BIOMARKERS AND THEIR APPLICATION TO THE STUDY OF PIGMENTS IN THE FOSSIL RECORD

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By

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Congratulations, Gibbet! I hear you’ve had a rough deal.

Oh yes, very exciting. It’s going to be called The Metaphoric Restoration Group: A Retrospective.


Don’t be silly, I’m deciding what fancy quote to put in the preface.
Abstract

The idea of studying the colour of organisms in the fossil record has for a long time seemed nothing more than the far flung dreams of a few idealistic palaeontologists. The last 5 years however have brought sweeping advances in this area, such that the idea is no longer hypothetical but an actual possibility. Recent studies on the preservation of fossilised pigment containing organelles, melanosomes, have hailed a new era of palaeontological study and reconstruction. In this work geochemical techniques are applied to study such structures in fossil feathers and an amphibian, to determine the necessary level of confidence that these structures are not bacterial, discuss the relative advantages and disadvantages of the techniques used in the study of fossil melanin, and extend the study of fossil colour to other pigments in fossil algae. Fourier transform infrared spectroscopy (FTIR) data is shown to provide some of the most consistent evidence of the presence of melanin, however it does not enable us to adequately distinguish between the two major types, eu- and pheomelanin. Determining the presence of bacteria in fossil stromatolites is shown to be most effective by using tetra-methyl ammonium hydroxide assisted pyrolysis gas chromatography mass spectrometry (Py-GCMS) to look for the distribution pattern of fatty acid methyl esters. Such analysis shows that samples identified as bacterial have identical patterns that differ significantly from those of fossil plants and animals. Such a biomarker is shown to be more reliable than hopanes, further commonly used bacterial biomarkers. The combination
of FTIR and Py-GCMS suggest the presence of the red algal pigment phycoerythrin in the Jurassic fossil *Solenopora jurassica*, strengthening its identification as a calcareous alga.
Declaration

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This thesis is dedicated to my father with all my love

Colin Barden

1949-2008
Chapter 1

Introduction

1.1 Biomarkers

1.1.1 What are biomarkers?

For the vast majority of living organisms, death marks the beginning of a chain reaction of destruction and decomposition that leaves nothing behind to attest to their former existence. Important components become recycled into the various organic and inorganic cycles, leaving nothing behind but occasional bone. However, some facets of an organism’s tissues can become incorporated into the sediments and soils and occasionally leave behind biomolecular remains as a result of their decomposition. Collectively these organic molecules are known as sedimentary organic matter. The term biomarker refers to a specific subset of the total preserved organic matter, and is defined as “an organic compound in natural waters, sediments, soils, fossils, crude oils or coal that can be unambiguously linked to specific precursor molecules made by living organisms” [55, p. 285].

Identifying organic molecules in a fossil organism can be very difficult, as they are
usually so altered that there is no way to reassemble their former structure \[31\]. However, under certain conditions, some molecules are able to retain a diagnostic chemical structure despite the effects of diagenesis, with only relatively minor changes of form. This process of diagenetic change, whereby over time biomolecules are stripped of their less tightly bonded and more reactive parts, such as certain functional groups, leaves what is known as a geomolecule. Geomolecules are usually simple carbon chain skeletons, those that are identifiable as having derived from specific precursor biomolecules are known as biomarkers. Usually these biomolecules have relatively little chemical reactivity, and in life served protective roles in cell walls and membranes, or were components of chemical pigments \[39\]. There are therefore two fundamental criteria that organic molecules must fulfil in order to become classed as biomarkers \[118\]:

1. The parent biomolecule must be relatively common within a certain group of organisms

2. The diagnostic structure of the geomolecule must be chemically stable

It should also be added, as per the definition, that the geomolecule must be able to be definitively associated with its parent biomolecule, and it should not be capable of abiotic generation \[137, 138\].

### 1.1.2 Biomarkers in the fossil record

Biomarkers are used in palaeontology to complement the traditional morphological approach to the fossil record. They can potentially provide information about organisms that leave no hard parts in the record, and are an additional source for those that do. Biomarkers have been discovered for many major groups and are routinely used to indicate the presence of higher plants and bacteria in sediments \[102\]. Sterols, such
as cholesterol, are characteristic of Eukarya, whereas hopanoids are specific to certain bacteria. Different biomarker molecules can even be used to distinguish between different bacterial groups, the carotenoid pigment isorenieratene is exclusively synthesised by green sulphur bacteria [32], whereas okenane is another carotenoid indicative of the presence of the purple sulphur bacteria family *Chromatiaceae* [18]; both also indicate the presence of photic zone euxinia. The structure of certain biomarkers can indicate the presence of oxidising or reducing conditions, and whether taphonomic processes such as preservation through sulphurization has taken place [47]. Specific spatial arrangements of higher plant biomarkers can demonstrate the presence of percolating waters which aid preservation [28]. They can also be used to distinguish marine, lacustrine and hypersaline environments [118].

The most commonly studied biomarkers are lipids, particularly phospholipids, which compose structural components in cell walls [23, p. 76-77]. Their hydrophobic nature gives them a high preservation potential [44], consequently they are generally the best preserved and most abundant organic molecules in the geological record [137]. Other types of biomarker include carbohydrates and proteins. DNA is the most sought after biomarker, however without the presence of exceptional environmental conditions, such as extreme cold, it only has a half life of about 521 years and is fully degraded by approximately 7 million years [3].

There is a strong correlation between the conditions necessary for the survival of organic molecules in the fossil record, such as rapid burial, anoxic environments [74] and extremes of salinity [44], and those that generally favour exceptional preservation. These conditions make the recovery of organic material more likely from soft tissues as opposed to bone, though such material can occasionally be found in bone [5, 135, 144].
1.2 Colour in the natural world

Colour and colour patterns serve many different functions in the natural world, both decorative and functional. In animals they can provide camouflage (Fig. 1.1a,b) to help predators or prey avoid detection [104] p. 83-86]. They can be used by organisms to display that they are unpalatable or poisonous(Fig. 1.1f), or by harmless animals to mimic more dangerous ones (Fig. 1.1e) [70]. They are used to help members of the same species recognise each other, those of the opposite sex and for display(Fig. 1.1c) [10,35]. Colour patterns can also signify health or social status [8], and even dynamically change to reflect mood or to aid camouflage [105].

Colour can be determined by various different chemical pigments or structural mechanisms. Though usually it is a combination of structure and pigment that bestows visible colour, certain colours, such as the blues of bird feathers, are solely caused by the scattering of light from their inner structure [54]. It is also worth noting that the way different animals perceive colour varies widely. Birds for example, are known to employ colours for display that are only visible in the ultraviolet spectrum, which they can see but humans cannot [21].

Among the most common chemical pigments are carotenoids, porphyrins and melanins [51] p. 8-12]. Whilst there have been studies to look at the presence of carotenoids and porphyrins in ancient sediments [4,75], it is melanin that has been used to study the colour patterns of extinct animals. This has been a major advance in palaeontology, and has provided a whole new dimension to our knowledge about ancient animals and their behavioural patterns.
Figure 1.1: (a, b) The stone flounder (Kareius bicoloratus) camouflaged on different substrates. (c) Male magnificent frigate birds (Fregata magnificens) inflate a bright red throat pouch to attract females. (d) The yellow banded poison dart frog (Dendrobates leucomelas) advertises its toxicity to potential predators by use of warning colouration. (e) The harmless hornet moth (Sesia apiformis) mimics the dangerous European hornet (f) (Vespa crabro) to deter predators from eating it. All images are in the public domain.
1.3. MELANIN

1.3.1 Structure

Melanin is an insoluble [81] granular protein [60] composed of many different types of monomer units linked by carbon-carbon double bonds [79][153]. The overall structure is highly conjugated (Fig. 1.2), leading to its wide spectral absorbance and colour properties [124]. The two main types of melanin that give visible colour in living organisms are eumelanin and pheomelanin [100]. They are synthesised within membrane bound organelles called melanosomes [25][124]; eumelanin is formed within eumelanosomes, and pheomelanin in pheomelanosomes. Eumelanosomes are elongate and approximately 1μm long, whilst pheomelanosomes are spherical and approximately 0.5 to 1μm in diameter [84].

