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(with contributions from B Boismer and D Keen)



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## Summary

This report documents the attempts to conduct amino acid racemization analysis for age estimation. It is concluded that it is not possible to age the site, as the shells were badly corroded.

Four major problems were encountered. Firstly we have had no previous experience of dating *Planorbis* – which were the main species available. Also, most of the shells were corroded, which is believed to reduce the potential for dating. Most of our dating effort was placed on unbleached material (to retain consistency with earlier studies). Bleaching clearly improved the analysis, however too few samples were obtained to permit extensive bleaching. Finally, it is difficult to provide dates in a cold stage, when there is only a small amount of racemization over long periods of time.

## Keywords

Amino Acid Racemization
Geochronology

## Author's address

**ne**aar, Biology S Block, Department of Biology, PO Box 373, York YO10 5YW
Tel: 01904 328806

## Introduction

In late February and early March 2002, an archaeological watching brief at Lynford Quarry, Mundford, Norfolk (Fig 1; TL 82399482) revealed a relic Middle Devensian palaeochannel with a dark organic fill containing *in situ* mammoth remains and associated Mousterian stone tools and debitage buried under two to three metres of bedded sands and gravels. The site was subsequently excavated by the Norfolk Archaeological Unit from the 8th of April to the 11th of September 2002 with funding provided by English Heritage through the Aggregates Levy Sustainability Fund.

## Lynford Quarry, Mundford, Norfolk

Site location and description of the sediments in the main palaeochannel can be summarised as follows.

The site comprised the surviving eastern end of a major palaeochannel feature filled with organic deposits within the current application area of Lynford Quarry, Mundford, Norfolk. The quarry is situated in south-west Norfolk *c* 2km north-east of the village of Mundford and *c* 500m to the south-east of the village of Ickburgh. It lies on the southern side of the floodplain of the River Wissey (centred at NGR TL 825 948) and comprises an overall area of some *c* 8.46ha. Soils for this area are predominately sandy and peaty soils of the Isleham 2 Association overlying glaciofluvial drift composed of stratified sand, gravel, and stones with rare inclusions of chalky gravel. In relief, the area generally slopes towards the north-west with surface elevations typically ranging between 12m and 15m AOD.

The workings in which the site occurred consisted of a *c* 1.20ha rectangular area located in the north-western part of the application area between the River Wissey to the north and a flooded former pit to the west (Fig 1). The palaeochannel was situated in the north-eastern part of these workings (centred at NGR TL 8239 9482) and survived for a length of *c* 21.0m with a maximum width of *c* 12.0m. No *in situ* channel deposits survived within the machine excavated area of the quarry. The feature appears to have been a meander cut-off acting as a small basin or oxbow lake originally orientated in an east/north-east to west/south-west direction.

Approximately 199m$^2$ of the palaeochannel survived *in situ* to depth of *c* 1.50m with the deposits, containing faunal and lithic material, composed of an organic sediment with very fine alternating organic/merogenic laminae and a minerogenic fine sand on which the organic sediment had accumulated. The organic sediment was characterised by fine alternating laminae (<5mm thickness) of dark brown to brown organic matter with silt and very fine quartz sand, and pale yellowish-brown to grey lenses of inorganic very fine to fine quartz sand. The upper 0.10 to 0.30m of the deposit comprised a series of discontinuous laminae of white to grey coarse/fine sand and brown to dark brown organic matter with silt and fine sand.

