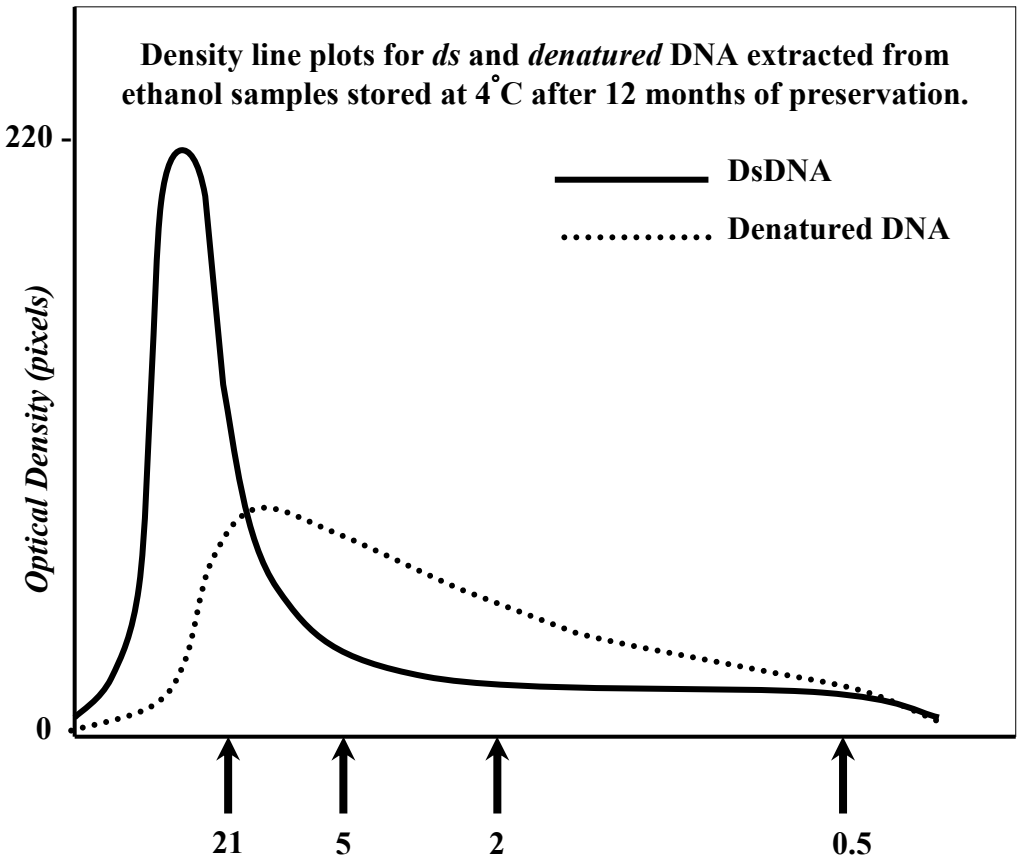


Figure 25: Density line plot for duplex and denatured DNA extracted from ethanol preserved specimens stored at 4°C after 12 months of preservation. The solid line represents the extracted dsDNA, and the dashed lined represents the alkaline denatured DNA samples.

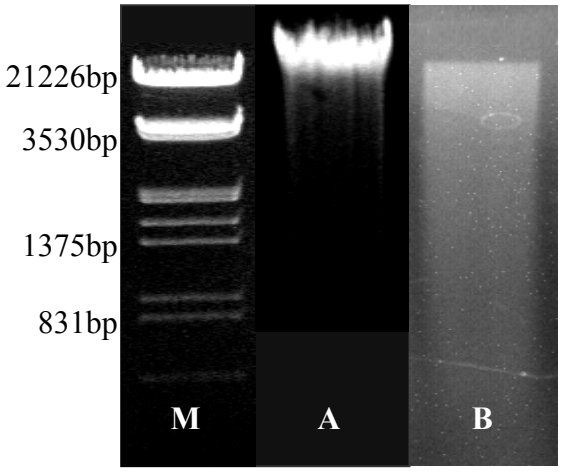


Figure 26: Agarose gel images of ethanol preserved dsDNA stored at 4°C after 12 months of preservation; A: dsDNA; B: denatured DNA; M: Molecular weight marker III (012-21.2kbp) from Boehringer Mannheim.

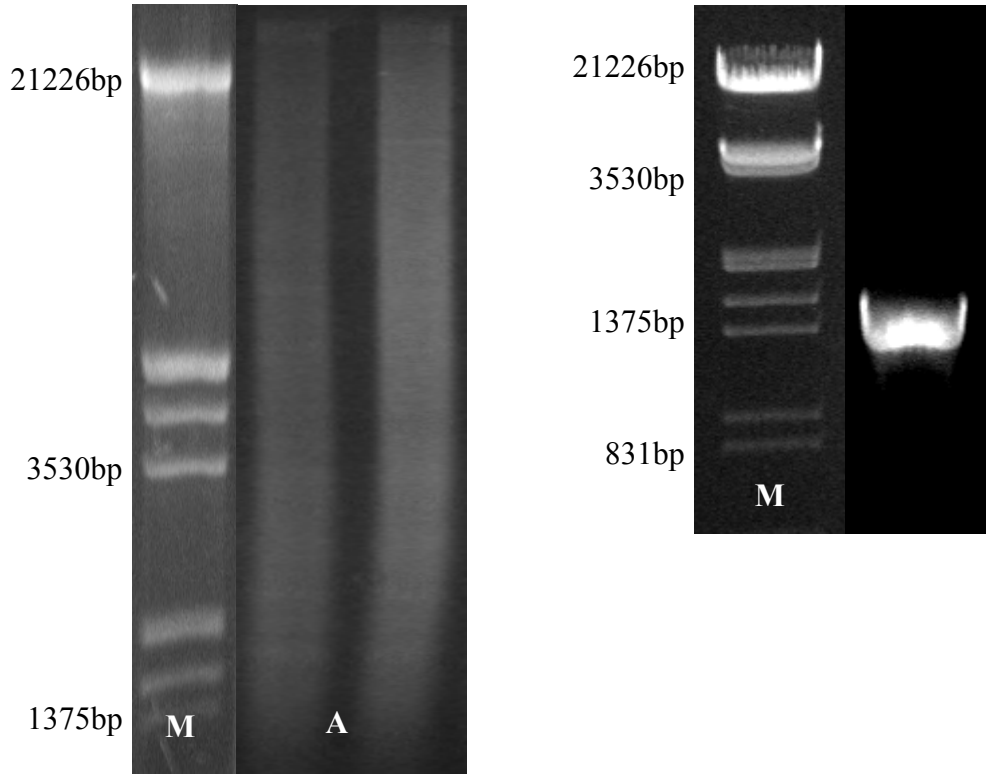


Figure 27: Agarose gel images of A: *Hin*f1 digested dsDNA; B: 18S PCR product (using NS3 and NS4 primers); M: Molecular weight marker III (012-21.2kbp) from Boehringer Mannheim.

4.2.3 The effects of changing the ethanol during preservation.

Ethanol preserved specimens had the ethanol changed after 1 week of preservation. The samples were then studied after 13 months of preservation. The quantity of DNA extracted, 53.4 μg ml^{-1} ±22.4, was comparable to the standard ethanol preserved samples. The density profile of the dsDNA demonstrated high molecular weight DNA to be present, although the profile of the denatured DNA shows much greater fragmentation (figure 28). The samples still gave successful PCR amplifications with both the 16S and 18S primers, whilst a good RE enzyme digest was also obtained with *Hin*f1 (data not shown).

It must be stressed that the effects of changing the preserving ethanol solution is dependent on the ratio of specimen volume to preserving fluid volume, and on the water content of the specimen.

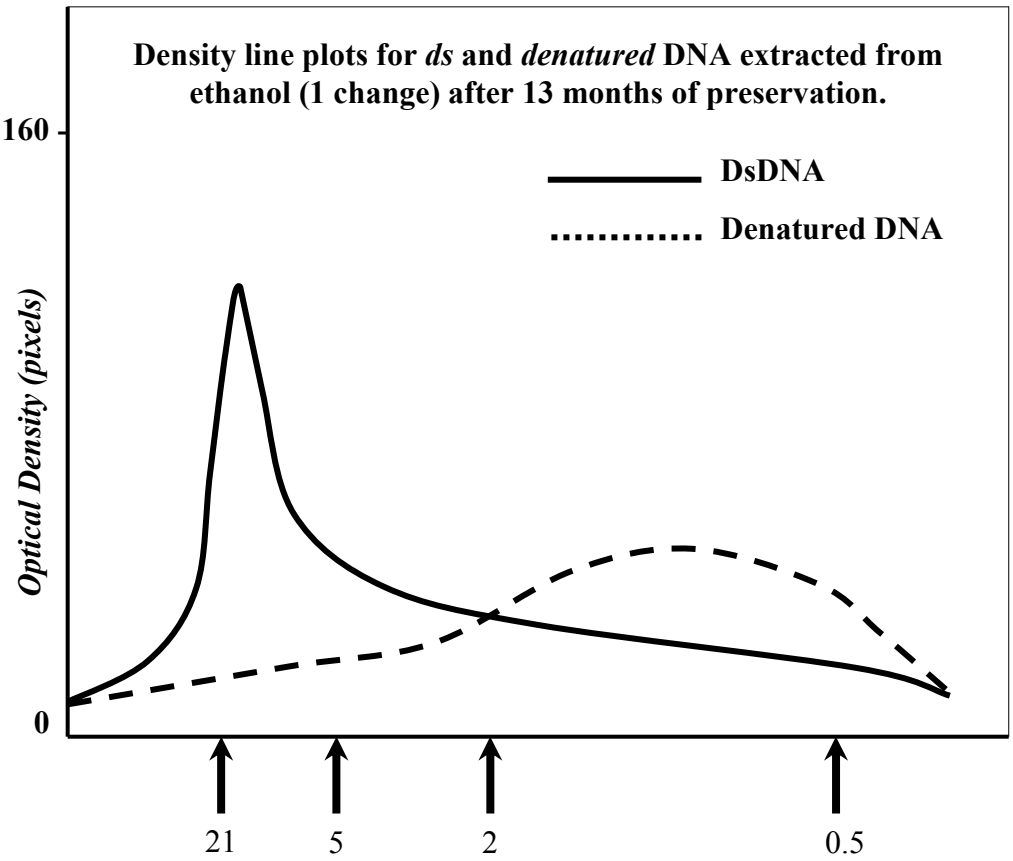


Figure 28: Density line plot for DNA extracted from ethanol (1 change) preserved specimens after 13 months preservation. The graph shows fragmentation has occurred in the denatured DNA.