Eumelanin has more carbonyl groups than pheomelanin, leading to increased absorption of the red part of the visible spectrum which gives it a darker black/brown
colour than pheomelanin, which produces lighter colours [72, 124]. Pheomelanin contains more sulphur (9-12%) than eumelanin (0-1%) and is also soluble in alkali solutions, whereas eumelanin is not [79]. Melanin is always associated with proteins [100], which provide increased rigidity and potentially help to retard degradation [124].

1.3.2 Function

Though the primary modern use of melanin seems to be for pigmentation, it has other functions that historically may have been more important. It acts in thermoregulation and provides some protection against ionizing radiation such as UV [25, 72]. Certain intermediate products of melanogenesis are also thought to have antibiotic properties, though research on this is conflicting and inconclusive, see Riley [124] for a review. Melanin also has significant chelating ability, and forms strong bonds to heavy metal cations [124] which when free can be toxic [25, 100].

Eumelanins are generally thought to endow structures with black, brown or grey colours, whereas pheomelanins confer yellows and reds [72]. Perceived colour is normally the result of a combination of both eu- and pheomelanin as well as other pigments and structural colours; significant amounts of eumelanin will generally overpower the pheomelanin contribution [81].

1.3.3 Melanin in the fossil record

The identification of fossil melanosomes was first proposed by Vinther et al. [151], who identified elongate bodies approximately 1-2 µm long in a visibly banded Lower Cretaceous fossil feather. The bodies were clustered within the darker bands of the feather and none were seen in the lighter bands. This, combined with the apparent alignment of the bodies and their similarity in form and size to those in modern feathers, led the authors to conclude that they were fossil eumelanosomes. This discovery
1.3. MELANIN

was extended in 2010 [150] when studies on an Eocene feather showed these bodies were preserved in dense layers surrounding loosely packed layers. This pattern is also seen in modern feathers that display iridescence, therefore the fossil feathers were also inferred to possess structural colour.

Zhang et al. [167] studied melanosome-like structures in Early Cretaceous feathers and the feather-like integument of *Sinosauropteryx* and *Sinornithosaurus*. They demonstrated the presence of a banded formation of pheomelanosomes in the tail filaments of *Sinosauropteryx*, suggesting it had a rust/orange and white striped tail. The presence of melanosomes within such filaments also showed that they were indeed epidermal structures and not degraded collagen fibres as had previously been suggested [97].

Further studies by Li et al. [95] reconstructed the plumage of the Late Jurassic bird *Anchiornis huxleyi* by statistically analysing the shape of the melanosomes found within its feathers. When compared to such analyses of modern melanosomes they were able to assign colour to differently shaped melanosomes, and could infer the plumage of the bird to be black, white, grey and brown/orange. Such techniques were also applied to Late Eocene penguin feathers [27], and isolated Upper Cretaceous feathers of the Ingersoll shale [87].

These studies predominantly used morphological analyses, using environmental or normal scanning electron microscopy (ESEM or SEM). Geochemistry was roughly analysed using energy dispersive x-ray spectroscopy (EDS), in order to demonstrate the preservation of specimens as carbonaceous films [87, 150, 151] and the absence of pyrite frambooids [27, 87].

The problem with relying solely on such techniques is that they are insufficient for distinguishing between fossilised bacteria and fossilised melanosomes, which have the same size and shape characteristics [2, p. 103, 103, p. 67-69] (Fig. 1.3). Therefore there was a subsequent shift to a predominantly geochemical approach to the study of
CHAPTER 1. INTRODUCTION

Figure 1.3: Images of fossil eumelanosomes (a) and pheomelanosomes (b) from *Confuciusornis sanctus*, and modern bacteria (c) *Staphylococcus aureus* and (d) *vibrio cholerae*. Long red arrows indicate eumelanosomes and short arrows indicate pheomelanosomes. Scale bars represent 2µm. The fossil melanosomes are remarkably similar in size and shape to the modern bacteria. Modern bacteria images are in the public domain. Fossil melanosome images adapted by permission from [Macmillan publishers Ltd: Nature] Zhang et al. [167], copyright 2010.
fossil melanosomes. A large variety of geochemical techniques have been used including infrared [6,59,96], chromatography mass spectrometry [6,59], x-ray fluorescence and absorbance [159], and magnetic resonance [6,59]. Such analyses have shown, among other results, the presence of eumelanin functional groups within fossil material [6,59,96] and how copper can be used as a eumelanin biomarker due to its strong chelation with certain metals [159].

There has been significant geochemical evidence demonstrating the presence of melanin in fossil feathers [6,159], a fish eye [96] and a cephalopod [59], and currently none to indicate the presence of bacteria, though there is still more work to be done in this area. To date, there has been much work on the study of bacterial biomarkers, but not in their detection within fossils over time. By knowing what bacterial molecules we can expect to find in fossil bacterial mats of different ages we can be more directed and certain of ruling out their presence in other fossil material.

The study of melanin in the fossil record has opened up the field of studying prehistoric colour, though at the time of writing it has been limited to melanin. However, there are many other pigments used by modern organisms that have not yet been studied in fossil material. Given the limitations previously discussed about the sole use of morphological techniques, the studies detailed in this thesis make use of a combined morphological and geochemical approach.

1.4 Aims

The aims of this thesis are to:

- Apply geochemical techniques to further our understanding of melanin preservation in the discrete fossil structures, and determine the relative usefulness of different geochemical analyses for its study.
• Broaden the study of melanin in the fossil record to other taxonomic groups. Currently most work has been done on fossil feathers or feather-like integuments with few studies also analysing cephalopods and fish. So far there has been no work done to study the preservation of melanin in amphibians.

• Expand the study of colour in the fossil record to pigments other than melanin. Currently the majority of work on pigments in macroscopic fossils has been limited to melanin, whereas there are many other chemical pigments that contribute to visible colour.

• Determine how useful biomarkers are at differentiating between bacterial and melanosome bodies in fossil tissues of different ages. In order to determine whether ancient bacteria have in any way contaminated a fossil and may therefore be responsible for any microscopic structures reliable biomarkers must be found to diagnose their presence. This would also help to determine whether bacteria have had any role in the preservation of the organism.

1.5 Approach and thesis structure

This thesis is written in the alternative format as the author has already published a paper from the research carried out for this doctorate (chapter 3). For this reason there may be a certain amount of repetition of some content, such as methodological details. It contains an introductory chapter, presenting the background for the research, five chapters written up as scientific papers, and a concluding chapter which summarises the results of the thesis. The chapters written as scientific papers are either published (chapter 5) or due to be submitted soon (chapters 2, 4, 5 and 6).

Chapter 2 comprises methodology information written up as a review paper. It gives information not only on the methodologies used in this thesis, but also in other
1.5. APPROACH AND THESIS STRUCTURE

studies on melanin in the fossil record. It critically reviews each technique, giving
details on what information the technique generates, how the technique is applied to
the study of fossil melanin and how destructive the technique is. The various methods
are discussed in terms of how useful they are and what combinations of analyses are
best for different types of samples.

The remainder of the thesis is separated into two main sections. The first (chapters
4 and 5) deals with evidence of the preservation of colour in the fossil record, and the
second (chapter 6) is a study of the reliability of the use of bacterial biomarkers to
indicate the presence of bacteria in fossils of different ages.