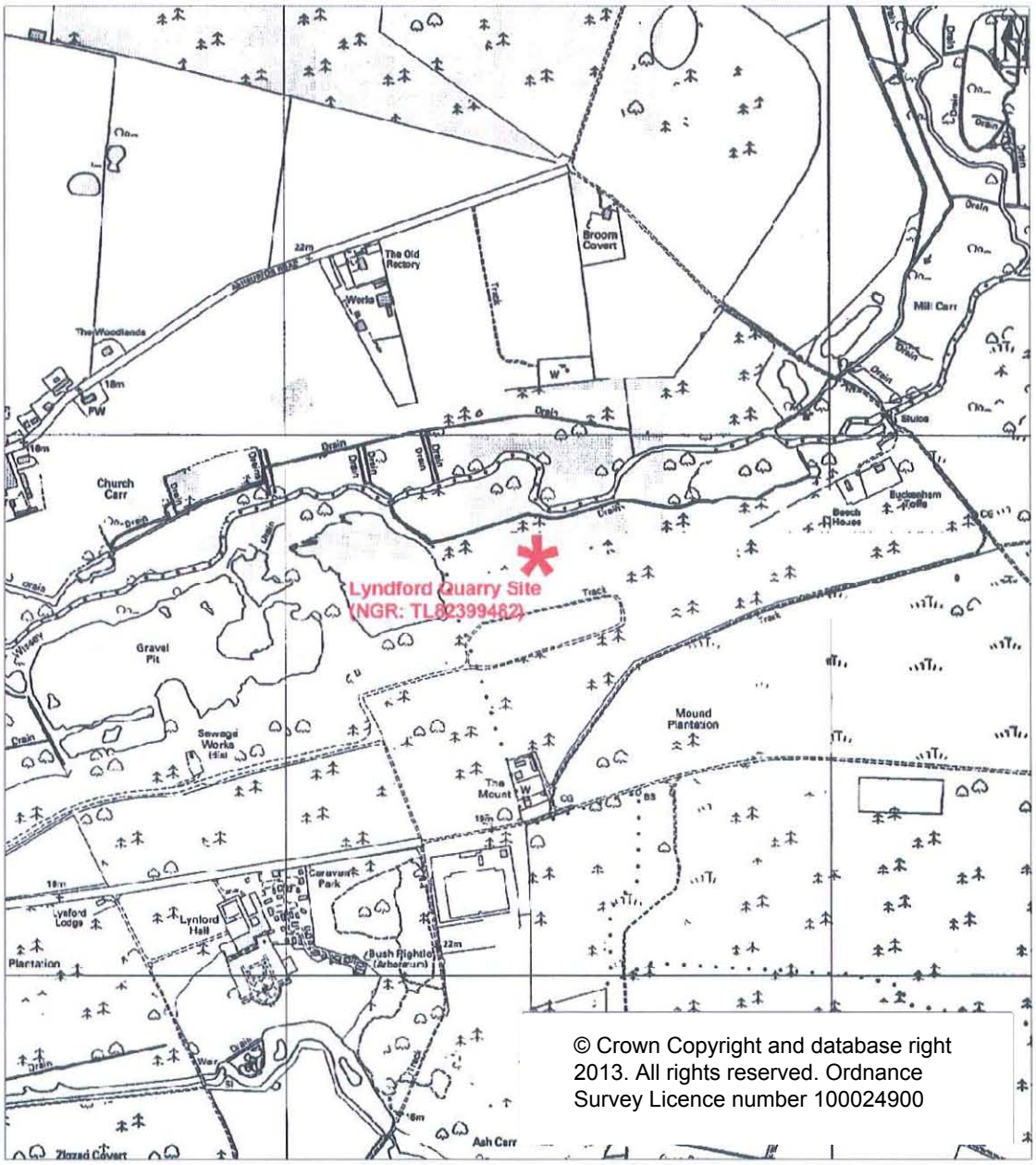


Figure 1: Map showing the location of the Lyndford Quarry Site (NGR: TL82399482)

The organic laminae appear to have been highly compressed with the organic component composed of either amorphous plant matter or possibly a fine detrital peat that had been subjected to some oxidation and impregnation of amorphous iron. Medium and coarse gravel were concentrated predominately in the south-east part of the deposit adjacent to the edge of the channel and suggested an origin involving the incorporation of bank material in the organic sediment.

The formation of the organic deposit within the channel may have been the result of alternating accumulations of organic matter and fine sediment produced by possible seasonal fluctuations of the water level in the channel. Deposition of the coarser eroded material (flint gravel and nodules) may have been produced by bank collapse and other disturbances caused by animal

movements to and from the channel and/or flood events. The origin of the alternating of inorganic and organic laminae in the upper part of the deposit was due to post-depositional diagenetic changes in sediment characteristics produced by the leaching of organic matter by groundwater flow.

Immediately below the organic sediment was a patchy deposit of soft greenish-brown clayey silt with rare lenses of white sand and sparse densities of medium to coarse gravel. This deposit occurred in the south-central and north-west parts of the palaeochannel and ranged between 0.10m-0.40m in thickness. The fine-grained texture of the sediment indicated that the deposit was largely formed by the settling of organic matter and fine sediment under still or gently flowing water conditions.

The base of the palaeochannel sequence was composed of a series of relatively thin beds of light grey-brown sandy clay with a high calcium carbonate content and brown silty sands with sparse densities of medium to coarse gravel. Physical processes involved in the origin of these deposits included the leaching of sulphate and organic matter from overlying organic deposits and dissolution of chalk bedrock and the settling of fine overbank material in standing or gently flowing water conditions.

## Description of the faunal and artefactual material recovered (Boismier)

In total, some 2,079 bones, tusks, antlers and teeth of mammoth (*Mammuthus primigenius*), woolly rhinoceros (*Coelodonta antiquitatis*), reindeer (*Rangifer tarandus*), horse (*Equus ferus*), bison (*Bison priscus*), wolf (*Canis lupus*), red or arctic fox (*Vulpes vulpes* or *Alopex lagopus*) and brown bear (*Ursus arctos*) were individually recorded and a further 25,000 bone, tooth and tusk fragments recovered from the 0.50m$^2$ subunits. Coprolites of scavengers (possibly the spotted hyena *Crocuta crocuta*) were also recovered from the organic sediments. No articulated skeletons were found. Bone fractures characteristic of marrow extraction by hominids have been identified on some of the reindeer and horse bones recovered from the deposit. Biostratigraphically, the faunal remains recovered from the palaeochannel are typical of the Pin Hole Mammal Assemblage Zone of the Middle Devensian.

Some 590 worked flint artefacts consisting of 44 handaxes (cordate, subcordate, and ovate forms), three cores, and a number of retouched, utilised, and waste flakes were individually recorded with over 1,000 pieces of small debitage recovered from the 0.50m$^2$ spit units. A number of the handaxes and flakes were found in direct association with bones and/or tusks. Typologically the assemblage falls within the Mousterian of Acheulean Tradition (MTA) facies of the Middle Palaeolithic.

Bone condition and skeletal element representation suggest that the vertebrate remains recovered from the palaeochannel represent a palimpsest or time-averaged accumulation of bone derived from a number of different

agents and event episodes. An accumulation of debris from multiple events may also be indicated for the lithic assemblage. Major taphonomic and behavioural factors responsible for assemblage formation appear to have included: natural mortalities; trampling by large animals; the modification, destruction, and removal of bone by predator-scavengers, and possibly hominids; and the production, use, and discard of stone tools. Other factors involved in the accumulation of objects within the channel probably included bank collapse, mud or debris flow, and fluvial activity.

Optical Stimulated Luminescence dates indicate a date for the organic sediments within the palaeochannel of between 64,000 to 67,000 years ago. This report details attempts to obtain age estimates using amino acid racemization

## Amino Acid Racemization Geochronology

*Theory*

Amino acids, the building blocks of proteins, occur as two isomers that are chemically identical, but optically different. These isomers are designated as either D (dextrorotary) or L (levorotary) depending upon whether they rotate plane polarised light to the right or left respectively. In living organisms the amino acids in protein are almost exclusively L and the D/L ratio approaches zero[1]. The potential application to geochronology arises from the fact that after death amino acid isomers start to interconvert. This process is commonly termed racemization. In time the D/L ratio approaches one. The proportion of D to L amino acids is therefore an estimate of the extent of protein degradation, and if this is assumed to be predictable over time can be used to estimate age.