4.2.4 The effects of using EDTA as an additive with the ethanol preservative.

The use of low concentrations of EDTA as an additive to the ethanol has been recommended (Dessauer *et al.*, 1996) to prevent the action of enzymes in the preserved specimens. A batch of specimens were preserved in ethanol to which a trace of EDTA had been added (to a concentration of about 100 μM). After 15 months the samples were analysed. The average DNA quantity extracted, 27.7 μg mol^{-1} ±9.0, was lower than equivalent ethanol samples. The dsDNA profile (figure 29) showed that high molecular weight DNA was present, but this showed extensive fragmentation when denatured.

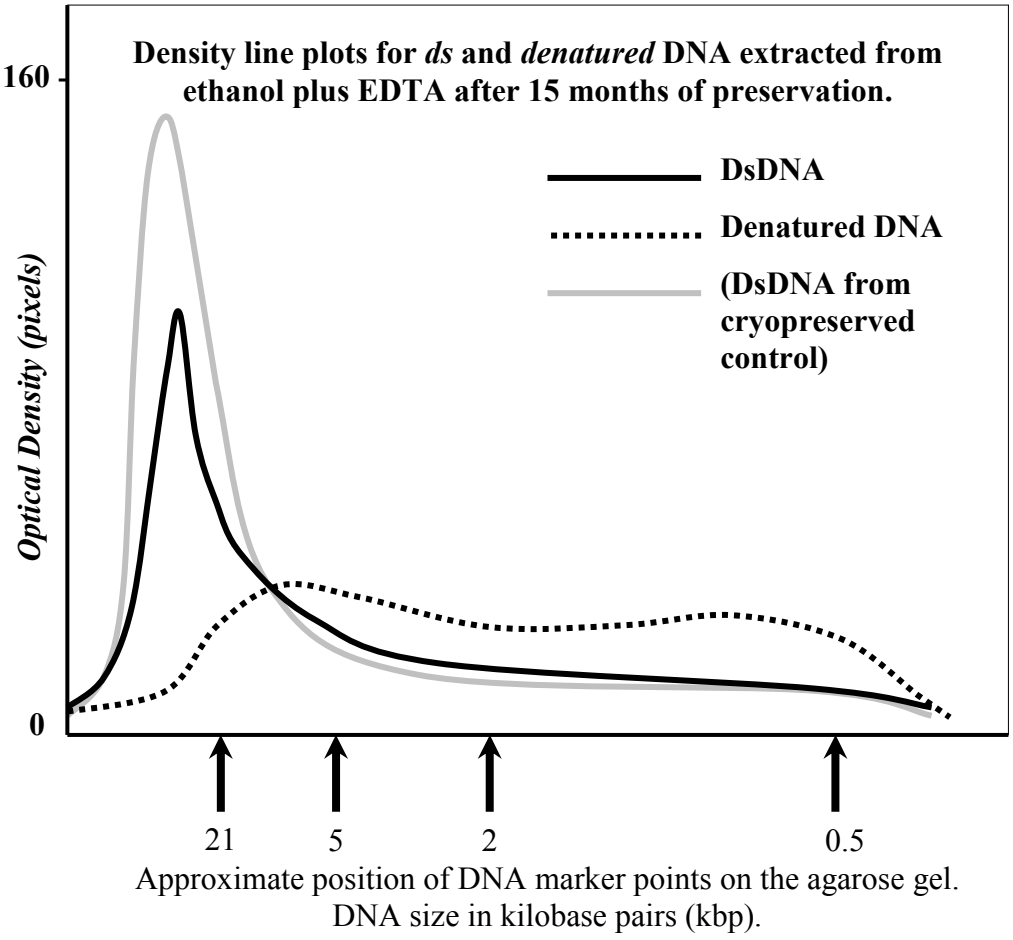


Figure 29: Density line plot for DNA extracted from ethanol plus EDTA preserved specimens after 15 months preservation.

Both 16S and 18S amplifications were successful, and the RE enzyme *Hin*f1 successfully digested the extracted DNA (data not shown).

4.3 The effects of Industrial Methylated Spirit (IMS) preservation on the integrity of the DNA.

This section considers the effect of undiluted IMS and 80% IMS preservation on the integrity of the DNA. As previously discussed, 80% IMS solutions are used as a standard preservative at NMGW.

4.3.1 The effect of 100% IMS preservation.

When compared to ethanol preservation, then the quantities of dsDNA extracted from 100% IMS preserved samples were broadly comparable. IMS specimens preserved between 15 months and 18 months yielded an average of 52.6 μg ml^{-1} ±20.5, compared to 56.4 μg ml^{-1} ±8.0 for ethanol preserved samples of a similar age range. The dsDNA profiles for the extracted DNA for both ethanol and IMS preserved samples exhibited a similar pattern. However when denatured the DNA patterns became significantly different (figure 30), suggesting that the IMS preserved DNA has been weakened by using IMS as a preservative when compared to ethanol. Figure 31A shows the gel image of dsDNA extracted from IMS preserved samples, while figure 31B shows the effect of denaturation on an extract of the same sample set.

The extracted DNA from IMS preserved samples gave positive results with both the 16S and 18S PCR amplifications, and was digested by the RE enzyme *Hin*f1 (figures 31C and D).

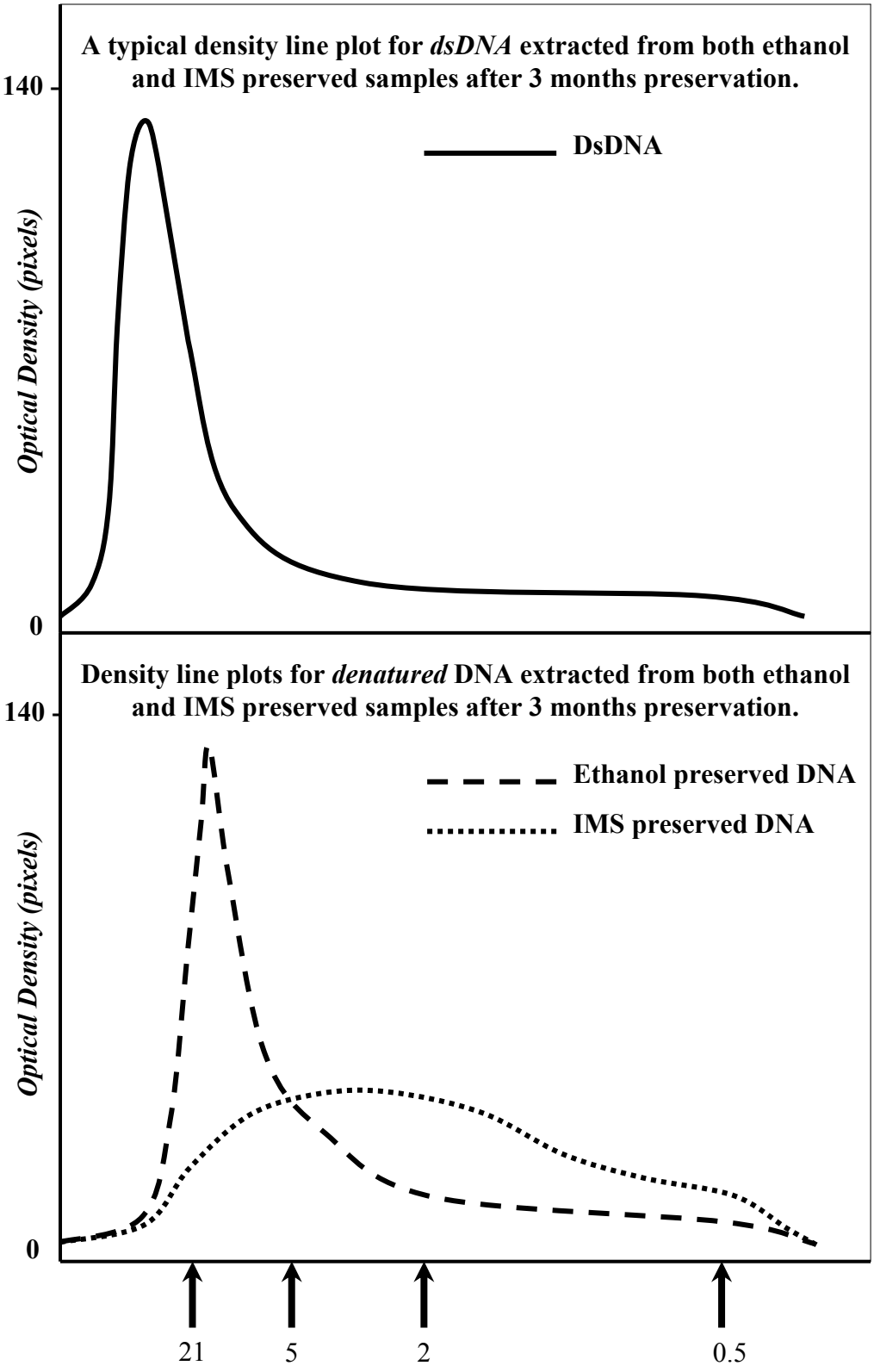


Figure 30: Density line plot for DNA extracted from ethanol and IMS preserved specimens after 3 months preservation. The upper graph shows a typical profile for dsDNA from both the ethanol and IMS preserved samples demonstrating that high molecular weight DNA is present. The lower graph shows the denatured DNA profiles for the two sample types. Note the increased fragmentation of the IMS preserved samples.