Chapters 3 and 4 deal with fossil melanin. Chapter 3 details a published study on
the preservation of eumelanin in the feathers of the Lower Cretaceous bird Gansus
yumenensis. Chapter 4 is a study on the potential preservation of pheomelanin within
an Oligocene tadpole from the Enspel Formation. Such studies were carried out to geo-
chemically analyse fossil material at a time when similar studies were predominantly
morphological, and to extend such studies to a taxonomic group that had thus far not
been studied.

The preservation of other pigments are covered in chapter 5, where a fossil alga is
analysed for the red algal pigment phycoerythrin. This chapter represents the first time
that such analyses have been applied to the study of pigments other than melanin in the
fossil record.

The research in chapter 6 was conducted in order to assess how much bacterial
biomarkers should be relied upon to determine the presence of bacteria in fossil ma-
terial. Thus far no systematic time step studies have been performed to determine
whether bacterial biomarkers are reliably detectable in fossils of different ages, or if
not, how they change over time. Without knowing what signals should be observed,
the presence of bacteria cannot be reliably ruled out when studying other fossils.
Chapter 2

Paper 1: A review of techniques used in the study of melanin in the fossil record

This chapter contains the following paper which is in preparation to be submitted to the *Journal of Analytical Techniques*:

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Abstract

The study of melanin in the fossil record has recently attracted much attention, and the techniques used have changed. Studies are now utilising a wide range of geochemical techniques rather than solely morphological ones to provide more convincing evidence of the preservation of melanin. The approach of combining morphological with multiple geochemical analyses is the most effective, as each technique gives slightly different data. However each has strengths and weaknesses regarding sensitivity, ease of use and access and sample destruction. The major techniques used in the study of fossil melanin are evaluated here, and their major advantages and disadvantages discussed.

2.1 Introduction

Melanin is a chemical pigment found in a wide variety of organisms [124, 162]. It comes in two main forms; eumelanin (Fig. 2.1a), which bestows dark blacks and browns, and pheomelanin (Fig. 2.1b), which gives lighter and more blonde or rust coloured tones [100]. There is another form called neuromelanin that is found in brain tissue, however as such material is not commonly preserved neuromelanin is not currently studied in fossils and will not be discussed further here. Melanin is an insoluble [81] granular protein [60] composed of many different types of monomer units linked by carbon-carbon double bonds [79, 153]. The overall structure (Fig. 2.1) is highly conjugated leading to its wide spectral absorbance and colour properties [124]. The pigment is formed within membrane bound organelles called melanosomes. Eumelanin is synthesised in eumelanosomes which are elongate in structure and approximately 1µm long, whereas pheomelanin is produced in pheomelanosomes which are spherical and can be up to approximately 0.5 to 1µm in diameter.

Such elongate and spherical structures have been observed in fossil material for
Figure 2.1: Structure of (a) eumelanin and (b) pheomelanin. Reprinted from the International Journal of Radiation Oncology*Biology*Physics, [134] with permission from Elsevier.

many years where they were usually interpreted as lithified bacteria [33], contributing to various hypotheses about their role in the promotion of exceptional preservation [164]. However, in 2008 they were reinterpreted as fossilised melanosomes [151] based on a structural similarity to modern melanosomes, and localisation within the dark areas of a visually banded fossil feather of the Crato Formation. Since then, there have been several studies on the subject which have purported to find melanosomes in the feathers of fossil birds [24, 59, 87, 94, 95], and the integuments of a fossil theropod [167]. On the basis of the geometric properties of the structures several authors have projected colour reconstructions of these extinct animals [24, 95, 167]. There was also an attempt to demonstrate evidence of iridescence in the fossil record by analysis of the orientation of layers of the preserved melanosomes [150]. These earlier studies principally analysed morphology using scanning electron microscopy (SEM) [27, 95], with some use of energy dispersive x-ray spectroscopy (EDS) for geochemical analysis [87, 94, 150, 151, 167].

There are problems with this approach, mainly that it is very difficult to differentiate between fossil bacteria and melanosomes based on structure alone as both are so
2.1. INTRODUCTION

similar [2, p. 103] [103, p. 67-69]. Accordingly, most studies produced in later years moved away from a morphologically heavy approach and focused instead on the combination of structure and geochemistry. Such geochemical techniques reveal traces of the melanin pigment itself as its structure lends itself well to preservation. The types of analyses used vary widely, from infrared [6, 59, 96] to chromatography mass spectrometry [6, 59], x-ray techniques [59, 159], and magnetic resonance [6, 59], with many studies utilizing a broad range of different techniques within the same study. Such analyses provide more data but are often, though not always, destructive to samples.

Herein lies one of the major considerations when carrying out this type of work on fossil material. Namely that by its very nature, fossils preserved well enough to allow these types of analysis are usually so rare that no form of sample destruction is allowed. There are varying degrees of destruction to each analysis, so that in some cases certain more mildly destructive techniques can be used. Therefore careful consideration must be given to exactly what can be done with a sample, and therefore what types of analyses can and cannot be performed. Further issues come from the nature of the sample itself, what it is composed of, how it was procured, how and where it has been stored, and the curation and conservation measures employed. This all speaks to the potential level of geochemical and organic contamination it may have experienced, which can significantly affect the quality of the data.

This review aims to discuss the major techniques used in the study of fossil melanosomes and melanin. It is not meant to be an in-depth description of each type of analysis, but to give a brief introduction to how they work, the specific type of information they give, how destructive they are, and their relative usefulness for different sample types.
2.2 Morphological techniques

2.2.1 Scanning electron microscopy (SEM) and environmental scanning electron microscopy (ESEM)

Whereas light microscopy makes use of visible light to study small objects, electron microscopes use a beam of electrons. This provides much higher resolution, as the wavelength of an electron is on the order of 100,000 times smaller than that of visible light. There are many different ways that the electrons can interact with an object and each type can give different information. One of the most common interactions commonly used in the study of fossil melanin is that of secondary electron imaging. This is an emission type that is exhibited by virtually all materials and is therefore very useful for imaging a variety of sample types [73, p. 35]. The secondary electrons are produced because the incoming electrons have sufficient energy to displace valance electrons from the surface atoms of the sample. Secondary electrons are produced from a small interaction volume of the sample and therefore give good spatial resolution [62, p. 166]. These are primarily used to create images of the sample surface.

The number of secondary electrons released is usually different to the number of electrons incident on the surface from the beam, which causes a current to flow. If the sample is an insulator, as almost all fossils are, this current is unable to dissipate and a charge builds up on the sample [73, p. 35]. This not only distorts the image but can potentially damage the sample. In SEM this problem is generally overcome by coating samples in a fine layer of an electrically conductive element, usually gold or carbon. However, there are problems with this approach, namely that further geochemical analyses, by techniques such as energy dispersive x-ray spectroscopy, are compromised. The coating will emit its own x-rays under the influence of the electron beam and potentially interfere with the true signals of the sample. An alternative to
coating a sample is to use an environmental chamber, ESEM. Whereas SEMs operate under a vacuum, ESEM chambers are filled with a small amount of gas, usually water vapour [61, p. 572], enabling the excess charge to be dissipated by the interactions of the electrons with the water molecules [62]. However, the scattering of electrons by water molecules can lead to some image degradation [61, p. 572]. This technique is therefore also useful for samples that cannot be exposed to a vacuum.

The choice of using an SEM or an ESEM depends very much on the sample. If the only object is to image, and the sample is small enough to fit in the chamber then SEM is preferable as it can be easier to obtain higher quality images. However, if any further geochemical analysis is required then ESEM should be used as it does not require the sample to be coated, and is therefore non-destructive. Coating is not physically destructive but will result in a covering of carbon or gold on the sample. This will significantly affect any further geochemical studies, and is therefore not to be used if such analyses are required. Another issue is that of sample mounting. Samples are usually mounted on carbon tape coated aluminium stubs, requiring that the sample has a relatively flat surface to adhere to. More difficult samples can require specially developed holders. Samples with severe height gradients can also be somewhat difficult to image and in general flatter samples are preferred. The size of the sample will also be limited by the size of the chamber. The largest samples the authors have managed to scan have been approximately 15cm$^2$ and 3cm thick. The other option that has been employed is to remove small flakes of the sample from the area of interest and study those. Obviously, how destructive this approach is depends very much on the sample.