*Mechanisms of racemization*

The rate of racemization is governed by a variety of factors, most of which have been studied in detail only for free amino acids. North-East Amino Acid Racemization (**ne**aar) analysed the intra-crystalline amino acid fraction and in this way, within a closed environment in which other factors (water content, concentration of cations, and pH) are constant, the extent of racemization is a function of time/temperature. If the thermal history of a site can be estimated, it is possible to estimate age; conversely if the age of a site is known the extent of racemization can be used to infer the integrated thermal history.

## Materials

The Mollusca were supplied by David Keen. The Molluscs present were moderately well, to well, preserved. The only particular problem of

[1] D-amino acids are synthesised by some organisms; they are found free in invertebrate body fluids where they play a role in osmoregulation and can occur peptide bound in bacterial peptidoglycan, where part of their function is resistance to proteases.

preservation has been caused by the acidity of the organic sands which has entirely removed the shell content at some levels. Amino acid racemization (AAR) analyses were conducted of shells from the following sections.

### *30331*

x co-ord: 582407.50; y co-ord: 294832.00 (grid unit co-ords); z co-ord: 8.27m aod

A spot sample for small vertebrates from within 20003 (organic deposit) which yielded *Pupilla muscorum* [from subsample 10]

### *30228*

x co-ord: 582409.53; y co-ord: 294834.928; z co-ord: 8.749m aod top of column

The sands are all lower fill of the palaeochannel. The sample sequence and associated contexts are:

| | | |
|---|---|---|
| 01 | 20003 | Organic sediment |
| 02 | 20003 | Organic sediment |
| 03 | 20003 | Organic Sediment |
| 04 | 20003/30139 | Interface of organic sediment and grey-brown sand |
| 05 | 20139 | Grey-brown sand |
| 06 | 20245 | Brown sand |
| 07 | 20245 | Grey-brown sand |

*Planorbis planorbis* [from subsample 01] was analysed for AAR

### *30198*

x co-ord: 582405.586; y co-ord:294837.563; z co-ord: 8.764m aod

*Planorbis planorbis* - subsample 40-50 cms, 20003 organic sediment

**Method**

*Sample Preparation*

Shells were examined under a low powered microscope and any adhering sediment removed. The shell samples were sonicated and rinsed several times in High Performance Liquid Chromatography (HPLC) grade water (ie low organic carbon content). The shells were then crushed to <100$\mu$m. Both bleached and unbleached (ie conventional preparation used by other laboratories; Bowen *et al* 1989), samples were analysed.

*Bleaching*

50µl of 12% solution of sodium hypochlorite (NaOCl) at room temperature was added to each milligram of powdered sample and the caps retightened. The powders were bleached for 48 hours with a shake at 24 hours. The bleach was pipetted off and the powders were then rinsed five times in HPLC-

grade water and a final rinse in HPLC-grade methanol ($CH_3OH$) to destroy any residual oxidant by reaction with the $CH_3OH$. The bulk of the $CH_3OH$ was pippetted off and the remainder left to evaporate to dryness.

*Hydrolysis*

Protein-bound amino acids are released by adding an excess of 7M hydrochloric acid (HCl) and hydrolysing at 110°C for six hours. The process involved adding 20µl (per milligram of sample) of 7M Hydrochloric Acid (HCl) to each Hydrolysis (H) sample in sterile glass vials. These samples were then flushed with nitrogen for 20 seconds to prevent oxidation of the amino acids and were then placed in an oven at 110°C for six hours. After ten minutes in the oven, the caps of the 3ml vials were re-tightened to prevent the escape of vapour.

After six hours, the samples were dried in a centrifugal evaporator overnight. When completely dry, they were rehydrated with 10µl per mg of Rehydration Fluid: a solution containing 0.01mM HCl, 0.01mM L-homo arginine internal standard, and 0.77mM sodium azide at a pH of 2. Each vial was vortexed for 20 seconds to ensure complete dissolution, and checked visually for undissolved particles.

Free amino-acid samples (F) were demineralised in cold 2M HCl, which dissolves the carbonate but minimises the hydrolysis of peptide bonds, dried in the centrifugal evaporator and then rehydrated as above (with 10µl of Rehydration Fluid per milligram of sample).

For each set of sub-samples a blank vial was included at each stage to account for any background interference from the bleach, acid, or rehydration fluid added to the samples.

Approximately 50µl of rehydrated sample was then placed in a sterile, labelled 2ml autosampler vial containing a glass insert, capped, and then placed on the autosampler tray of the high performance liquid chromatographer (HPLC). Samples were separated using a Hypersil BDS column.

*Analysis of Free and Hydrolysed Amino Acids*

Amino acid enantiomers were separated by reverse phase HPLC. North-East Amino Acid Racemization uses the method outlined in Kaufman and Manley (1998), which uses an automated reverse phase high pressure liquid chromatography system. This method achieves separation and detection of L and D isomers in the sub-picomole range. Samples (2$\mu$l) were derivitised with 2.2$\mu$l *o*-phthaldialdehyde and thiol *N*-isobutyryl-L-cysteine automatically prior to injection. The resulting diastereomeric derivatives were then separated on Hypersil $C_{18}$ BDS column (sphere d. 5 µm; 250 x 3mm) using a linear gradient of a sodium acetate buffer (23mM sodium acetate, 1.3mM $Na_2EDTA$; pH6), methanol and acetonitrile on an integrated HP1100 liquid chromatograph (Hewlett-Packard, USA).

The fluorescence intensity of derivitised amino acids was measured (Ex=230nm, Em=445nm) in each sample and normalised to the internal standard. All samples and blank extracts that had been subjected to identical preparation procedures were run in triplicate. Quantification of individual amino acids was achieved by comparison with the standard amino acid mixture.

External standards containing a variety of D and L- amino acids, allowing calibration with the analyte samples were analysed at the beginning and end of every run, and one standard was analysed every ten samples. Blanks were randomly interspersed amongst the standards.

*Reverse Phase High Performance Liquid Chromatography*

A Hewlett-Packard 1100 Series HPLC was used to analyse the samples for amino-acid molecules.