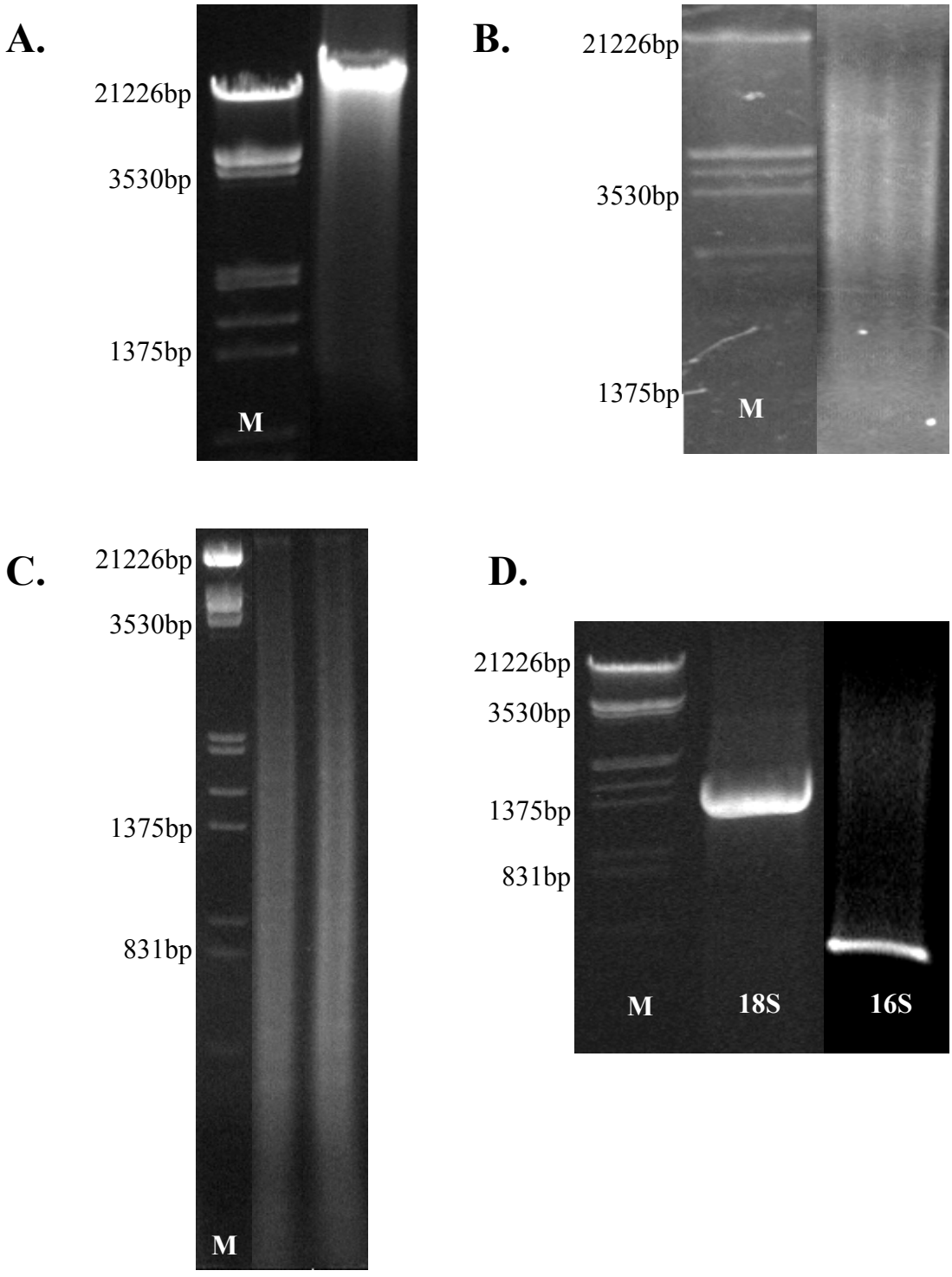


Figure 31: Agarose gel images of DNA extracted from IMS preserved samples; A: whole dsDNA; B:denatured DNA; C: Hinf1 digested DNA; D: 18S and 16S PCR amplifications; M: Molecular weight marker III (012-21.2kbp) from Boehringer Mannheim.

4.3.2 The effect of 80% IMS preservation.

There is an immediate loss in overall DNA quantities that could be extracted from the 80% IMS samples when compared with ethanol preserved samples. Figure 32 compares the average DNA quantities extracted from 80% IMS preserved samples relative to the preservation age of the sample. The dsDNA profile and agarose gel images (figures 33 and 34) reflect this pattern of DNA quantity loss, and also shows an increased fragmentation of the dsDNA over time. This pattern of fragmentation is also reflected in the denatured DNA profile (data not shown).

The 80% IMS samples all gave positive 16S PCR amplifications. However the archival 80% IMS samples failed to give 18S PCR amplification products. Successful *Hin*f1 RE digestions were obtained with all the extracted 80% IMS DNA samples, except the archival 80% IMS sample in which the DNA was too highly fragmented for a whole extract RE digest.

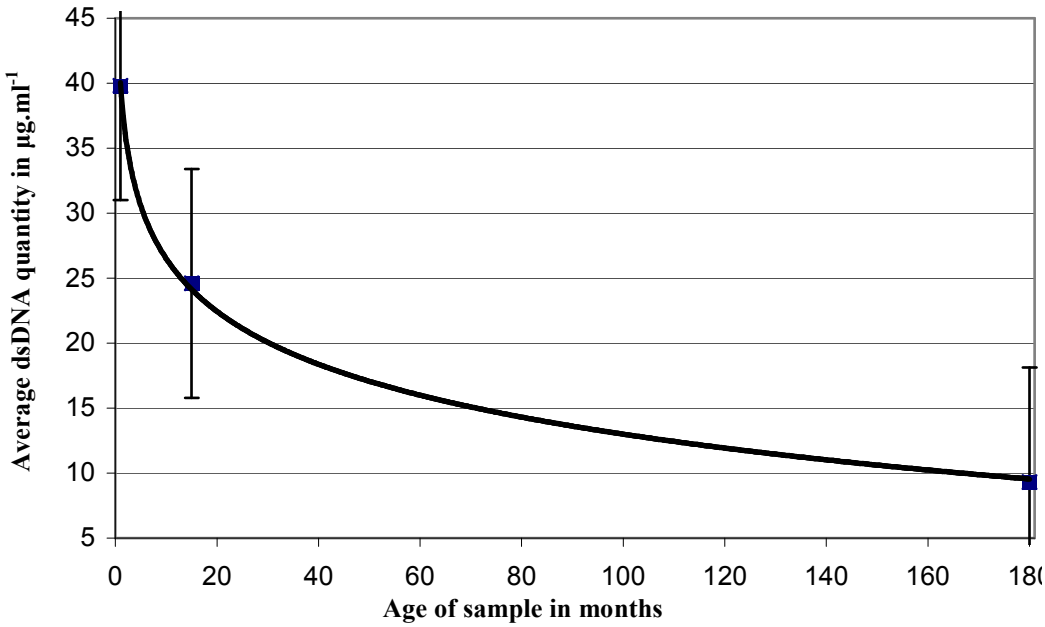


Figure 32: The average DNA quantities extracted from 80% IMS preserved samples over time. Error bars relate to Standard Error.

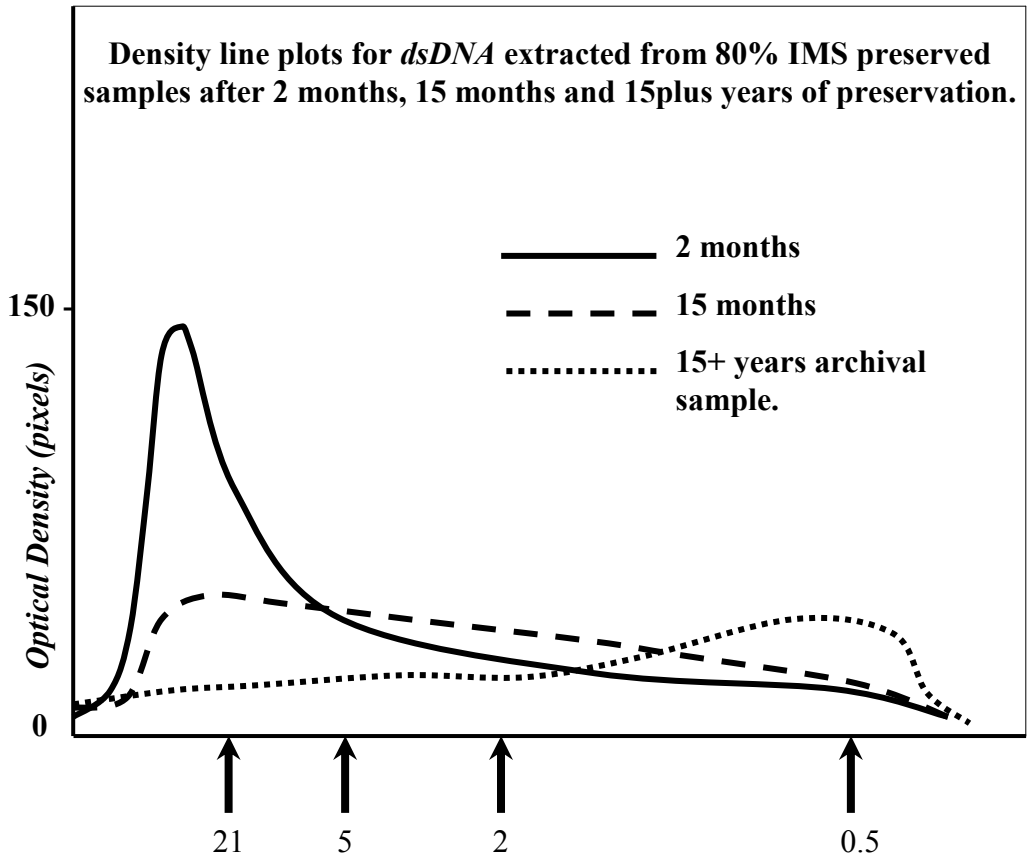


Figure 33: Density line plots for dsDNA extracted from 80% IMS preserved samples after 2 months, 15 months and 15+ years of preservation. The graph shows extensive fragmentation in the dsDNA over time.

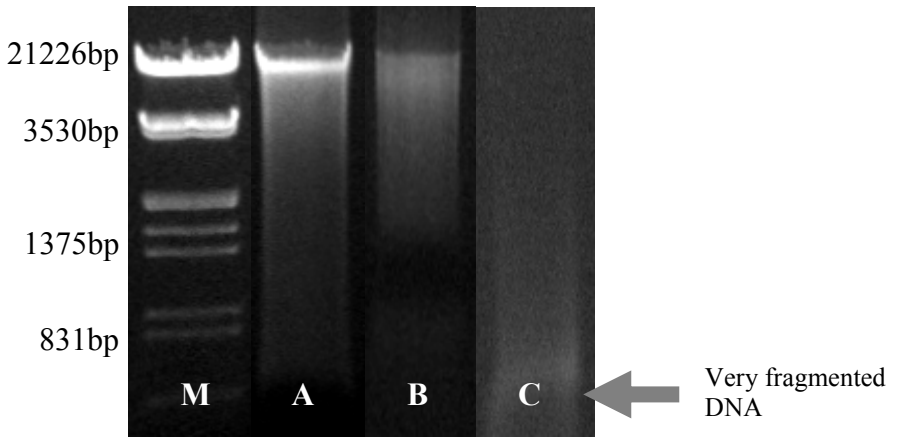


Figure 34: Agarose gel images of dsDNA preserved in 80% IMS after; A: 2 months preservation; B: 15 months preservation; C: 15+ years archival preservation. M: Molecular weight marker III (012-21.2kbp) from Boehringer Mannheim.

4.4 The effects of other chemicals used as additives in preservation fluids.

This part of the study considered the effects of a number of chemicals used either as additives to the preservative solution, or as part of the preservation process. Three chemicals were considered; propylene glycol, 2-ethoxy ethanol and ethyl acetate. Specimens were preserved in the three fluids for 12 months prior to DNA extraction. Both the propylene glycol and 2-ethoxy samples yield extractable dsDNA (23.4 ±6.6 μg ml^{-1} and 20.4 ±9.2 μg ml^{-1} respectively,) whilst the ethyl acetate sample yielded no detectable dsDNA. The dsDNA profiles (figure 35) and gel images (figure 36) demonstrate that some high molecular weight dsDNA is present in the propylene glycol and 2-ethoxy ethanol samples.