2.3 Geochemical techniques

For all geochemical techniques there are a few caveats to bear in mind before performing an analysis. First, it is not only the geochemical nature of the sample itself that is
CHAPTER 2. PAPER 1

of interest, but the geochemistry in comparison to that of its surrounding matrix. Without analysing a matrix sample it is unclear whether any of the chemical components of the sample are endogenous, originating from the organism itself, or have been transported into it over time from the surrounding environment (exogenous). By properly comparing analyses of a sample and its matrix such effects can be studied. Another consideration is whether or not the sample has been exposed to potential sources of contamination. These can be environmental, as discussed above, or the result of human interaction. There exists a particular risk for deleterious contamination when studying organic remains, as any direct human interaction can leave organic traces that could potentially be mistaken for geochemical remains. It is very important therefore to minimise contamination risks to the sample at the earliest possible opportunity. This involves avoiding touching the sample at all times, by wearing gloves and manipulating it with tools, rather than holding it directly. The method of sample containment and storage is also important, as plastics contain organic compounds which can also be a source of contamination. Therefore the sample should be stored in aluminium foil, or some other such metal container, not in plastic wrapping.

2.3.1 X-ray techniques

This family of techniques is based on the phenomenon of photoelectron or fluorescent x-ray emission from a sample when it is exposed to incident electromagnetic energy. A summary of the key differences and similarities of the techniques discussed here and their basic operation can be seen in Fig. 2.2.

2.3.1.1 Energy dispersive x-ray spectroscopy (EDS)

During EDS analysis, a beam of electrons is directed at a sample. These electrons interact with the surface atoms causing an inner shell electron to be dislodged and
Figure 2.2: A summary of the basic similarities and differences between the x-ray techniques discussed in this review. Other interactions of x-rays with matter, such as auger emissions, are possible but not shown here for simplicity.
emitted. The loss of this electron leaves the atom in an excited state, at which point an outer shell electron drops down to fill the hole in the inner shell. In doing so it loses energy which is emitted from the atom as an x-ray [73, p. 35] [61]. Determination of the elements present in the sample is done by measuring the energy of the x-rays, which are characteristic for each element. By measuring the number of each type of x-ray emitted per unit time a determination of the abundance of each element present can also be calculated [62]. As well as being able to determine elemental levels at a particular point with a sensitivity of 100s of parts per million (ppm), EDS can also be used to produce elemental maps of an area.

This technique is non-destructive, however as with ESEM and SEM there may be restrictions on sample size due to the size of the chamber of the equipment available. The topography of the sample can also strongly affect the accuracy of elemental quantitation. Unless a thin section can be made and a standard included for calibration, the quantitation of elements is useful for comparison of relative quantities rather than absolute. Whilst the use of a vacuum or partial vacuum allows the analysis and mapping of a wide range of elements, it is not possible to map them in different oxidation states.

2.3.1.2 X-ray photoelectron spectroscopy (XPS)

In XPS, a sample held in a vacuum is bombarded with x-rays which, when of sufficient energy, are capable of dislodging electrons from its surface atoms. This is known as the photoelectric effect, and the emitted electrons are also called photoelectrons. The incident energy must be above the binding energy of the electron it is trying to excite in order to eject it from the atom. Because each element produces a characteristic set of binding energy peaks, the measurement of the kinetic energy of the emitted electron and the incident energy can be used to determine which elements are present in the sample [13][115] [45, p. 3-5]. The number of electrons detected for a specific set of binding energies can also be used to determine the abundance of each element. The
2.3. GEOCHEMICAL TECHNIQUES

Technique can produce such data from point analyses [59] but can also be used to elementally map an area.

XPS is technically non-destructive, and can be used to analyse up to approximately 10nm below the sample surface. However, samples are again limited on size and must be on the order of a few cm$^2$ and ground flat.

2.3.1.3 Synchrotron rapid scanning x-ray fluorescence (SRS-XRF)

This technique is in principle very similar to EDS, involving the liberation of x-rays from an atom, as a result of an outer shell electron dropping to replace one removed from an inner shell by an incident energy beam. The energy source in this case though is synchrotron radiation, specifically high intensity x-rays, rather than electrons [83, p. 3-4]. However, the determination of elemental composition is the same, measuring the energy of the emitted x-rays and their abundance.

Synchrotron radiation is produced by the acceleration of electrons [129, p. 22] (Fig. 2.3). The electrons are produced by thermionic emission in an electron gun, a process by which heat is applied to a material until its binding energy is overcome causing it to release electrons or ions. They are then fired into a booster ring, where they are accelerated by a combination of high strength bending magnets and radio frequency electromagnetic radiation. Once up to a sufficient speed they are injected into the storage ring. As they are forced to travel in a curved path by bending magnets, they accelerate and thence give off electromagnetic radiation tangentially to their path of travel. This energy is directed along structures tangential to the storage ring (beam lines) where it is used for a variety of experiments [129, 157, 158]. Synchrotron radiation is incredibly useful partly because it is high intensity, but also because it has a broad but finely tunable energy spectrum, ranging from infrared to hard x-rays [158]. It is the finely tunable nature of the incident energy that allows its use for the analysis and mapping of different oxidation states (see section 2.3.1.4).
Figure 2.3: An example diagram of the basic features of a synchrotron. 1) Electrons are produced in an electron gun and fired into the booster ring. 2) The electrons are accelerated in a path around the booster ring by bending magnets and radio frequency (RF) cavities until they reach suitable velocities. 3) They are then injected into the main storage ring and kept flowing around it by bending magnets and RF cavities. 4) As the electrons are accelerated by the bending magnets they give off electromagnetic radiation at a tangent to their path, this energy is channeled into beamlines where it is manipulated and used for experiments.
This technique can be used to determine elemental composition by point analysis, but can also give elemental distribution. By moving the sample through the beam line by line (rastering), an elemental map of the sample can be constructed (Fig. 2.4). This technique was used to great effect by Wogelius et al. [159] who used it to demonstrate the presence of copper within the dark feathers of *Confuciusornis sanctus*. The presence of identical elemental distribution patterns on the part and counterpart of one of the specimens showed that the pattern was not the result of differential organic remains on each part as a consequence of their separation (Fig. 2.4). The copper chemistry was later shown to have the same coordination as that of copper in modern eumelanin (see Section 2.3.1.4).

The analysis is non-destructive and unlike EDS there are theoretically no limits to the sample size although the surface must be relatively flat with a relief of <10mm, however samples of ~70mm relief have been scanned. The major difference is that
as the sample is not held in an airtight container a vacuum cannot be imposed, and therefore there is a limit to the elements that can be measured. Heavier elements (i.e. calcium and above) are easily detectable as they are not significantly attenuated by the atmosphere. However, to analyse lighter elements some form of low density environment must be present around the sample. So far, this has been achieved by placing the sample within a helium gas chamber, but elements such as carbon are still too light to detect, and will be attenuated by the helium. The significantly higher intensity of the synchrotron beam compared to a standard electron beam technique means that trace elements can be detected with considerably more sensitivity, to levels of a few ppm.

2.3.1.4 X-ray absorption spectroscopy (XAS)

XAS is a blanket term that covers techniques which analyse the inner structure of the atoms of a sample by detecting their absorbance as a function of incident x-ray energy. Here we will discuss the two major branches of this analysis: x-ray absorbance near edge structure (XANES) and extended x-ray absorption fine structure (EXAFS; Fig. 2.5) spectroscopy.