Individual amino-acids are separated on a non-polar stationary phase according to their varied retention times: a function of their mass, structure, and hydrophobicity. A fluorescence detector is used to determine the concentrations of each amino-acid and record them as separate peaks on a chromatogram. A gradient elution programme was used to keep the retention time to below 120 minutes.

## Results

In total we conducted 75 analyses, most of which (70%, 60 runs) were on unbleached samples. As anticipated bleaching reduced the yields of amino acids, and also increased reproducibility.

Detailed results are provided in Appendix IV. The key findings were as follows:

- Amino acid yields from *Pupilla* were higher than those from *Planorbis*
- Amino acid yields were reduced following bleaching as expected, however the extent of reduction was greater for *Planorbis* than for *Pupilla* (Fig 2)
- Reproducibility was greater for bleached than for unbleached shells

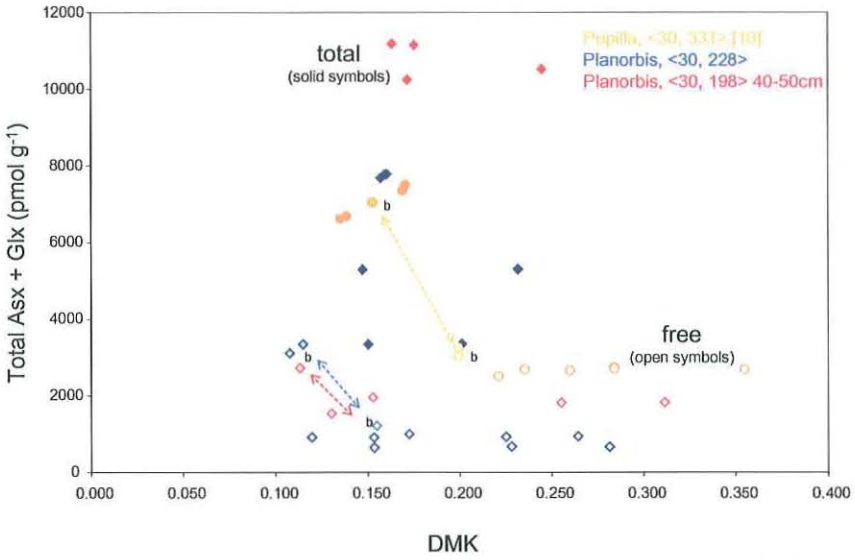


Figure 2: Comparison of amino acid contents (Glx + Asx) from *Planorbis* (diamonds) and *Pupilla* (circles) from unbleached and bleached samples (the latter indicated by the b symbol and lighter colouration - paired samples of total and free amino acids linked by arrows). Note the decrease in scattering in both concentration and range of DMK values following bleaching. The individual shells are colour coded as illustrated in the key.

- Higher levels of total amino acids remained in *Pupilla* compared to *Planorbis*

- Most shells fail to provide a series of D/L ratios which are consistent with the DMK model (even with a generous 20% allowance for the standard deviation – ie those with a standard deviation of predicted versus observed values of less the 20% considered acceptable)

- Due to the small sample sizes only 19 runs were of bleached samples, and of these only eight bleached values, from two specimens, fitted the DMK model ($\sigma$ < 15%), these are listed in Table 1, below:

| NCL No | Sample | H | Age | F | Age |
|---|---|---|---|---|---|
| 466 | <30, 198> 40-50cm | 0.115 | 52,900 | 0.158 | 80,700 |
| | *Planorbis planorbis* | 0.113 | 50,500 | | |
| | | 0.112 | 49,100 | | |
| mean | | 0.113 | **50,833** | 0.158 | **80,700** |
| 2 σ | | 0.003 | 3,844 | | |
| 469 | <30, 228> [01] | 0.106 | 49,700 | 0.152 | 78,000 |
| | *Planorbis planorbis* | 0.112 | 39,300 | 0.157 | 80,400 |
| mean | | 0.109 | **44,500** | 0.155 | **79,200** |
| 2 σ | | 0.008 | 14,708 | 0.007 | 3,394 |

From these values the model derives very different age estimates for Hydrolysed (H) and Free (F) fractions. These values can be compared with data from *Valvata* and *Bithynia* from the two sites we have studied which potentially age bracket Lynford, the Holocene site of Stanwick and the MIS5a(?) sites at Cassington. The differential between the H and F fractions is much lower for the two prosobranch gastropods, even at the site of Cassington. The differences may be the result of different patterns of racemization (in which case the DMK model would not be valid) or alternatively may be due to the dissolution of the shells.

| **Sites** | **Species** | **H** | **2 σ** | **F** | **2 σ** | **Age** |
|---|---|---|---|---|---|---|
| Stanwick | *Valvata* | 0.098 | 0.005 | 0.095 | 0.021 | 5145 bp |
| Stanwick | *Bithynia* | 0.087 | 0.019 | 0.094 | 0.008 | 5145 bp |
| Cassington | *Valvata* | | | 0.157 | 0.004 | MIS 5a? |
| Cassington | *Bithynia* | 0.151 | 0.009 | 0.179 | 0.004 | MIS 5a? |

We conclude that **no reliance can be placed upon our age estimates and the material we examined is considered undateable.**

## Discussion

*Effects of dissolution*

Free amino acid racemization levels produce older, often an order of magnitude older, Degradation Model Kinetic (DMK) age estimate values than the total amino acids from the **same** shell. One possible explanation for high *free* and low *total* DMK values is the extent of corrosion observed in shells recovered from the site. Keen (in unpublished report submitted to Boismier, page 1) reports "in other samples partial dissolution has primarily left the calcite plates of Limacid slugs, but only vestigial remnants of the aragonite shells of gastropods and bivalves, the latter sometimes preserved only as internal casts. There are however, enough samples in which acid-attack has been sufficiently restricted to allow considerable shell content to remain. As with all freshwater molluscan assemblages, large numbers of juvenile shells occurred which could only be identified to generic level".