Positive 16S PCR amplifications were obtained for both the propylene glycol and 2-ethoxy ethanol samples. Both treatments also yielded positive 18S PCR amplifications, but with the 2-ethoxy ethanol sample PCR product could only be obtained with the NS primer pair that gave a shorter product (data not shown). *Hin*f1 RE enzyme digests of the whole DNA extract were inconclusive due to the lower DNA quantities extracted and the already fragmented condition of the DNA.

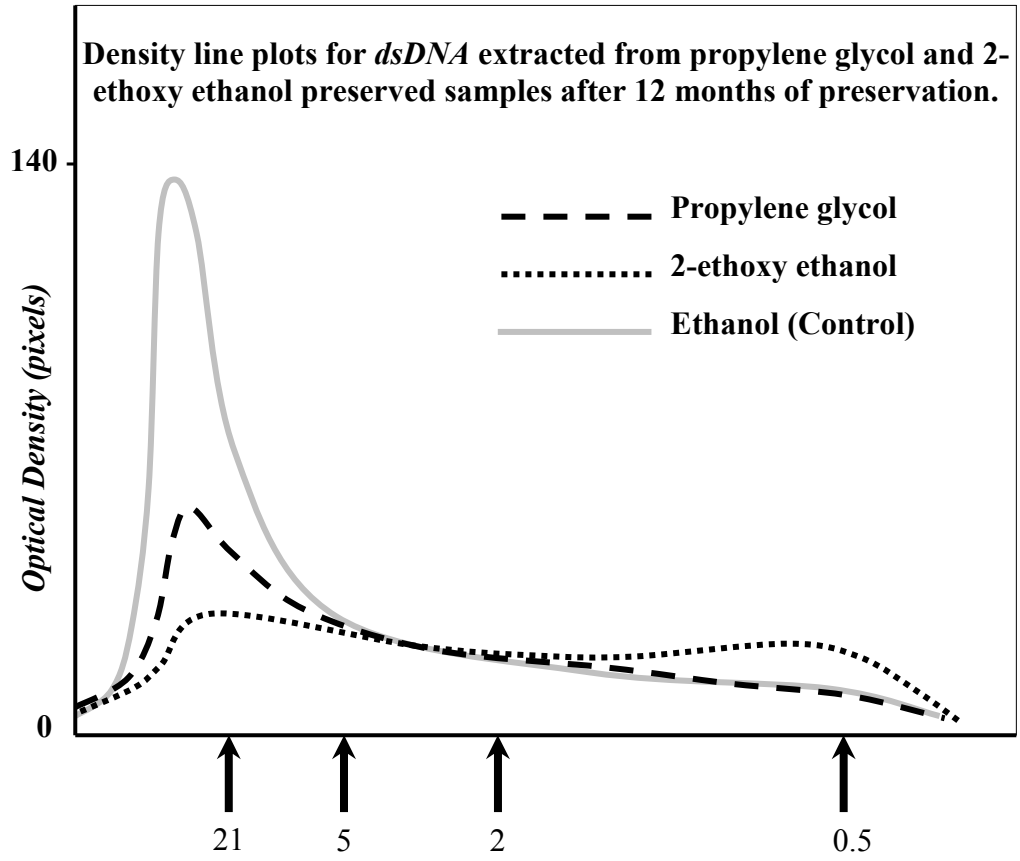


Figure 35: Density line plots for dsDNA extracted from propylene glycol and 2-ethoxy ethanol preserved samples after 12 months of preservation. The graph shows fragmentation in the dsDNA, although high molecular weight dsDNA is still present. The control shows the dsDNA profile from ethanol preserved samples after 1 week of preservation.

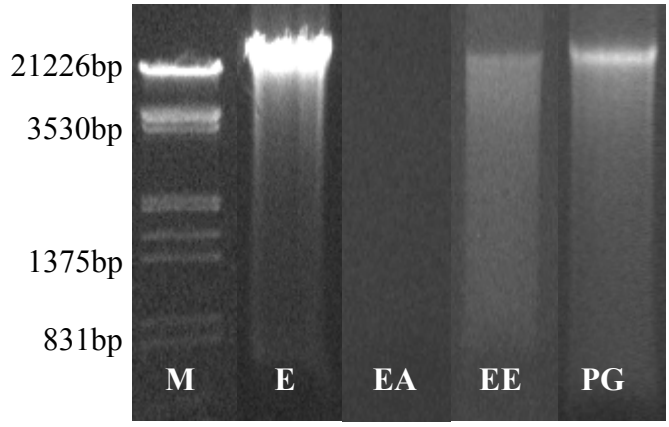


Figure 36: Agarose gel images of; A: Ethanol preserved dsDNA; EA: ethyl acetate treated dsDNA; EE: 2 ethoxy ethanol treated dsDNA; PG: propylene glycol treated dsDNA; M: Molecular weight marker III (012-21.2kbp) from Boehringer Mannheim.

4.5 The effects of other fluid preservatives.

This section considers two other fluid preservative solutions utilised at NMGW, 4% formaldehyde and Steedman's solution. The effect of 4% formaldehyde was explored by comparing samples in a 4% formaldehyde solution using de-ionised water; 1:1 de-ionised water : propylene glycol; and propylene glycol. This was to consider whether replacing the use of water as a solvent could improve DNA preservation with formaldehyde.

The samples were examined after 12 months of storage in the preservatives. No extracted DNA product was observed with the Steedman's preserved samples. The formaldehyde samples all produced very low quantities of very degraded DNA (figure 37). There was no notable difference in the quantity of dsDNA extracted between the formaldehyde samples using water or propylene glycol. The quantity of extracted dsDNA was less than 5 $\mu g\ ml^{-1}$ in each of the three sample sets, but was too low to be accurately measured by the methods used in this study.

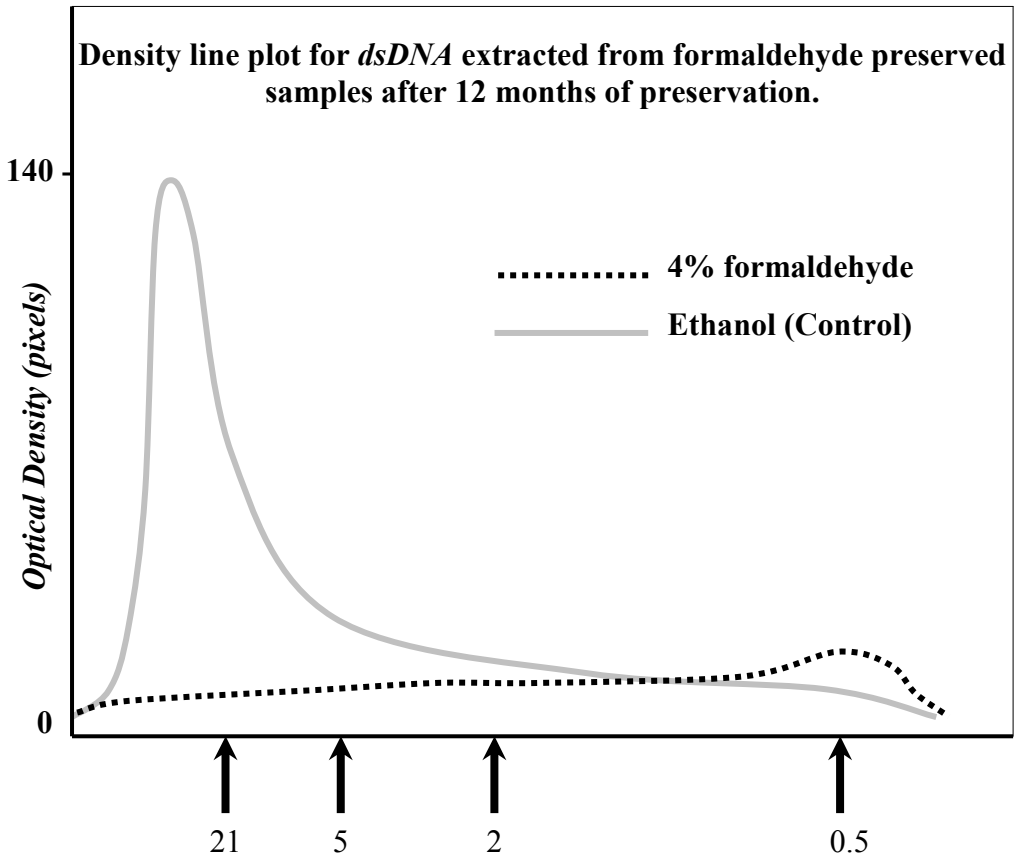


Figure 37: Density line plots for dsDNA extracted from 4% formaldehyde preserved samples after 12 months of preservation. The graph shows extensive fragmentation of the dsDNA, with very low quantities of DNA remaining. The control shows the dsDNA profile from ethanol preserved samples after 1 week of preservation.

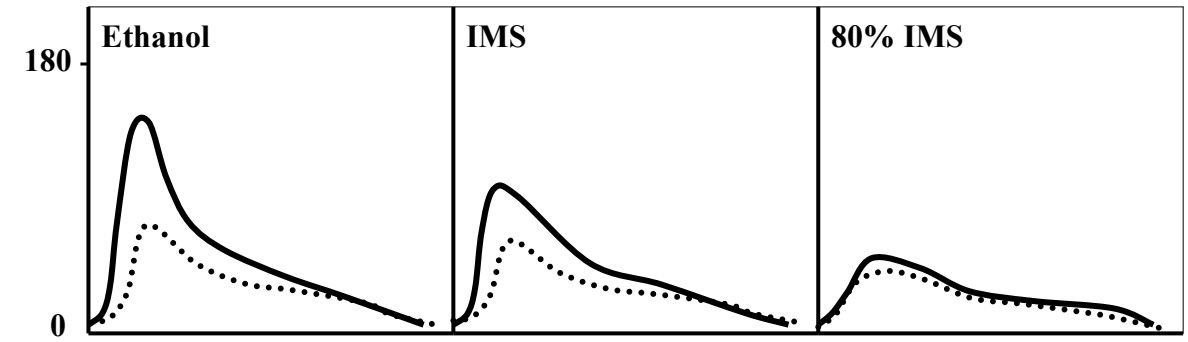
4.6 The rehydration of dried material using Decon90.

Specimens preserved in ethanol, IMS and 80% IMS for 18 months were allowed to air-dry. After dry storage for 1 month at ambient conditions, half of the sample sets were removed and the DNA extracted. The other half of the sample sets were then rehydrated in a 4% solution of Decon90 for 24 hours, after which the DNA was extracted. The effects of the rehydration process on DNA integrity was then compared with the dried samples. Table 7 summarises the results.