XANES spectroscopy refers to the portion of the x-ray absorption spectrum approximately 50eV either side of the absorption edge (Fig. 2.5). The edge refers to the sharp peak on the spectrum caused by the sudden increase in absorption that occurs as the incident energy corresponds to the binding energy of the electron of a core shell [147]. XANES can give information about the electronic state of the absorbing atom [90]. The cut off point distinguishing XANES from EXAFS spectroscopy is somewhat arbitrary, but conventionally XANES spectroscopy stops at about 50eV above the edge [157] p. 233-234]. XANES spectroscopy can be used to interpret the oxidation state of an atom by looking at the position of the edge in comparison to that of a known standard. As the oxidation state becomes more positive it will take more energy to dislodge a core electron and therefore the edge will shift to a higher energy.
It is possible to map a sample for an element of a specific oxidation state \[41\], though this has not thus far been used to study fossil melanin.

EXAFS refers to the oscillatory structure of the x-ray absorption spectrum from approximately 50 to 1000eV above the edge \[166\]. It is the interference patterns produced by the backscattering of the emitted x-rays interacting with neighbouring atoms that produces the complex EXAFS patterns \[157, 166\]. EXAFS can therefore give information on the type and number of surrounding atoms, as well as the bond distances between to them. This has been used by Wogelius et al. \[159\] to demonstrate that the copper present in the feathers of *Confuciusornis sanctus* was coordinated in the same manner as copper in modern eumelanin (Fig. 2.6). Thereby establishing that the copper was most likely bonded to the remnants of the eumelanin in the original animal, and hence that copper could be used as a biomarker to indicate the presence of...
These techniques are usually performed with synchrotron radiation and require the x-ray beam to be directed at the sample at a single point for several minutes. As the radiation is very intense there is a chance of photoreduction, which can cause some minor discolouration of geological samples at that point as a result of the analysis. However the technique is generally considered non-destructive. These analyses are generally performed under the same set up as with XRF, hence there are similarly no theoretical limits on sample size. As with SRS-XRF mapping however, there are limitations on the elements that can be analysed due to the surrounding environment, with lighter elements requiring a helium environment or preferentially a vacuum.
2.3. GEOCHEMICAL TECHNIQUES

2.3.2 Electron paramagnetic resonance (EPR)

EPR is a technique used to study paramagnetic species (those that have an unpaired electron, including organic free radicals, inorganic molecules with unpaired electrons, metals and transition metal ions). Samples are held within a magnetic field and exposed to microwave frequency electromagnetic radiation. The frequency is generally held constant whilst the magnetic field strength is varied, and the absorption of electromagnetic energy is measured as a function of magnetic field strength. All electrons have an intrinsic angular momentum, also called spin, which gives rise to a magnetic moment. When exposed to an external magnetic field these moments can either align themselves parallel or antiparallel to the field. Each state has a characteristic associated energy and the electrons move between these two states by absorbing or emitting a photon leading to resonance. The magnitude of the energetic difference between the two states rises with increasing magnetic field strength [9, p. 9]. It is the pattern of electromagnetic absorption as a function of magnetic field strength that is characteristic of paramagnetic matter, and allows its identification. Though EPR is not technically destructive, the sample needs to be powdered before analysis. This technique was used by Barden et al. [6] and Glass et al. [59]; though found to be ineffectual in the former it was used in the latter to demonstrate the presence of eumelanin.

2.3.3 Fourier transform infrared spectroscopy (FTIR)

In FTIR, organic molecules and functional groups are identified by studying the interactions of infrared radiation with a sample. When the radiation is of a certain energy it can cause the bonds to resonate. The bonds can vibrate in a number of different ways, including stretching, twisting and wagging. The measurement of the absorption as a function of incident infrared energy by the bonds forms a characteristic spectrum that
Figure 2.7: FTIR point analyses of *Gansus yumenensis* show not only that the spectrum of the feather is different from that of the matrix, but that it is very similar to that of modern eumelanin. C=O represents a group from a carboxylic acid, C=O” represents a group from either a carboxylic acid or a ketone, NH” represents a group from a secondary amine. Both the feather and melanin spectra show the two C=O peaks as well as the broad OH peak. When one of the eumelanin peaks (the carboxylic acid C=O symmetric stretch at 1415 cm⁻¹) is mapped, it shows that it only occurs within the feather and not the matrix. (A) is diagrammatic representation of the area of the feather (B) mapped to guide the eye, (C) shows the map of the peak at 1415 cm⁻¹. Figure adapted from Barden et al. [6].

can be used to identify the parent molecule [133]. This energy level will vary depending on the types of atoms bonded together and their spatial coordination [29, p. 4]. Point analyses were used by Glass et al. [59], Barden et al. [6] (Fig. 2.7) and Lindgren et al. [96] to show the similarities between the various samples and modern eumelanin.

This is a non-destructive technique, though there are caveats to this. Whilst infrared analysis can be done on powdered samples, it can also be done using an attenuated total reflectance crystal (ATR). This crystal contacts the sample surface such that an evanescent wave of infrared radiation penetrates a few µm deep. The crystal can be rounded, in which case establishing good contact with the sample is difficult, or pointed and lowered onto the site of interest. The latter can leave small indentations on the sample if it is composed of a relatively soft material, but hard rocks will show no evidence of the analysis. This technique can also be used to collect absorption intensity maps in addition to single point analyses.
2.3.4 Pyrolysis gas chromatography mass spectrometry (Py-GCMS)

Chromatography techniques involve the separation of complex mixtures of compounds. They contain two phases: a stationary and a mobile phase. A sample is flushed through the system in the mobile phase and the individual components are differentially retarded based on their adsorption to the stationary phase [113, p. 43]. In gas chromatography the stationary phase is a solid, or liquid coated solid, and the mobile phase is a gas [143, p. 1-6]. Different stationary phases can be used depending on the type of compounds one is interested in studying. Once separated, the moieties (parts of molecules) are swept through to a mass spectrometer where they are bombarded with electrons that knock another electron from the moieties and turn them into ions. These ions are deflected around a curve with magnets which allows their mass to be calculated. Heavier ions require a stronger magnetic field in order to follow a set path than lighter ions. Ions are identified by their mass to charge ratio, and compounds are identifiable by their resultant ions [34, p. 1-4]. Modern machines generally use a slightly different version of this technique known as a quadrupole mass spectrometer. In such spectrometers 4 metal rods are held in two parallel pairs with a space running between them (Fig. 2.8). Opposite rods are electrically connected and a voltage is applied that is composed of a direct current and radio frequency component. Ions travel between these rods and at a given radio frequency ions of a given \( m/z \) ratio will be able to travel through the rods to the detector. If the radio frequency is not right for the ion travelling through its path will be unstable and it will crash into the rods and not be detected. Therefore you can scan for an ion of particular \( m/z \) value by holding the radio frequency steady, or vary it to scan for a range of \( m/z \) values [106, p. 51-57]. The use of a known amount of an internal standard can allow the calculation of the abundance of individual compounds from the GCMS spectrum. Otherwise the only abundance
In GCMS samples, volatile components are extracted into a solvent which is injected directly onto the separation column. However, there are situations where this is not possible. Firstly where the compounds of interest are in the non-extractable material, and secondly when the amount of the sample available for analysis is too small for extraction to be effective. In these instances we can instead use Pyrolysis. In Py-GCMS a sample is rapidly heated to a predetermined exact high temperatures in the absence of oxygen; this ensures that the sample is broken down to volatile components but not combusted. These components are then flushed with the carrier gas onto the GC column and the technique carries on as before [131, p. 150]. It is important to note that the products detected by Py-GCMS are the breakdown products of macromolecular complexes in the sample. Therefore the breakdown products must be looked at in combination to assess what was present in the original sample.