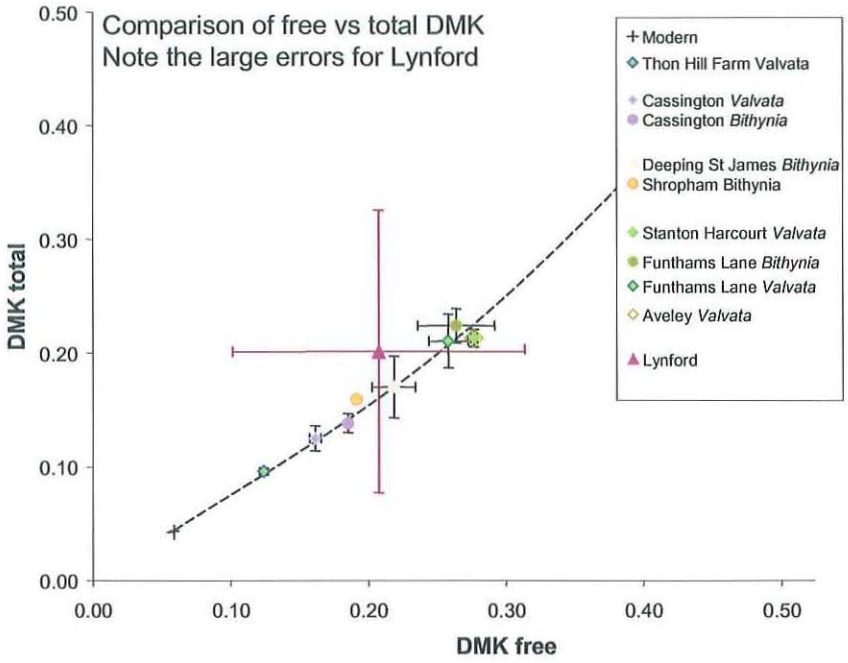


Figure 3: Comparison of free and total DMK for a variety of Maritime Isotope Stages (MIS) 4 (purple) MIS 5 (orange) and MIS 7 (green) sites with the range of values from Lynford. Note the large error bars on the data from Lynford and the offset from the anticipated values indicated by dotted line.

Low pH has two opposing effects on an amino acid age estimate. Firstly the low pH accelerates protein decomposition (increasing the extent of racemization and hence over-estimate age). Secondly dissolution increases the rate of leaching of the most highly racemized and mobile free amino acids, which depresses the extent of racemization in the *total* fraction, and has the effect of underestimating age. The very low DMK of total fractions in some shells suggest that some of this corrosion has occurred in the recent past.

*Age estimation in cold stages*

A further problem with AAR age estimation of Lynford are the low rates of racemization observed in cold stages, such as MIS 4. Amino acid racemization rates are reduced as temperatures fall. Consequently over a long time period there is only a minor increase in the DMK (compared with warm stages; Fig 4).

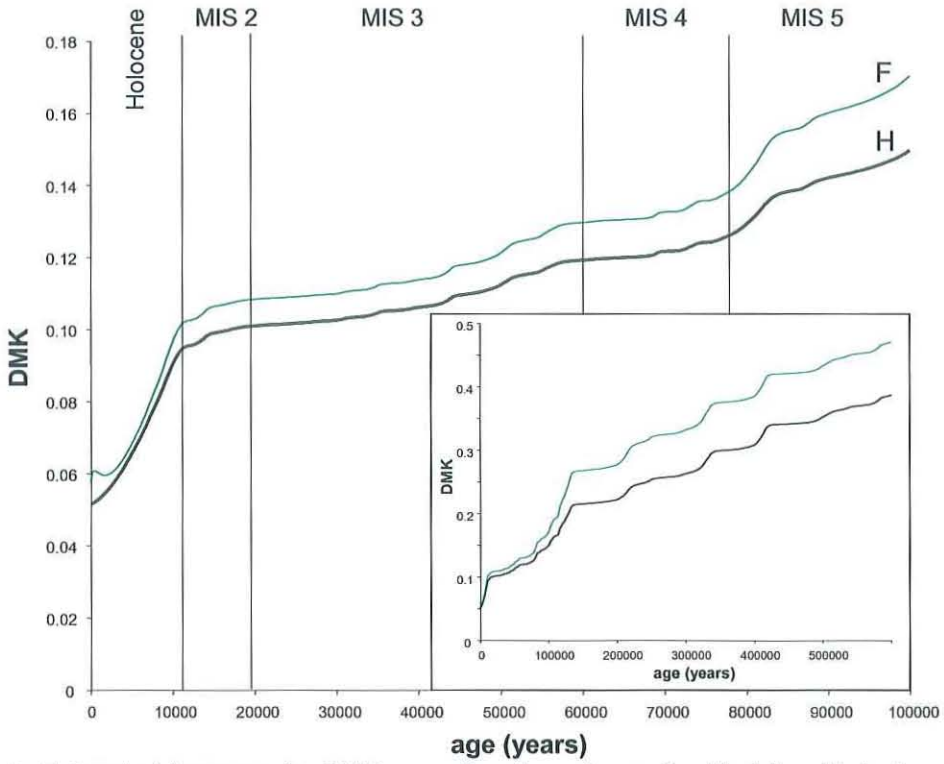


Figure 4: Estimated increase in DMK as a function of age for Norfolk. Note the lack of increase in DMK in stage 4, compared with rapid rises in both stage 5 and the Holocene. The temperature model (which is still in development) is based a model of protein composition linked to terrestrial proxy temperature data for the last cycle and extrapolated back using marine and ice records. The model fails to account for the large variation on the DMK of the H and F fractions observed in *Planorbis* from Lynford.

We therefore conclude that it is **not possible** to provide a reliable age estimate of the shells from Lynford based upon the current AAR data. In itself this is a highly instructive finding for the application of the AAR method but not for an age estimate of this site.