There is an overall loss in DNA quantity following rehydration in all three sample sets, but this is most noticeable with the ethanol-preserved samples. With both the IMS and 80% IMS samples the extracted DNA quantity differences are close to, or within the errors of the study. Figure 38A shows the dsDNA profiles from the ethanol; IMS; and 80% IMS samples respectively. Figure 39 shows the agarose gel images of the dsDNA extracted from the same samples. The profiles show that whilst there is a loss in DNA quantity, a peak of high molecular weight dsDNA remains, especially in the ethanol and IMS samples. When denatured (figure 38B) all the samples demonstrated a greater

Sample	DNA Quantity (µg ml^{-1} ± SE)	RE *Hin*f1 digestion?	16S PCR Product	18S PCR Product
Ethanol – dried	61.8±12.3	Yes	Yes	Yes
Ethanol – rehydrated	30.9±4.6	Yes	Yes	Yes
IMS – dried	38.0±8.5	Yes	Yes	Yes
IMS –rehydrated	32.2±16.1	Yes	Yes	Yes
80% IMS – dried	25.0±14.2	Yes	Yes	Yes
80% IMS - rehydrated	24.0±25.9	Yes	Yes	Yes

Table 7: Summary of rehydration results.



B: Density line plot for *denatured* DNA extracted from ethanol. IMS and 80% IMS preserved samples before and after Decon90 rehydration.

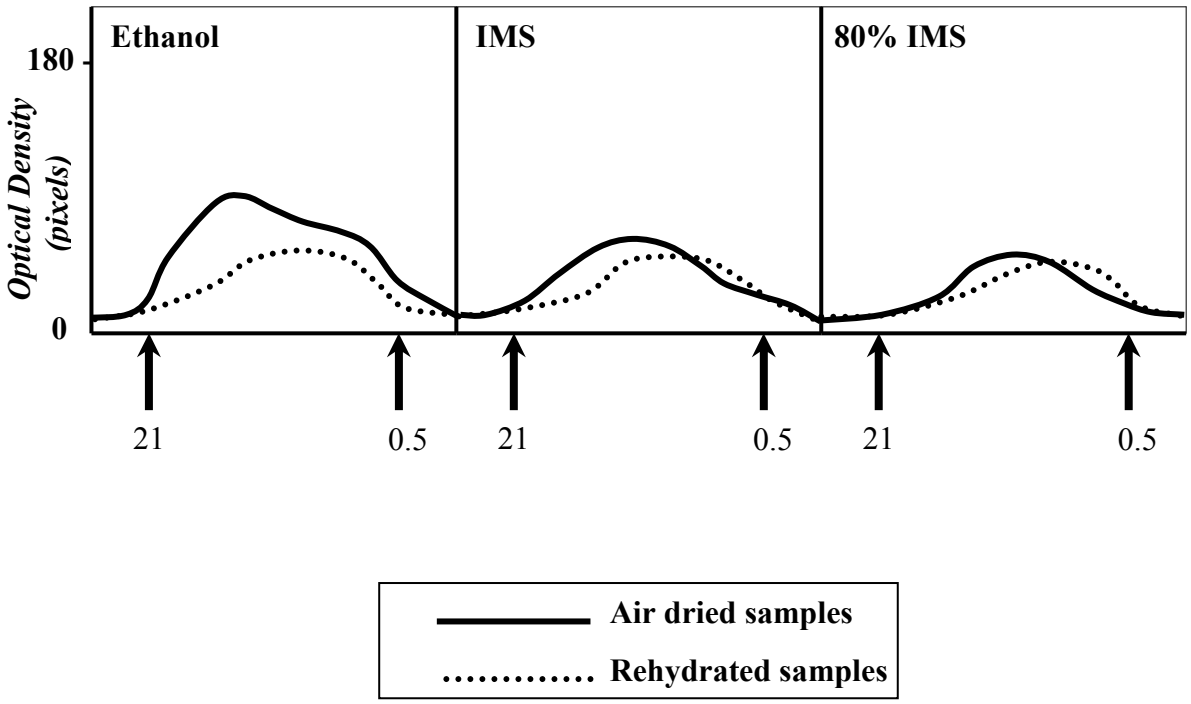


Figure 38A & 38B: Density line plots for dsDNA extracted from ethanol, IMS, and 80% IMS preserved samples before and after rehydration in Decon90. A shows the dsDNA density profiles before and after rehydration, whilst B shows the denatured DNA density profiles before and after rehydration.

degree of fragmentation than that shown with the dsDNA profile. When comparing the denatured DNA profiles between the dried and the rehydrated samples, a downshift in the overall sizes of the DNA fragments present occurs.

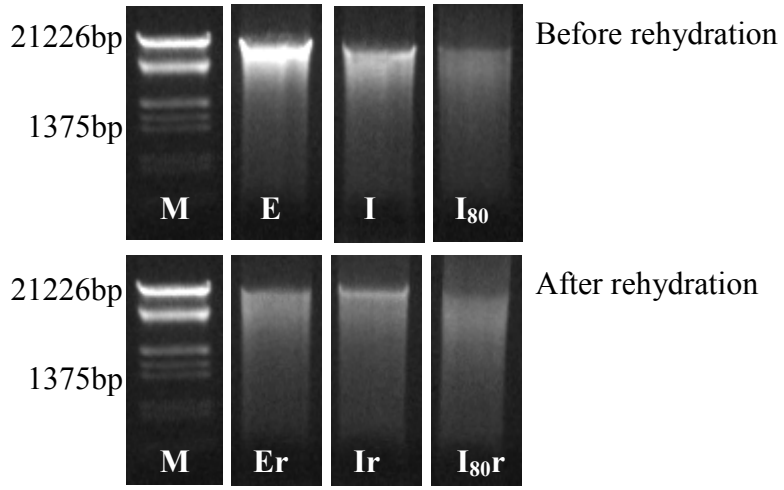


Figure 39: Agarose gel images of dsDNA from samples preserved for 18 months and then allowed to air dry. After storage for 1 month in a dry condition the DNA was extracted from half the sample group; E: ethanol preserved; I: IMS preserved; $\mathbf{I_{80}}$: 80% IMS preserved prior to drying. The other half of the sample group was then rehydrated (**r**) in Decon90 solution, and the DNA extracted. M: Molecular weight marker III (012-21.2kbp) from Boehringer Mannheim.

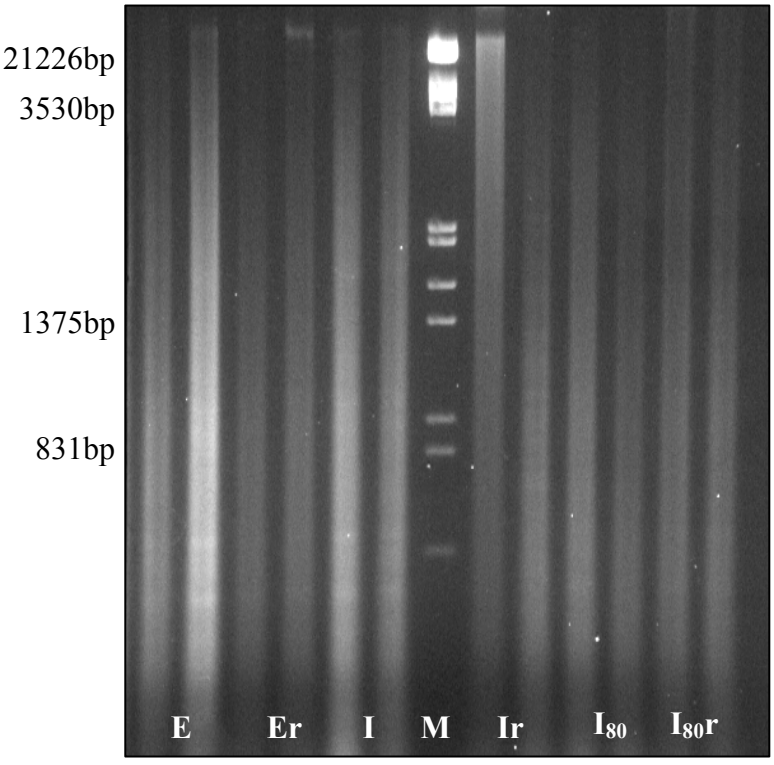


Figure 40: *Hin*f1 digested dsDNA from samples preserved for 18 months and then allowed to air dry. After storage for 1 month in a dry condition the DNA was extracted from half the sample group; E: ethanol preserved; I: IMS preserved; I_{80}: 80% IMS preserved prior to drying. The other half of the sample group was then rehydrated (r) in Decon90 solution, and the DNA extracted. M: Molecular weight marker III (012-21.2kbp) from Boehringer Mannheim.

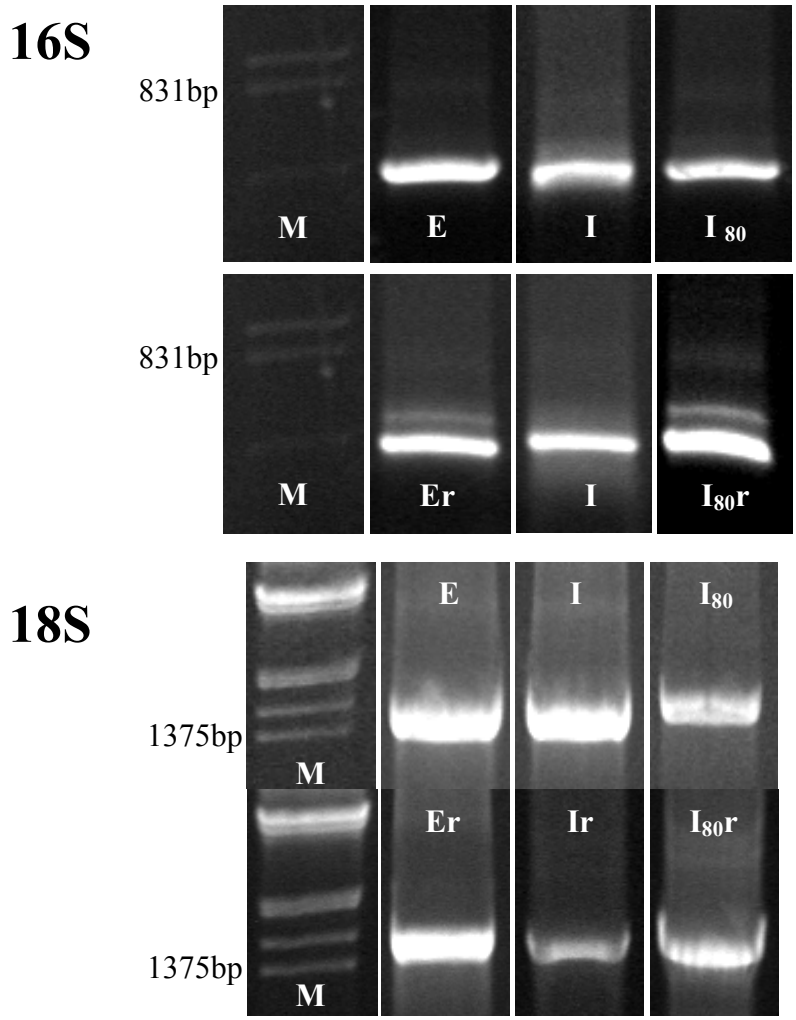


Figure 41: 16S and 18S (using NS3 and NS4 primer pair) amplification products from dsDNA samples preserved for 18 months and then allowed to air dry. After storage for 1 month in a dry condition the DNA was extracted from half the sample group; E: ethanol preserved; I: IMS preserved; I_{80}: 80% IMS preserved prior to drying. The other half of the sample group was then rehydrated (r) in Decon90 solution, and the DNA extracted. M: Molecular weight marker III (012-21.2kbp) from Boehringer Mannheim.