Py-GCMS is a destructive technique that uses a few mg of a powdered sample (Fig. 2.9). However, it is a very powerful technique for the identification of component organic compounds and has been used successfully in the study of fossil melanin [59].
Figure 2.9: Images of a *Gansus yumenensis* feather before and after a sample was removed for Py-GCMS, indicating the level of destruction to a sample in order to carry out the analysis [6].

Py-GCMS is also particularly useful for identifying complex macromolecular structures, which are easily broken down by intense heat. Problems arise when the sample of interest is preserved as a thin film, in which case the whole sample may have to be scraped off the block in order to gain enough material for analysis (1-5mg depending on concentration) [42]. For this reason if the technique is to be used at all, it is obviously performed last.

### 2.3.5 Alkaline hydrogen peroxide oxidation

This technique has only been used once thus far to identify eumelanin within fossil material [59]. It is based on a method that was developed in 1983 [77] for the rapid identification and quantification of both eu- and pheomelanin for the study of melanoma. The method has been improved and adapted over the years [78], but the basic idea has
remained the same. Originally permanganate oxidation of eumelanin was performed, which yields pyrrole-2,3,5-tricarboxylic acid (PTCA), a compound characteristic of eumelanin \[77\]. A value of eumelanin abundance can be calculated because natural eumelanin gives a constant yield of approximately 2% PTCA \[79\]. When applied to fossil samples \[59\], alkaline hydrogen peroxide oxidation of eumelanin yields not only PTCA but also pyrrole-2,3- dicarboxylic acid (PDCA), pyrrole-2,3,4-tricarboxylic acid (isoPTCA), and pyrrole-2,3,4,5-tetracarboxylic acid (PTeCA). All these products are characteristic of eumelanin and are separated and identified using high performance liquid chromatography, another chromatography technique where both the stationary and mobile phases are liquids. This technique is again destructive and requires a few mg of powdered sample for analysis, however it is currently the best and most definitive technique for showing the presence of melanin.

### 2.4 Discussion

When choosing a method for analysing melanin in fossil material the key considerations are the nature of sample itself, whether destructive sampling of any form is permissible, and the type of data you require. It is usually a matter of balancing the data you want with your sample limitations. Where possible, morphological techniques should be combined with geochemical ones in order to verify the presence of melanin with sufficient confidence. This is now the most common approach, and is necessary due to the structural similarities between melanosomes and bacteria.
### Table 2.1: A summary of the techniques discussed in this review

<table>
<thead>
<tr>
<th>Technique</th>
<th>Data type</th>
<th>Mapping functionality</th>
<th>Destructiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEM</td>
<td>Imaging</td>
<td>NA</td>
<td>Yes - coating required</td>
</tr>
<tr>
<td>ESEM</td>
<td>Imaging</td>
<td>NA</td>
<td>No</td>
</tr>
<tr>
<td>EDS</td>
<td>Elemental</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>XPS</td>
<td>Elemental</td>
<td>Yes</td>
<td>Yes - sample must be ground flat</td>
</tr>
<tr>
<td>SRS-XRF</td>
<td>Elemental</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>XANES</td>
<td>Elemental oxidation state</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>EXAFS</td>
<td>Elemental coordination environment</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>EPR</td>
<td>Paramagnetic species</td>
<td>No</td>
<td>Yes - sample powdered</td>
</tr>
<tr>
<td>FTIR</td>
<td>Organic and inorganic molecules, and functional groups</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>GCMS</td>
<td>Volatile organic compounds and moieties</td>
<td>No</td>
<td>Yes - sample extracted</td>
</tr>
<tr>
<td>Py-GCMS</td>
<td>Non-extractable organic compounds, moieties and macromolecular organic complexes</td>
<td>No</td>
<td>Yes - sample powdered</td>
</tr>
<tr>
<td>Alkaline oxidation with H$_2$O$_2$</td>
<td>Melanin breakdown products</td>
<td>No</td>
<td>Yes - sample extracted</td>
</tr>
</tbody>
</table>
2.4.1 Imaging

Electron microscopy is currently the best option for imaging melanosomes, as they are too small for conventional light microscopy. If the sample is small enough, or there is access to a large enough ESEM chamber, the whole sample can be analysed without destruction. This cannot always be guaranteed, and a good alternative is to carefully remove small pieces of the area of interest and analyse these [95][167]. Another consideration when using electron microscopy is whether to use SEM or ESEM. It is usually easier to obtain higher quality images from samples coated with either carbon or gold, but this precludes the use of these samples from further geochemical analyses.

Destructive analysis should be considered a relative term. Technically, an analysis is destructive when the sample is not completely destroyed during analysis and can thus be used in further studies. However, if you are attempting to study a precious palaeontological sample then anything that damages the specimen, however slightly, is considered destructive. This includes cutting off part of the sample to allow it to fit within certain pieces of equipment.

2.4.2 Geochemical analysis

2.4.2.1 Elemental analysis

EDS is generally found in association with SEM and ESEM machines and the set up is therefore primarily for imaging, with geochemical analysis being of secondary importance. It also has the same sample size limitations as SEM and ESEM, though sufficiently small samples can be analysed whole in an ESEM with no coating. XPS samples must be even smaller and the surface must be ground flat, whereas SRS-XRF analysis can be performed on samples of any size. However, SRS-XRF is not able to study the lightest elements, such as carbon, as there is no vacuum environment, while both EDS and XPS can. Though all three techniques offer quantitative as well as
2.4. DISCUSSION

qualitative analysis, that of EDS will suffer from reduced accuracy if the sample is not absolutely flat. EDS is therefore most useful for assessing relative rather than absolute abundances.

The spectrum of electromagnetic radiation produced by the excitation of matter using an electron beam (as in EDS) is made up of two parts: sharp fluorescence peaks and Bremstrahlung radiation. The latter is a type of background radiation, and is released by the electrons as they decelerate rapidly upon colliding with atoms. When the source of radiation is an x-ray beam (as in SRS-XRF and XPS) there is only fluorescence \[83,157\]. This means that EDS spectra generally suffer from more background radiation than XRF spectra, though this can be accounted for reducing background radiation is always preferable. EDS and XPS however, are both much more readily available analyses than SRS-XRF. The use of synchrotron techniques is generally much more costly and harder to access, though it is the only realistic option for dealing with large samples that cannot be destructively sampled, and has a greater sensitivity which allows trace elements to be more easily analysed.

Because melanin has been shown to chelate a variety of elements, such as calcium, zinc, copper and iron \[107\], finding out which elements are present in a fossil thought to contain melanin is only the first step in a full analysis. The next step is to ascertain whether that element is organically or inorganically bound, and therefore whether it may actually be part of the original organism or not; this is usually done with XANES and EXAFS spectroscopy. XANES spectroscopy is used to probe the oxidation states of elements and compare them to known standards, whilst EXAFS gives more detail on the specific geometry of the atom in relation to those surrounding it. Wogelius et al. \[159\] used such techniques to demonstrate that the copper found within the feathers of *Confuciusornis sanctus* had the same coordination environment as the copper found in the feathers of modern eumelanin. These techniques can only be conducted using synchrotron radiation, and therefore are not that easily accessible. However, their use
is vital in order to use elements to be able to verify the presence of melanin.

One of the key features that all of the above techniques provide is the ability to map elemental distribution. Whilst being able to see melanosomes under the electron microscope at a few key locations on a sample is good, being able to map elements that perform as a melanin biomarkers is especially useful. This is the major bonus of using SRS-XRF, as it is able to map the surface of large samples with no damage. Although mapping is also a feature of EDS and XPS, the maps will be of a much smaller area. ESEM imaging of melanosomes uses a fraction of a samples surface area, whereas SRS-XRF can cover up to 100% of the surface. This method helps to verify the data recovered using the structural approach, as well as allowing larger elemental distribution patterns to be seen.