## Appendix 1: Glossary

**18MΩ water**: The water has a resistivity of 18MΩ/cm, indicating a lack of ions.

**HPLC grade water.** In addition to low ion content, HPLC grade water has a low organic content (typically < 2 ppb).

**Amino acids**: the building blocks of proteins and consist of an alpha carbon atom ($C_\alpha$) which has four different groups bonded to it: an amino group (-$NH_2$), a carboxyl group (-COOH), a hydrogen atom (-H), and a side chain, (often called an R group). About 20 amino acids normally occur in nature and some of these can undergo further modification (eg the hydroxylation of proline to hydroxyproline). The amino acids are commonly known by three letter codes (see Appendix 3: Abbreviations). They exist free in the cell, but are more commonly linked together by **peptide bonds** to form proteins, peptides, and sub-components of some other macromolecules (eg bacterial peptidoglycan).

**Amino acid isomers:** amino acids occur as two stereoisomers that are chemically identical, but optically different. These isomers are designated as either D (dextrorotary) or L (levorotary) depending upon whether they rotate plane polarised light to the right or left respectively. In living organisms the amino acids in protein are almost exclusively L and the D/L ratio approaches zero. Two amino acids, isoleucine and threonine, have two chiral carbon atoms and therefore have four stereoisomers each. As well as racemization, these two amino acids can undergo a process known as epimerization. The detection of the L-alloisoleucine epimer (derived from L-isoleucine) is possible by conventional ion-exchange chromatography, and was thus the most commonly used reaction pathway in geochronology.

**Asx:** Measurements of aspartic acid following hydrolysis also include asparagines, which decomposes to Asx. This combined signal of aspartic acid plus asparagine (Asp +Asn) is referred to as Asx (Collins *et al* 1999).

**D-amino acid**: dextrorotary amino acid, formed following synthesis of the protein as it degrades over time (remember as "dead amino acid").

**DMK** Conventional racemization analysis tends to report an allosioleucine / isoleucine (A/I or D/L ratio). This amino acid ratio has the advantage of being relative easy to measure and also sufficiently slow to be used to "date" sediments in the European Quaternary.

Our DMK approach utilises multiple amino acids. However we have avoided trying to give a whole series of D/L values for each amino acid in each sample. Instead we are using a theoretical model of protein degradation. The model outputs are then used to compare observed D/L vales of any amino acid against the A/I value at the same stage of protein decomposition. The relative rate of racemization of any amino acid (its D/L ratio) is then reported as an A/I equivalent - which as a working title we have named the

Degradation Model Kinetic value (or DMK) (Collins Penkman and Kaufman in prep).

Instead of getting a single A/I ratio we obtain a series of (DMK) values, currently $DMK_{Asx}$, $DMK_{Glu}$, $DMK_{Phe}$, $DMK_{Ala}$, $DMK_{Val}$, and a (pretty unreliable) A/I ratio ($DMK_{A/I}$ = A/I). Other ratios, notably $DMK_{Ser}$, are not currently of implemented in the model – ie we don't have a good degradation model for this amino acid yet.

Because each amino acid has its own particular characteristics, only in a well behaved system will $DMK_{Asx}$ = $DMK_{Glu}$ = $DMK_{Phe}$ = $DMK_{Ala}$ = $DMK_{Val}$ = A/I. If an amino acid has an unusually low ratio (due to modern contamination) or unusually high racemization (due to inclusion of bacterial cell wall contaminants) either some or all of the amino acids will no longer fit to the idealized degradation model. Indeed we can use elevation of $DMK_{Asx}$ = $DMK_{Glu}$ and = $DMK_{Ala}$ to provide a bacterial contamination index. We have not done so in this case as there was no evidence of contamination.

**DMK values:** Degradation Model Kinetic, a summary value obtained from multiple amino acid D/L values from a single sample all normalised to a common model of protein degradation and racemization.

**Enantiomers / optical isomers:** mirror image forms of the same compound that cannot be superimposed on one another.

**Epimerisation:** the inversion of the chiral $\alpha$-carbon atom.

**Free amino acid fraction:** The fraction of amino acids directly amenable to racemization analysis. Only amino acids which have already been naturally hydrolysed (over time) are measured. These are the most highly racemized amino acids.

**Hydrolysis:** A chemical reaction involving water leading to the breaking apart of a compound (in this case the breaking of peptide bonds to release amino acids).

**L-amino acid:** levorotary amino acid, the constituent form of proteins (remember as "living amino acid").

**Peptide bond**: an amide linkage between the carboxyl group of one amino acid and the amino group of another.

**Racemization:** the inversion of all chiral carbon atoms, leading to the decrease in specific optical rotation. When the optical rotation is reduced to zero, the mixture is said to be racemized.

**Stereoisomers:** molecules of the same compound that have their atoms arranged differently in space.

**Total amino acid fraction:** The extent of racemization of all amino acids in a sample, determined following aggressive high temperature hydrolysis with strong mineral acid, which has the effect of breaking apart all peptide bonds so that the total extent of racemization in all amino acids both free and peptide bound are measured.

**Zwitterion:** A dipolar ion containing ionic groups of opposite charge. At neutral pH the ionic form of amino acids which predominates is the zwitterions

**DMK = Glx not alle / Ile?**

Due to the problem of being unable to accurately measure A/I in our current system, we have switched to a version DMK which is normalized for Glutamic acid. Although D/L Glu ≡A/I, we have not yet fully established this relationship.

**What does the date estimated from DMK mean?**

The date is our best estimate based upon the temperature history of the site. If we wanted to constrain this further we would need reliable independent dates. There are considerable differences in racemization rates between different molluscs. This reflects differences in rates of decomposition of proteins within the shell – the so-called species effects (Lajoie *et al* 1980).

## Appendix 2

### Past Use of Amino Acid Racemization Dating

Amino acids were first reported in fossils by Abelson (1954). Later, it was discovered that the systematic changes of amino acids in an organism after its death could be used to determine the age of the fossil (Abelson 1956). Hare and Mitterer (1967) analysed fossil samples from the Miocene and discovered that the proportion of D-amino acids was significantly larger than those found in younger fossils.