All of the samples gave successful 16S and 18S PCR amplifications (figure 41), although the occurrence of secondary bands was possible more of a problem with the rehydrated 16S samples. The samples were also digested by the *Hin*f1 RE enzyme (figure 40).

4.7 Subsequent treatments; the effects of specialist drying methods.

Two key methods used for the subsequent preparation of fluid preserved invertebrate material were examined. These were Critical Point Drying (CPD) and chemical drying using HMDS. Sample specimens were dried from 24 month ethanol-preserved specimens using the two methods. Following preparation the DNA from the specimens was extracted and analysed, and compared to the DNA extracted from the batch of ethanol preserved specimens the dried samples had been prepared from.

The quantity of DNA extracted from the specimens prepared by the two batches was high, equivalent or potentially even greater than the amounts obtained with the original ethanol preserved material (table 8).

The dsDNA profile from the samples (figure 42) shows high molecular weight DNA to be present. When compared to the original ethanol sample, from which the samples were prepared, the CPD and HMDS sample DNA shows less dsDNA fragmentation. When denatured, the DNA from all three-sample sets showed a very similar DNA profile in which little difference could be perceived (results not shown).

All three sample sets gave successful 16S and 18S PCR amplifications, and were digested by the *Hin*f1 RE enzyme (results not shown).

	Ethanol standard	**HMDS sample**	**CPD sample**
Quantity±95% C.L. (μg ml^{-1})	54.9 ±8.0	78.6 ±12.0	62.2 ±22.1

Table 8: Comparison of the DNA quantities extracted.

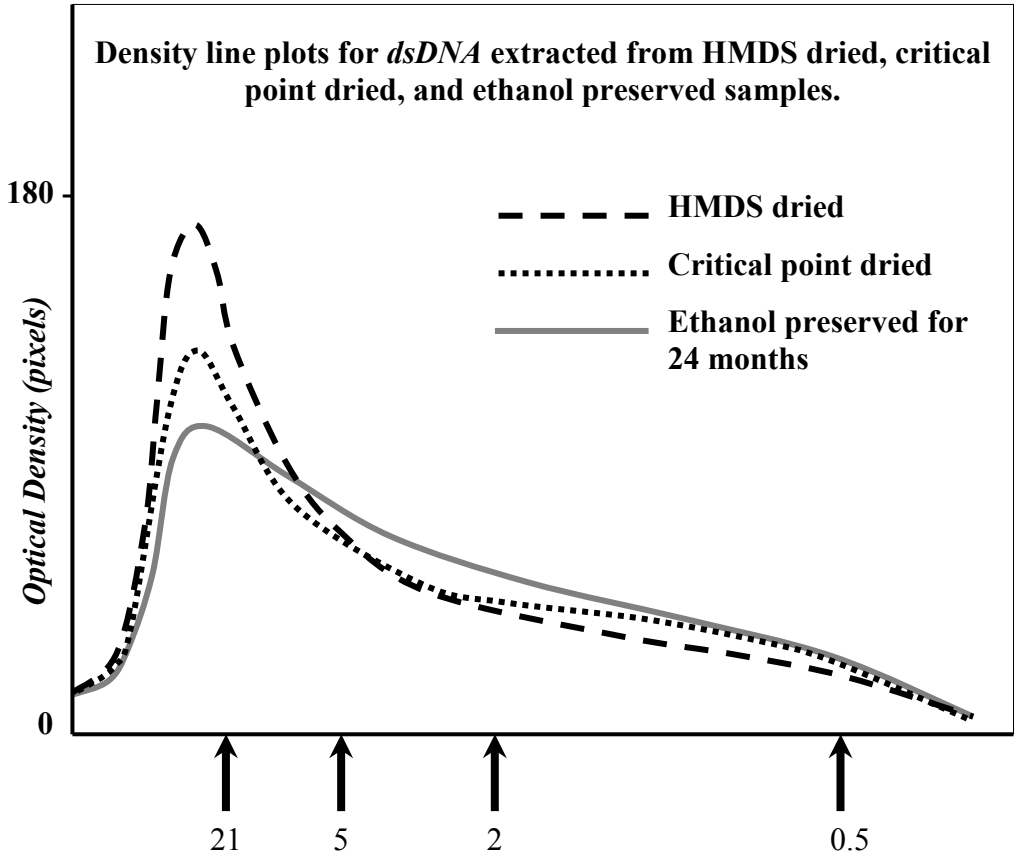


Figure 42: Density line plots for dsDNA extracted from HMDS dried, critical point dried and ethanol preserved samples. The dried samples were all prepared from the ethanol preserved sample after 24 months of preservation.

4.8. Summary of the main results obtained from this study.

Sample treatment	Preservation Age	Average DNA Quantity, ±95% C.L. (µg.ml^{-1})	*Hinf*1 RE enzyme digest	16S PCR product	18S PCR product	No. of Samples (N)	No of Replications
Frozen (-30^{o}C)	20 months	78.7±40.5	Yes	Yes	Yes	5	-
Ethanol	1 day	54.2±14.7	Yes	Yes	Yes	6	2
Ethanol	3 months	49.4±6.2	Yes	Yes	Yes	6	1
Ethanol	24 months	56.4±8.01	Yes	Yes	Yes	4	-
Ethanol (1 change)	13 months	53.4±22.4	Yes	Yes	Yes	6	1
Ethanol (4^{o}C)	12 months	67.2±19.04	Yes	Yes	Yes	6	-
Ethanol plus EDTA	15 months	27.7±9.0	Yes	Yes	Yes	6	-
IMS	15 –18 months	52.6±20.5	Yes	Yes	Yes	6	1
80% IMS	1 month	39.8±17.2	Yes	Yes	Yes	6	1
80% IMS	18 months	24.6±14.2	Yes	Yes	Yes	6	1
80% IMS archival	15 + years	9.3±7.8	Insuff. DNA	Yes	No	6	-
Decon 90 rehydrated.	18 months	*See Table 6*	Yes	Yes	Yes	18	-
Ethyl acetate	12 months	None detectable	Insuff. DNA	No	No	4	-
2-ethoxy ethanol	12 months	20.4±9.2	In-conclusive	Yes	Yes	4	-
Propylene glycol	12 months	23.4±6.6	In-conclusive	Yes	Yes	4	-
4% Form-aldehyde	13 months	> 4.0±2.3	Insuff. DNA	No	No	6	-
Steedman's	1 month	None detectable	Insuff. DNA	No	No	4	-
Critical Point Dried	24 months	62.2±22.1	Yes	Yes	Yes	5	-
Chemical Dried	24 months	78.6±12.0	Yes	Yes	Yes	6	-

Table 9: Summary of the key results obtained in the study.

Chapter 5.

Discussion

5.0 Discussion

The results obtained in this study have reaffirmed some of the findings of previous studies that have considered some aspect of molecular preservation (Greer *et al.*, 1991a; Goebel and Simmons, 1992; Post *et al.*, 1993; Reiss *et al.*, 1995; Dillon *et al.*, 1996; Hammond *et al.*, 1996; Austin and Dillon, 1997; Dawson *et al.*, 1998; Douglas and Rogers, 1998; Fukatsu, 1999; Quicke *et al.*, 1999). The study has also raised a number of further issues relating to the effects of preservation treatments on the condition of the DNA. Section 1.2 discussed the factors affecting the preservation of DNA in museum collections. These factors will now be considered with the results of the study.

Ethanol-preserved samples are regularly used in DNA studies, and the oldest recorded specimen used in a successful molecular analysis appears to date from the 1850's (Criscuolo, 1994). As this study further confirmed, the use of ethanol is useful for preserving material for use in molecular studies. It is fortunate that the use of ethanol as a preservative has a 300 year old history (Simmons, 1992 *pers. comms*.). However, the quality of the alcohol used can play a large part in the condition of the DNA in a specimen. Preservation in absolute ethanol can preserve DNA of a high molecular weight and of high quality. Over the time of this study the quantity of extractable DNA remained high. However it did begin to show signs of degradation through increased fragmentation, especially when compared with the cryo-preserved DNA samples. The temperature and general storage environment must effect this rate of degradation, but so would chemical components within the preserved specimen that have been incompletely preserved or become extracted by the preserving medium (Steedman, 1976a; Pearse, 1980; Von Endt, 1994). Out of the methods considered in this study (apart from cryo-preservation) ethanol currently offers the best means of preserving both the DNA and

the gross morphology of a specimen. Despite this, ethanol preservation does present problems with morphological preservation. It can cause extensive tissue shrinkage, colour loss and can extract cellular components such as lipids. In addition ethanol is expensive to purchase and is highly flammable.

One of the main aims of this study was to try and establish whether the use of IMS as a preserving fluid was as good as ethanol preservation. The results of this study suggest that IMS preservation does have an effect on the preservation of the DNA within a specimen. While high molecular weight DNA can be extracted, the denatured alkaline gel electrophoresis results suggest that the preservation process has effected the overall stability of the dsDNA. Despite this the DNA remains open to analysis with RE and polymerase enzymes. Using IMS also causes similar morphological preservation problems to absolute ethanol. These problems have been reduced by diluting the IMS solutions to 70-80% IMS, and by using additives such as propylene glycol. The use of 80% IMS solutions caused an immediate drop in the quantity of DNA that can be extracted, and whilst high molecular weight DNA is present, significant degradation has occurred. The use of propylene glycol in the solution does not appear to effect the overall preservation of the DNA in 80% IMS solutions. Archival samples from the NMGW collections that had been preserved in 80% IMS for over 15 years still yielded extractable DNA, but this was significantly degraded. From this it can be deduced that the action of water is likely to be the contributing factor to the degradation of the DNA. Thus the evidence in this study suggests that although the concentration of denaturant additives in IMS are low, their effect on the DNA is potentially significant, especially over time. Further study on the effects of IMS preservation is warranted.