2.4.2.2 Organic analyses

EPR has proved a very effective tool for identifying and distinguishing between eu- and pheomelanin in modern samples [11,92,136]. When first applied to fossil feathers it was unsuccessful [6], though this was most likely because the amount of material available for analysis was very low. When Glass et al. [59] applied it to a Jurassic cephalopod they were able to successfully resolve eumelanin. Though the technique requires a powdered sample and is therefore morphologically damaging, it is not chemically destructive and the sample can be re-used for example with Py-GCMS, the *de facto* organic compound separation and identification tool.

Py-GCMS has been used to demonstrate the presence of eumelanin breakdown products in the fossil record [59]. However, the quality and concentration of the sample is a big factor in the success of this analysis. Barden et al., were unable to resolve evidence of melanin using this technique on Early Cretaceous feathers [6]. Both Py-GCMS and FTIR attempt to identify melanin based on the presence of a range of
breakdown products, of which it is the group that is diagnostic rather than the individual moieties.

In contrast, alkaline hydrogen peroxide oxidation is based on a technique that is able to diagnose the presence of eumelanin by the presence of a single compound, pyrrole-2,3,5-tricarboxylic acid (PTCA) \[77\]. The technique has since been modified to include more compounds \[59\] but they are highly diagnostic of eumelanin and unlikely to be confused with other molecules \[79\]. However, it is both morphologically and chemically destructive.

Chemical mapping has been very useful in demonstrating eumelanin functional groups occurring solely within fossil material \[6, 159\], however neither EPR nor Py-GCMS offer any mapping functionality. As with SRS-XRF, whilst it is useful to be able to demonstrate melanin functional groups at certain points within a fossil sample, it is more conclusive if it can be shown that these groups occur only in the sample or in certain parts of the sample.

### 2.4.3 Sample condition

One of the most important considerations before starting any work on a sample is whether enough is known about its history. It is important to know how the sample was collected, where and how it was stored and whether anything was done to it, such as preservation with glues or casting. All these factors can affect the geochemical integrity of a sample. Where possible a sample should be collected cleanly, with workers wearing gloves to minimize contact with skin, no consolidants or glues used to repair any breaks, and stored in a metal bag or container. Plastics are to be avoided as they contain organic molecules which can contaminate the sample, as can transfer from human skin, hair etc. Sometimes it is difficult to ensure this and some samples may come from museum collections. Such samples must be treated with severe caution as they
are often treated with varnishes to ensure they are in the optimum condition for display. This often depends on the age of the sample, with older fossils much more likely to have been treated this way than more modern specimens. This can cause problems with organic and inorganic analyses as the varnishes often contain metals as well as organic molecules. It is also important to note that anything that comes into contact with the surface of a sample can geochemically contaminate it, this includes such processes as molding. It is easy to think that just because nothing is visibly left on a sample after such techniques that the fossil is fine to be subject to geochemical analysis. However, anything that contacts the fossil will most likely compromise its geochemistry. If such a sample is all that is available the techniques used to study it must take account of such activity and where possible try to compensate for it, by analysing similar samples to correct for traces of preservatives for example. For this reason it is also imperative that the geochemistry of the fossil be compared to the enclosing matrix, this is another way to account for potential damage by conservation measures.

2.5 Conclusion

In order to be confident of the identification of melanin in the fossil record a combined morphological and geochemical analytic approach is required. The question then becomes which techniques are best suited to analyse your sample.

Imaging is currently limited to electron microscopy. The type and size of the equipment available will thus determine whether the sample can be imaged whole, or whether small pieces will have to be removed for imaging. If the sample is too precious to be destructively analysed at all then a combination of EDS, SRS-XRF, XANES and EXAFS spectroscopy, and ATR FTIR is the most useful geochemical approach. If the rock is too soft to allow mapping by FTIR, single point analyses can be taken which
are minimally damaging, if at all. This gives a good indication of the elemental composition and distribution within the sample, oxidation state and coordination of those elements to determine whether they are organic or inorganic, the organic functional groups in the sample, and potentially their spatial distribution as well.

If some destruction is permitted then expanding the analyses to include some combination of Py-GCMS, EPR, or alkaline hydrogen peroxide oxidation makes for a very robust study. As both Py-GCMS and alkaline hydrogen peroxide oxidation are destructive, a choice may have to be made as to which one to use if there is little material available for analysis. This often occurs if a sample is preserved as a thin carbonaceous layer overlaying the matrix; when removed the powdered sample is often only a matter of a few mg. If the sample is very concentrated then 1mg per analysis may be sufficient, but if not it is more advisable to pick one technique and use all the material for a single analysis in order to ensure efficacy. This is obviously not ideal but is a frequent occurrence when dealing with precious palaeontological samples. Completely destructive techniques such as Py-GCMS and alkaline hydrogen peroxide oxidation should, of course, be performed last. Further analyses that have been used to study fossil melanin but that have not been covered here include nuclear magnetic resonance spectroscopy (NMR) [59] and time of flight secondary ion mass spectroscopy (ToF-SIMS) [96]. NMR is similar to EPR, though it uses the spin of nuclei rather than electrons; and ToF-SIMS uses an ion beam to liberate ions from the surface of a sample which are then measured and identified according to the time it takes them to reach the detector.

The best analytical approach to the study of melanin in the fossil record is the combination of not only morphological and geochemical techniques, but multiple geochemical techniques. Each gives a slightly different type or quality of data, and careful thought should be applied to selecting those most appropriate for the sample in question.
Chapter 3

Paper 2: Morphological and geochemical evidence of eumelanin preservation in the feathers of the early Cretaceous bird, *Gansus yumenensis*

This chapter contains the following published paper:

Morphological and Geochemical Evidence of Eumelanin Preservation in the Feathers of the Early Cretaceous Bird, *Gansus yumenensis*

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Abstract

Recent studies have shown evidence for the preservation of colour in fossilized soft tissues by imaging melanosomes, melanin pigment containing organelles. This study combines geochemical analyses with morphological observations to investigate the preservation of melanosomes and melanin within feathers of the Early Cretaceous bird, *Gansus yumenensis*. Scanning electron microscopy reveals structures concordant with those previously identified as eumelanosomes within visually dark areas of the feathers but not in lighter areas or sedimentary matrices. Fourier transform infrared analyses show different spectra for the feathers and their matrices; melanic functional groups appear in the feather including carboxylic acid and ketone groups that are not seen in the matrix. When mapped, the carboxylic acid group absorption faithfully replicates the visually dark areas of the feathers. Electron Paramagnetic Resonance spectroscopy of one specimen demonstrates the presence of organic signals but proved too insensitive to resolve melanin. Pyrolysis gas chromatography mass spectrometry shows a similar distribution of aliphatic material within both feathers that are different from those of their respective matrices. In combination, these techniques strongly suggest that not only do the feathers contain endogenous organic material, but that both geochemical and morphological evidence supports the preservation of original eumelanin pigment residue.