The presence of proteins in archaeological remains has been known for some time. Nearly fifty years ago Abelson (1954) separated amino acids from subfossil shell. He suggested the possibility of using the kinetics of the degradation of amino acids as the basis for a dating method (Abelson 1955). In 1967 Hare and Abelson measured the extent of racemization of amino acids extracted from modern and sub-fossil *Mercenaria mercenaria* shells (edible clam). They found that the total amount of amino acids present in shell decreased with the age of the shell. The amino acids in recent shell were all in the L configuration and over time the amount of D configuration amino acid increased (Hare and Abelson 1967). However, even after 35 years this method of dating is still subject to vigorous debate, with the application of AAR to date bone being particularly controversial (Bada 1990; Marshall 1990). Major reviews of AAR include: Johnson and Miller (1997), Hare *et al* (1997), Rutter and Blackwell (1995), Murray-Wallace (1993), Bada (1991), and Schroeder and Bada (1976). Racemization is a chemical reaction and a number of factors influence its rate (Rutter and Blackwell 1995). These include; amino acid structure, the sequence of amino acids in peptides, pH, buffering effects, metallic cations, the presence of water, and temperature. To establish a dating method the kinetics and mechanisms of the racemization (and epimerization) reaction of free and peptide bound amino acids need to be established. To this end various workers in the late 1960s and the 1970s studied free amino acids in solution and carried out laboratory simulations of *post mortem* changes in the amino acids in bone (Bada 1972a) and shell (Hare and Abelson 1967; Hare and Mitterer 1969). Attempts have also been made to relate the kinetics of free amino acids, with those in short polypeptides and the proteins in various archaeological samples (Bada 1982; Smith and Evans 1980).

The ability of this technique to be used as a geochronological and geothermometry tool has led to its use in many environmental studies. Goodfriend (1991) analysed terrestrial gastropods, other studies have investigated bivalves (Goodfriend and Stanley, 1996), foraminifera (Harada *et al* 1996), ostrich egg shells (Miller *et al* 1992, 1999) and even speleothems (Lauritzen 1994)

## Appendix 3

### Abbreviations used in this report

| Abbreviation | 1-letter code | number of chiral centres | Expanded name |
|---|---|---|---|
| Ala | A | 1 | Alanine |
| Arg | R | 1 | Arginine |
| Acn | | | acetonitrile |
| AA | | | Amino acid (n) |
| Asn | N | 1 | Asparagine |
| Asp | D | 1 | Aspartic acid |
| Asx | | | Aspragine + Aspartic acid + Succinimide |
| Asu | | | Succinimide |
| Cys | C | 1 | Cysteine |
| DCM | | | Dichlormethane |
| GABA | | | γ-Aminobutyric acid |
| Gln | Q | 1 | Glutamine |
| Glu | E | 1 | Glutamic acid |
| Glx | | | Glutamine and Glutamic acid |
| Gly | G | 0 | Glycine |
| His | H | 1 | Histidine |
| HPLC | | | High-Performance Liquid Chromatography |
| Hyp | | | Hydroxyproline |
| IBD(L) C | | | N-Isobutyryl-D(L)-Cysteine |
| Ile | I | 2 | Isoleucin |
| Leu | L | 1 | Leucine |
| Lys | K | 1 | Lysine |
| MeOH | | | Methanol |
| Met | M | 1 | Methionine |
| Nle | | | Norleucine |
| OPA | | | ortho-Phthaldialdehyde |
| Orn | | | Ornithine |
| Phe | F | 1 | Phenylalanine |
| Pro | P | 1 | Proline |
| Ser | S | 1 | Serine |
| Thr | T | 2 | Threonine |
| Trp | W | 1 | Tryptophan |
| Tyr | Y | 1 | Tyrosine |
| Val | V | 1 | Valine |