The use of a variety of chemicals used as additives or pre-preservation treatments was also considered. EDTA has been used as an additive to ethanol to enhance DNA preservation (Dessauer *et al.*, 1996). However the DNA extracted was lower in quantity and demonstrated greater fragmented than the DNA in ethanol preserved specimens. The reasons for this are unclear and the results require further replication. Both propylene glycol and 2-ethoxy ethanol preserved some high molecular weight DNA when used as preservative solutions. The results from propylene glycol are particularly encouraging as this is used as an additive at 2-4% by volume in IMS based preserving solutions. Its use in this study suggests that propylene glycol is not adequate as a preservative on its own, but its properties as an additive enhancing morphological preservation is not detrimental to the preserving solutions overall ability to preserve dsDNA. This study used 2-ethoxy ethanol as a gross preservative and demonstrated that it has the potential to preserve some high molecular weight dsDNA that is utilisable in PCR-based studies. Again, like propylene glycol, it is not the ideal long term preservative on its own. However it may prove to be a useful additive to a preserving solution especially if a high fat and oil content proves detrimental for long term preservation, although problems with its toxicity and long term stability is a concern. Ethyl acetate is not recommended for use in material being preserved for possible DNA studies. The results showed that ethyl acetate appears to be highly damaging to DNA. This may be due to the possible breakdown products of ethyl acetate such as acetic acid and acetaldehyde, (Deshusses *et al.*, 1999) degrading DNA. This is in agreement with the findings of previous key studies on DNA preservation in fluid preserved material (e.g. Reiss *et al.*, 1995; Dillon *et al.*, 1996).

When considering formaldehyde preserved samples, the DNA extraction method used in this study did manage to extract low quantities of degraded DNA. However, the extraction method used had not been optimised for extracting DNA from formaldehyde-fixed material (Vachot and Monnerot, 1996). Thus higher yields of DNA could be possible. Substituting propylene glycol as the solvent, rather than using deionised water, did not improve the preservation of DNA within the formaldehyde preserved specimens. This suggests that the difficulty in extracting good quality DNA is linked in with mechanisms of formaldehyde fixation rather than from the effects of hydrolysis. Further study would be of benefit.

Steedman's solution had a very detrimental effect on the DNA as no product was extracted by the method used in this study. The main component of Steedman's is the phenol derivative, Phenoxetol, combined with propylene glycol and a trace of formaldehyde. This study has shown that both propylene glycol and formaldehyde should result in some DNA being extractable, thus the phenoxetol is considered to be very damaging to the molecular integrity of the DNA.

The results suggest that the rehydration of material using Decon90 will have some degradative effect on the condition of the dsDNA, but not to the extent of rendering the dsDNA unusable in molecular studies. DNA of a high molecular weight and that is usable in PCR and RE studies was still extractable, provided it was not damaged by any previous preservation processes. It is considered that the longer the period of contact the Decon90 solution has with the specimen, the greater the damage will be to the DNA. However detergents are frequently part of the DNA extraction process, and some have even been recommended for DNA storage (Ulrich *et al.*, 1999). It is notable from the

results in this part of the study that the specimens originally preserved in absolute ethanol had the most notable decrease in extracted DNA quantity on rehydration in Decon90. This is an observation that will require further study as it suggests that part of the DNA may be more open to degradation than other parts of the DNA molecule, which rapidly degrades when preservation conditions are unsuitable. Such factors are being considered in ancient DNA research (Lindahl, 1993). It is also possible that a portion of the DNA is more readily extracted by the detergent solution. Further investigation with other detergents would be desirable, especially with commonly used reagents such as SDS whose detergent action is used to aid the maceration of cells in many DNA extraction protocols.

The use of methods such as critical point drying and chemical drying offer excellent possibilities for preserving invertebrate material (see section 1.3). Previous studies suggest that these techniques can preserve high quality DNA (Austin and Dillon, 1997), although the mechanism by which this is achieved is largely unknown. The results in this study confirm these findings as both CPD and HMDS prepared specimens yield high molecular weight DNA of good quality. It was notable that the quantity of DNA extractable by these methods was equivalent, if not higher than the quantities extractable form the original ethanol preserved control specimens the samples had been prepared from. There also appeared to be less fragmentation of the extracted DNA in the CPD and HMDS samples than when compared to the ethanol samples. This suggests the possibility that the two drying methods stabilise the DNA molecules in some way, enabling the DNA to survive the extraction process better. Overall the final condition of the dsDNA in such samples is dependent on the condition of the dsDNA prior to the drying treatments. The results of both this and previous studies strongly demonstrate

that both CPD and HMDS treatments are very good for both morphological and DNA preservation.

In summary the discussion notes a number of points:

- For fluid preservation absolute ethanol preserves high quality DNA and currently offers the best compromise between preserving a specimen for it gross morphology and its DNA content within the collections at NMGW. Storing at a lower temperature such as 4°C or –18°C (achievable with domestic fridge's and freezers) will enhance long term molecular storage without the expense of very low temperature storage facilities.
- The use of IMS for DNA preservation does not conserve DNA to the same quality as absolute ethanol preservation. The addition of water to the preserving fluid notably degrades the DNA further. Additives such as propylene glycol or 2-ethoxy ethanol did not notably cause further degradation of the DNA.
- The use of ethyl acetate should be avoided as it appears to either significantly degrade or chemically alter the DNA to the extent that it is either not present of cannot be extracted for molecular study.
- Formaldehyde solutions extensively degrade the DNA, although it may be possible to extract some DNA for molecular studies.
- Steedman's solution is not beneficial to DNA preservation, though whether the preservation treatment breaks down or chemically alters the DNA so it is not readily extracted, is unknown.
- Rehydration in Decon90 further degrades the DNA, but not to the extent that the DNA is unusable in subsequent molecular studies.

- The drying methods of CPD and HMDS preserve the DNA within a specimen very well, and may even stabilise the DNA in some way.

5.1 Future Considerations

It is evident from the results of this study, and the work of other researchers, that an 'ideal' fluid preservative that will retain both morphological and molecular integrity is still to be developed. The challenge is now to consider methods of improving the preservation potential of ethanol. With the wide range of quality refined chemicals now available it is worth considering the possibility of developing an improved ethanol-based preservative that counters some of the problems described earlier. The same principles could also be used to improve the performance of other solutions such as 4% formaldehyde, which has many desirable fixative properties, but is poor for the preservation of accessible dsDNA.

There are a number of potential ways that the performance of a fluid preservative could be improved. This could be through the use of antioxidants, osmoprotectants or stabilisers. The aim would be to create a storage fluid that preserved the chemical environment of an organism, but prevented the degradative effects of biological and physiological agents. Ideally a fluid preservative would:-

- Stabilise the specimen
- Maintain a lifelike appearance and form
- Preserve molecular information
- Protect from autolysis, decay and dehydration
- Minimise shrinkage or swelling

Currently the methods available are a compromise of these ideals. There is a growing understanding of the effects that damage and degrade fluid preserved material. It should now be possible to enhance and improved the abilities of a fluid preservative to preserve biological tissue for both its morphology and molecular information.

One of the most damaging effects on gross morphological form is the effect of shrinkage. This is especially noticeable with ethanol-based preservatives, which caused shrinkage through dehydration of the biological tissue. One method would be through the potential use of osmoprotectants to reduce the problems caused by the osmotic changes that occur during the preservation of biological tissue. Osmoprotectants are termed compatible solutes since they do not interfere with protein function, even at high intracellular concentrations (Swan, 1999). Osmolarity is an important property of the primary fixative or preservative, but is probably one of the least understood factors due to the complex interaction between the components in the fixative and in the organism (Swan, 1997). Typically osmoprotectants are low molecular weight organic molecules such as betaine, trehalose and mannitol. Betaine is used to enhance ultrastructural preservation in TEM studies (Swan, 1997) and is also considered to improve PCR success with difficult DNA templates (Henke *et al.*, 1997). It is thought that these compatible solvents stabilise the native conformations of proteins, preventing secondary structure formation and protecting against the denaturing effects of high ionic strengths. It is also possible that compatible solvents, such as betaine, are able to enter cells through cellular membranes that have been compromised by the fixation process, without active transport systems being necessary (Swan, 1999). The functionality of compatible solutes could also have additional effects. Mannitol has the potential to act as a quencher of oxygen radicals. Trehalose can aid in the dehydration resistance of

drought resistant organisms. It is thought this is achieved by trehalose replacing bound water, thereby stabilising macromolecules and biological membranes. The use of sodium bicarbonate has also been found to reduced tissue shrinkage problems in formaldehyde-based solutions (Artvinli, 1975). This is thought to be due to the bicarbonate-formaldehyde solution having no polyvalent ions, reducing osmotic stress. Further study is required, but compatible solutes such as betaine could well provide a valuable role in enhancing the preservation properties of fluid preservatives.

There is also some suggestion that the addition of salt to a preserving solution could stabilise dsDNA preservation. The use of high salt lysis solutions have already been commented on (see section 1.2), and generally only seem to be useful with homogenised material and for short term field preservation. Dessauer *et al.* (1996) recommended the use of small amounts (100 μM) of EDTA in ethanol solutions to aid the inactivation of DNAases. This has been briefly considered in this study but with mixed results. This may relate to the problem that not all enzymes are inactivated by the use of EDTA. This includes some DNAases (Adams *et al.*, 1999). There have also been reports of the use of NaCl to improve the preservation of DNA with formaldehyde-based solutions (Skrecky, 1996). Examples have included the addition of 25 mM sodium chloride or 30 mM potassium chloride. The addition of salt is considered to stabilise the hydrogen bonds between DNA strands thereby protecting the dsDNA from denaturation. High intracellular salt concentrations can also further protect dsDNA by inhibiting depurination especially from thermal degradation effects (Marguet and Forterre, 1998). Thus the use of salts could well have a value in developing long term-fluid preservatives.

The pH value of the preserving fluid is likely to have effects on the long-term stability of the preserved material. A low pH gives rise to protein embrittlement, whilst a high pH causes the possible gelatinisation of proteins (Steedman, 1976b). A low pH can also significantly degrade dsDNA, and this has been noted in attempts to obtain amplifiable DNA from acidic-based fixatives such as Bouins, Formol Acetic Acid (FAA) and Carnoys Reagents (O'Leary *et al.*, 1994; Douglas and Rogers, 1997; Longy *et al.*, 1997). Low pH levels in preserving solutions can also led to accelerated degradation of other molecular components such as lipids (Jones, 1976), leading to conversion to other compounds, such as glycols, and subsequent loss from the organism. If solutions such as formaldehyde are used as a preserving fluid, then the control of acidity is important. Formaldehyde solutions acidify over time due to the oxidation of formaldehyde to formic acid and fixative reaction effects (Steedman, 1976b). The pH level of a preserving solution thus needs to be near physiological levels, usually around pH7. At this point proteins are at their least soluble, also known as the iso-electric point. Many buffers have been recommended for use with aqueous based solutions such as formaldehyde (Steedman, 1976b; Carter, 1997). A buffer solution resists a change of pH on dilution or on the addition of small amounts of acid or alkali by controlling the concentration of hydrogen ions. A neutral pH can significantly reduce dsDNA damage with cytological fixatives (Douglas and Rogers, 1997), and its control is a concern for long term fluid preservation especially with solutions that utilise polar solvents such as water. Pure ethanol solutions are only very weakly acid, but the ethoxide ion formed has a strong negative charge located on the oxygen atom. This remains highly attractive towards H^+ ions, and thus the pH tends not to alter. However, when used as preservatives, ethanol solutions tend to change in pH, due to solvent effects dissolving cellular components such as lipids. This can promote their degradation, forming acidic

bi-products such as fatty acids (Von Endt, 1994). As the ethanol solution becomes more acidic the rate of degradation increases. The use of buffers would prevent the lowering of the pH, although it would not prevent the solvent action of ethanol removing lipids.

Another means of improving the action of fluid preservatives could be to review the solvents used to form the preservative. Solvent mixes are already utilised in preparing fluid preservatives. Examples are the dilution of IMS solutions with water to reduce shrinkage effects, and the use of propylene glycol as an additive to IMS solutions. Solvents such as water need to be avoided due to the long-term hydrolytic damage that can occur. It also provides a good media for the continued action of enzymes. Some of the most obvious substitutes are already used in fluid preservation methods, but are also commonly used as cryoprotectants in cryopreservation methods. This includes chemicals such as glycerol, trehalose, propylene glycol and dimethylsulphoxide (DMSO). These low molecular weight compounds are considered non-toxic and can easily permeate cellular membranes. The collections at the NMGW already utilise propylene glycol as an additive in diluted IMS solutions. The propylene glycol acts as a humectant, protecting the specimens form the loss of the preserving fluid, whilst also enhancing morphological preservation by imparting flexibility and reducing shrinkage (Boase and Waller, 1994). The results of this study suggest that propylene glycol would not be detrimental to the preservation of the dsDNA in ethanol based solutions. Thus replacing the water content of IMS based preserving solutions with propylene glycol could potentially enhance long term morphological and molecular preservation.

The addition of scavengers or antioxidants to the preserving fluids could also be used to enhance fluid preservation. Such methods have been utilised in order to try and preserve

colour in zoological fluid preserved material (Lee, 1989; Harris, 1990). They are also being increasingly used in medical and molecular studies to improve factors such as cell survival and DNA protection (e.g. Gründemann and Schömig, 1996; Karran and Legge, 1996; Lopes *et al.*, 1998; Dinara *et al.*, 2001). The range of chemicals that have been utilised as scavengers or antioxidants is quite diverse. Some of the early colour preservation methods developed for traditional morphological preservation utilised the powerful reducing agent sodium dithionite to remove oxygen from the solution and thus reduce oxidative damage during preservation (Lee, 1989). There have also been attempts to use antioxidants to enhance colour preservation (Harris, 1990). This has included the use of ascorbic acid and hydroxytoluene, but these methods have been developed for use with mammalian or anatomical collections and not for overall molecular preservation. More recently scavengers and antioxidants have been used to improve the protection of DNA during molecular analysis or cryopreservation. It appears that nucleosides such as cytidine or guanosine have the potential to protect DNA from UV-B damage during electrophoresis gel analysis (Gründemann and Schömig, 1996). It is thought that cytidine or guanosine protect dsDNA by a specific interaction that modifies local DNA geometry or internal motions preventing UV light damage. Amino acids, such as cysteine, have also been utilised as scavenging agents for formaldehyde in cryoprotectant solutions (Karran and Legge, 1999), improving mouse oocyte preservation and subsequent survival. The addition of antioxidants in cryopreservatives has also been found to improve the preservation of DNA. Lopes *et al.,* (1998) found pre-treatment with antioxidants significantly reduced DNA damage. Dinara *et al.,* (2001) also found that the addition of antioxidants, such as superoxide dismutatase and catalase, significantly improved the survival rates of cryopreserved mouse oocytes.

The use of antioxidants, and other scavenging additives, has to be approached with some caution. Antioxidants can have pro-oxidant properties under certain conditions. The amino acid cysteine can induce the oxidative degradation of deoxyribose in the presence of Copper(II) ions through the formation of reactive species such as $OH\bullet$ and H_2O_2 (Muñiz, *et al.*, 2001). It has also been shown that Copper(II) ions can cause tannic acid to exhibit pro-oxidant properties through the generation of reactive oxygen species (Khan *et al.*, 2000). Thus, whilst the use of antioxidants has great potential in enhancing the properties of fluid preservatives, the long-term effects of using such additives requires further study.

The preserved condition, both physical and chemical, is a result of the specimens original state and the changes undergone during preservation. However good the preserving fluid is, the environmental storage conditions will have an effect on this condition. The environment can lead to a series of interactions between the fluid preservative, the specimen and the storage container (Horie, 1994; van Dam, 1997). Significant external influences can arise from a series of factors such as:

- Temperature.
- Relative humidity.
- Light and UV radiation.
- Oxygen.

Temperature effects the state of the preserving fluid. As the temperature rises, the vapour pressure within the storage container increases putting stresses on the storage containers and their seals. Temperature increases also increase the rates of reaction. Thus the lower the temperature the better the molecular preservation, hence the use of

cryopreservation methods. Some studies have considered the effects of storage conditions (Cushwa and Medrano, 1993; Marshall *et al*, 1993) confirming lower temperatures enhanced DNA preservation. This study also considered the use of low temperature storage for ethanol preserved material and found the use of domestic refrigerators operating at 4^{o}C to improve general dsDNA preservation.

In a perfectly sealed storage container the relative humidity should have little effect. However, most storage containers have imperfect seals leading to fluid loss through evaporation and hence open to being affected by relative humidity levels. Diluted alcohol solutions, such as 80% IMS, will tend to evaporate ethanol or absorb water, driving the concentration to a more dilute solution. This will reduce the preservation properties of the fluid over time (Carter, 1995). This dilution effect will depend on the relative humidity of the environment. The lower the relative humidity the more likely the water evaporates with the alcohol. This is due to the vapour pressure of the water in the alcohol solution becoming equivalent with the atmospheric relative humidity (Horie, 1994).

Light and UV radiation can provide energy to the internal storage environment within the storage jar. This can have an effect on any water and dissolved oxygen within the storage fluid causing the production of reactive radicals. The effects of light can cause degradation and discoloration of the storage fluid, with subsequent degradative effects on the preserved material. Light can also cause the fading and degradation of dyes and pigments that give the preserved specimens colour and patterns in life. Thus the elimination of light and UV exposure during storage is important for the long term preservation of fluid preserved material.

The influence of oxygen is one of the main factors that the use of fluid preservation is designed to remove. In theory a good storage container should eliminate the diffusion of oxygen into the preserving fluid by providing an oxygen barrier. However the seals on storage containers are rarely completely airtight, and many of the materials used for the construction of jar lids, such as polypropylene, are themselves very permeable to oxygen (van Dam, 1997). The provision of oxygen into the fluid preservative can have numerous effects. Oxidation can promote the pH of formaldehyde solutions to fall, and can degrade lipids, amino acids and pigments (Stoddart, 1989). Whatever the fluid preserving medium, the prevention of oxidation effects will be of great benefit for the long-term preservation of biological material.

All of these effects can have a direct bearing on collection management decisions. The monitoring and maintenance of the collection storage environment is essential for the long-term morphological and molecular preservation of biological material, as is selecting the most appropriate storage containers for the storage environment. Even if an 'ideal' fluid preservative can be developed, a poor storage environment would still promote the degradation of fluid preserved collection material. Currently, the general recommendations for the storage of fluid preserved collections are around 18^{o}C and 40% RH (Paine, 1992). Such levels are reasonably practical and are potentially achievable by most UK institutions holding fluid preserved collections.

5.2 Summary

This study provides further information on how current methods of fluid preservation can effect the preservation of dsDNA in biological material. Whilst there are many variables that need to be considered to trying to assess the factors affecting the condition of the dsDNA (preservation methods used, type of organism, storage environment etc), the study has also highlighted potential future possibilities for improving the effectiveness of fluid preservatives. This includes the use of:-

- Osmoprotectants / Stabilisers e.g. betaine, salts etc
- Replacement solvents to water – e.g. propylene glycol.
- Antioxidants / free radical scavengers.
- pH buffers with aqueous based solutions.

The development of improved methods of fluid preservation will need careful study in order to replicate the effects of time. This can be done through the use of artificial ageing methods, which use heat and light to accelerate degradative reactions. With suitable resources other methods could be utilised to examine the condition of the DNA to those utilised in this study:

- Quantitative PCR / molecular beacons (Zimmermann and Mannhalter, 1996; Cayouette *et al.*, 1999). Quantitative PCR enables the amplification product to be monitored without resorting to gel electrophoresis. This method would allow accurate comparison of the effectiveness of DNA from different preservation treatments to be used in PCR based studies.
- Denaturing Gradient Gel Electrophoresis, DGGE. (Myers *et al.*, 1987; Lessa, 1993). This is a method that can be used to detect small changes in DNA sequences, particularly single base substitutions, deletions and insertions. Such changes can point to damage caused by the preservation treatments.

- Comet assay or Single Cell Gel Electrophoresis, SCGE. (Piperakis *et al.*, 1999). This provides a very sensitive method for detecting strand breakages or cross-links at the level of the single cell. The method allows intracellular differences in DNA damage to be assessed. The comet assay could be a useful method in the initial screening of proposed fluid preservatives.
- Mutation rates in sequences. The use of DNA sequencing and close examination for changes in the base sequences can be used to detect base damage caused by the preservation treatment (Douglas and Rogers, 1997; Hansen *et al.*, 2001).

While our current methods of preservation do have the potential to preserve DNA there are still many problems. The main problem is that on the death of an organism the cellular protection mechanisms are no longer effective. Preservation methods can significantly slow subsequent degradation pathways, but the preserved specimen is now missing its protective mechanisms. If this issue can be partially addressed then the potential to enhance morphological and molecular preservation would be significant. In the meantime the standard methods used at the NMGW are likely to generate usable DNA in potential molecular studies, especially as the methods utilised in molecular studies continue to improve. Our biological collections are an important resource, and the continued development of molecular analytical techniques further improves the value of this resource.

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