# Appendix 4 Amino acid data Lynford

| Code | Genus | species | location | Glx D/L | Asx D/L | Ser D/L | | Phe D/L | Leu D/L | Ile D/L | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0466bF | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | | 0.45 | 0.67 | 0.23 | | 0.12 | | |
| 0466bF | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | | 0.43 | 0.56 | 0.42 | 0.20 | 0.18 | | |
| 0466bF | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | | 0.44 | | | | | | |
| 0466bH | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | 0.10 | 0.36 | 0.40 | 0.17 | 0.07 | 0.14 | | |
| 0466bH | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | 0.10 | 0.36 | 0.41 | 0.15 | 0.07 | 0.14 | | |
| 0466bH | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | 0.10 | 0.36 | 0.40 | 0.15 | | | | |
| 0466uF | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | | 0.41 | 0.35 | 0.26 | | | | |
| 0466uF | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | | 0.38 | 0.29 | 0.40 | 0.24 | | | |
| 0466uF | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | 0.31 | 0.40 | 0.35 | 0.28 | | | | |
| 0466uH | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | 0.12 | 0.41 | 0.18 | 0.27 | 0.14 | | | |
| 0466uH | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | 0.12 | 0.41 | 0.18 | 0.31 | 0.32 | | | |
| 0466uH | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | 0.12 | 0.43 | 0.18 | 0.24 | 0.31 | 0.21 | | |
| 0468uF | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | | 0.45 | 0.58 | 0.22 | | 0.15 | | |
| 0468uF | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | 0.17 | 0.42 | 0.35 | 0.43 | 0.13 | 0.26 | | |
| 0468uF | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | | | | | | | | |
| 0468uH | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | 0.14 | 0.47 | 0.36 | 0.18 | 0.13 | 0.15 | | |
| 0468uH | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | | 0.48 | 0.35 | 0.15 | 0.11 | 0.12 | | |
| 0468uH | *Planorbis* | *planorbis* | Lynford, <30, 198> 40-50cm | 0.14 | 0.47 | 0.36 | 0.18 | 0.12 | 0.12 | | |
| 0469bF | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.14 | 0.46 | 0.72 | 0.25 | 0.06 | 0.19 | | |
| 0469bF | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.13 | 0.44 | 0.68 | 0.25 | 0.09 | 0.25 | | |
| 0469bF | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | | 0.45 | 0.69 | 0.23 | | 0.23 | | |
| 0469bH | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.10 | 0.33 | 0.42 | 0.16 | 0.08 | 0.13 | | |
| 0469bH | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.10 | 0.32 | 0.42 | 0.16 | | 0.13 | | |
| 0469bH | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.10 | 0.32 | 0.40 | 0.19 | | 0.14 | | |
| 0469uF | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | | | | | | | | |
| 0469uF | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | | | 0.24 | 0.56 | | | | |
| 0469uF | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | | | 0.40 | 0.18 | | | | |
| 0469uH | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.13 | 0.47 | 0.32 | 0.19 | | | | |
| 0469uH | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.13 | 0.48 | 0.37 | 0.18 | | | | |
| 0469uH | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.13 | 0.48 | 0.33 | 0.20 | | | | |
| 0470uF | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | | 0.43 | 0.56 | 0.24 | | | | |
| 0470uF | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | | 0.42 | 0.57 | 0.23 | | 0.14 | | |
| 0470uF | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | | 0.39 | 0.40 | 0.34 | | 0.20 | | |
| 0470uH | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.12 | 0.42 | 0.27 | 0.21 | | | | |
| 0470uH | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.13 | 0.43 | 0.30 | 0.18 | 0.32 | | | 0.19 |
| 0470uH | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.13 | 0.43 | 0.30 | 0.25 | 0.25 | 0.05 | | 0.16 |
| 0471uF | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | | 0.43 | 0.48 | 0.24 | | | | |
| 0471uF | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | | 0.40 | 0.45 | 0.40 | | | | |
| 0471uF | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | | 0.42 | 0.52 | 0.21 | | | | |
| 0471uH | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.12 | 0.44 | 0.19 | 0.17 | | | | |
| 0471uH | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.12 | 0.45 | 0.21 | 0.16 | 0.10 | | | |
| 0471uH | *Planorbis* | *planorbis* | Lynford, <30, 228> [01] | 0.12 | 0.45 | 0.21 | 0.20 | 0.10 | 0.07 | | |
| 0472bF | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.21 | 0.39 | 0.77 | 0.47 | 0.17 | 0.36 | | |
| 0472bF | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.21 | 0.39 | 0.82 | 0.39 | | 0.39 | | |
| 0472bF | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.21 | 0.39 | 0.82 | 0.39 | | 0.39 | | |
| 0472bH | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.13 | 0.36 | 0.59 | 0.29 | 0.07 | 0.20 | | |
| 0472bH | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.14 | 0.36 | 0.59 | 0.29 | 0.09 | 0.20 | | |
| 0472bH | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.13 | 0.35 | 0.59 | 0.29 | | 0.21 | | |
| 0472uF | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | | 0.39 | 0.35 | 0.46 | | | | |
| 0472uF | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.27 | 0.39 | 0.36 | 0.47 | 0.50 | | | |
| 0472uF | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.27 | 0.39 | 0.36 | 0.45 | | | | |
| 0472uH | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.14 | 0.44 | 0.40 | 0.27 | | | | |
| 0472uH | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.14 | 0.44 | 0.46 | 0.31 | 0.08 | 0.13 | | |
| 0472uH | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.14 | 0.44 | 0.44 | 0.29 | 0.08 | 0.12 | | |
| 0473uF | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | | 0.42 | 0.51 | 0.52 | | | | |
| 0473uF | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.31 | 0.42 | 0.67 | 0.54 | | 0.27 | | |
| 0473uF | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.30 | 0.43 | 0.68 | 0.54 | | 0.22 | | |
| 0473uH | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.11 | 0.37 | 0.33 | 0.23 | | | | |
| 0473uH | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.12 | 0.38 | 0.35 | 0.21 | | 0.12 | | |
| 0473uH | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.11 | 0.37 | 0.36 | 0.20 | | 0.10 | | |
| 0474uF | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | | 0.40 | 0.42 | 0.31 | | | | |
| 0474uF | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | | | 0.27 | 0.59 | | | | |
| 0474uF | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | | | | | | | | |
| 0474uH | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | 0.19 | 0.40 | 0.31 | 0.38 | | | | |
| 0474uH | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | | | | | | | | |
| 0474uH | *Pupilla* | *muscorum* | Lynford, <30, 331> [10] | | | | 0.34 | | | | |
| | | | | | | | | | | | |
| 0466bFm | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | | | | | | | | |
| 0466bFm | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | | | | | | | | |
| 0466uFm | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | | | | | | | | |
| 0466uFm | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | | | | | | | | |
| 0467bFm | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | | | | | | | | |
| 0467uFm | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | | | | 0.60 | | | | |
| 0467uFm | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | | | | | | | | |
| 0468uFm | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | | | | | | | | |
| 0468uFm | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | | | | | | | | |
| 0466bH6m | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | | | | | | | | |
| 0466bH6m | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | | | | 0.09 | | | | |
| 0466uH6m | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | 0.09 | | 0.12 | 0.08 | | 0.06 | | |
| 0468uH6m | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | 0.09 | | 0.12 | 0.08 | | 0.06 | | |
| 0467bH6m | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | 0.09 | | | | | | | |
| 0467bH6m | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | 0.09 | | | 0.10 | | | | |
| 0467uH6m | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | 0.08 | | 0.11 | 0.08 | | 0.04 | | |
| 0467uH6m | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | 0.08 | 0.24 | 0.11 | 0.08 | | 0.04 | 0.06 | |
| 0468uH6m | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | 0.10 | | 0.12 | 0.09 | | 0.05 | | |
| 0468uH6m | Planorbis | planorbis | Lynford, <30, 198> 40-50cm | 0.10 | 0.25 | 0.12 | 0.09 | | 0.07 | | |

Code is a 4 digit NCL number followed by, **b** for bleached or **u** for unbleached, **F** for Free or **H** for hydrolysed.
Where D/L values are missing from the spreadsheet this was because the peaks were not satisfactorily resolved or one of the two peaks was missing.

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