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Introduction

Melanin is a common chemical pigment found ubiquitously throughout the natural world [1,2]. As well as having a key role in display [3] and camouflage [1], melanin also provides UV protection [4,5], thermoregulation [1], sequestration of potentially toxic metal ions [6,7] and acts as a free radical sink [5,6]. Modern studies of animal pigmentation provide insights to behavior, life history and evolution [3] that have typically been thought to be beyond the realm of palaeontology. Recent studies of fossil feathers [8–12] and a theropod dinosaur integument [8] however have shown evidence of microscopic structures that have been interpreted as melanosomes, the melanin containing organelles in extant organisms. This interpretation is based on cogent arguments regarding size, form [8–12], spatial arrangement [9], organisation [10] and apparent embedding within soft tissue [10] for the presumed melanosomes. Whilst these arguments are somewhat persuasive, the problem remains that bacteria are capable of replicating each one of these characteristics. This is a particular problem when trying to identify pheomelanosomes; their spheroid shape and lack of distinctive spatial organization make them even harder to distinguish from similarly sized coccoid bacteria. In addition, many modern bacteria are not only the same shape but also the same size as purported eumelanosome structures. Rod shaped bacillus bacteria range in length from 0.5 to 20 µm [13], and the average diameter of coccus bacteria is 0.5 to 1.5 µm [14]. In a previous investigation on a fossil feather such structures were indeed thought to be bacteria covered with a glycocalyx (Figure 1a[15]), however in light of the recent studies on fossil feathers this assessment has been re-evaluated and the structures have since been identified as melanosomes surrounded by the remains of decaying β-keratin fibres [9]. In addition, some images of purported melanosomes clearly display the alveolus structure that is characteristic of microbial mats (Figure 1b,c [8]) [16,17]. The fact that melanosomes are only found within darker areas of fossilized feathers is probably not coincidental, though it is possible that this pattern could be replicated by bacteria, as there are still conflicting reports about whether feather degrading bacteria preferentially feed on melanin or not [18–20].

This study combines both morphological (imaging) and organic geochemical techniques to analyse feathers from the early Cretaceous Chinese bird *Gansus yumenensis*. The results are compared to those of extant feathers and a standard melanin sample to try and determine the extent of organic preservation.
within the fossil feathers and whether any geochemical evidence of melanic or bacterial biomarkers can be identified.

Materials and Methods

Samples

The two fossil feathers are attributed to the Lower Cretaceous (early Aptian/Aptian, 125 to 112 Ma) amphibious bird *Gansus yumenensis* [21,22]. Both specimens (MGSF318 and MGSF317, Fig. 1A and B respectively) were discovered in the Xiaogou Formation near Changma in Gansu Province of northwestern China [21]. Originally from the Gansu Geological Museum, the fossils are currently housed in the collections of the School of Earth Atmospheric and Environmental Sciences, University of Manchester, and will be returned to China after research. Both specimens have had minimum handling, MGSF317 has not been touched with any glue or consolidants, there is a thin layer of consolidant (cf polyvinyl butyral) on the underside of the matrix in MGSF318 though nothing compromising the chemical integrity of the fossil itself. Both specimens were handled with gloves at all times and stored in foil envelopes within sealed bags to minimize contamination. Samples of extant black (Marabou Stork (*Leptoptilos crumeniferus*)) and white (White-naped Crane (*Grus vipio*)) feathers were supplied from Birdland in Gloucestershire. Natural melanin from *Sepia officinalis* was supplied by Sigma-Aldrich. All fossil material, extant samples and standards were stored in isolation of one another, reducing possible cross-contamination.

Sample preparation and analyses

All samples were analysed by variable pressure field emission gun scanning electron microscopy (VP-FEG-SEM) with energy dispersive X-ray spectrometry (EDS), Fourier transform infrared spectroscopy (FTIR), electron paramagnetic resonance (EPR) and pyrolysis gas chromatography mass spectrometry (Py-GCMS). VP-FEG-SEM provides microscopic analysis of the morphology of the samples and allows the visual identification of potential melanosomes without the need for coating the sample or subjecting it to high vacuum conditions as would be the case with traditional...
electron microscopes. EDS, FTIR, EPR and Py-GCMS are all geochemical techniques that give information about the elemental composition, functional groups, organic free radicals and involatile macromolecular complexes, respectively, of a sample. EPR and Py-GCMS are both destructive techniques requiring additional sample preparation of the extant feathers and fossil material. Extant feather samples were finely chopped into small pieces and were prepared for FTIR analysis by cleaning in distilled water in an ultrasonic bath for 10 minutes 3 times and then air-dried without heating. Powdered fossil material was obtained by carefully scraping fossil and matrix samples from the main block under negative pressure from a flow hood. Sterile scalpels were used in both cases and gloves were worn at all times.

Scanning electron microscopy (SEM)/Energy dispersive X-ray spectrometry (EDS)

All specimens were analysed using variable pressure-field emission gun-scanning electron microscopy (VP-FEG-SEM). Extant specimens were prepared according to the technique in Zhang et al. [8], though they were not coated, and then imaged using an FEI XL30 instrument. Secondary electron images were taken at 10.0 keV, at a working distance of 10 mm. Elemental composition was analysed using an EDAX energy dispersive X-Ray spectrometer (EDS). Fossil specimens were analysed using a Zeiss Supra40VP instrument. Secondary electron images were taken at low accelerating voltage (2–3 keV) with a working distance of 9–10 mm. Standardless EDS spectroscopy was carried out using an Oxford Instruments machine to examine elemental composition via point analyses. All EDS spectra were taken at an accelerating voltage of 15 keV, a working distance of 15 mm, and were collected for 100 s. Errors on the standardless EDS analyses are estimated to be approximately 30% of the value reported.

Fourier transform infrared spectroscopy (FTIR)

Spectra from the fossil specimens were taken using a Spotlight 400 Perkin Elmer FTIR Imaging System (wavenumber range 4000 to 800 cm$^{-1}$). Point analyses and maps were collected in Attenuated Total Reflectance mode (ATR) with a 20$\times$20 µm aperture and 4 cm$^{-1}$ resolution; all final spectra were an average of 10 scans and were background subtracted. Maps are composed

Table 1. EDS determined elemental composition of extant and fossil samples show in weight percent.

<table>
<thead>
<tr>
<th>Weight percent (Wt. %)</th>
<th>C</th>
<th>O</th>
<th>F</th>
<th>Na</th>
<th>Mg</th>
<th>Al</th>
<th>Si</th>
<th>P</th>
<th>S</th>
<th>Cl</th>
<th>K</th>
<th>Ca</th>
<th>Ti</th>
<th>Fe</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marabou stork (Leptoptilus crumeniferus)</td>
<td>41.01</td>
<td>29.59</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.045</td>
<td>0.08</td>
<td>0.08</td>
<td>0.07</td>
<td>0.05</td>
<td>0.04</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>White-naped Crane (Grus vipio)</td>
<td>58.83</td>
<td>27.17</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.056</td>
<td>13.76</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>MGSF317 feather – dark part</td>
<td>42.74</td>
<td>31.83</td>
<td>0.08</td>
<td>0.05</td>
<td>0.42</td>
<td>0.036</td>
<td>0.076</td>
<td>0.10</td>
<td>0.08</td>
<td>0.42</td>
<td>0.01</td>
<td>0.48</td>
<td>0.03</td>
<td>0.84</td>
<td>0.67</td>
</tr>
<tr>
<td>MGSF318 feather – dark part</td>
<td>18.99</td>
<td>42.94</td>
<td>–</td>
<td>0.32</td>
<td>0.08</td>
<td>0.057</td>
<td>0.031</td>
<td>0.07</td>
<td>0.05</td>
<td>0.06</td>
<td>0.10</td>
<td>0.16</td>
<td>0.20</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>MGSF317 feather – light part</td>
<td>24.93</td>
<td>33.22</td>
<td>0.02</td>
<td>0.06</td>
<td>0.25</td>
<td>0.067</td>
<td>0.14</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
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<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>MGSF318 feather – light part</td>
<td>05.35</td>
<td>46.51</td>
<td>0.18</td>
<td>0.09</td>
<td>0.40</td>
<td>0.04</td>
<td>0.07</td>
<td>0.02</td>
<td>0.00</td>
<td>0.60</td>
<td>0.10</td>
<td>0.30</td>
<td>0.12</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>MGSF317 matrix</td>
<td>01.88</td>
<td>44.37</td>
<td>0.15</td>
<td>0.40</td>
<td>0.07</td>
<td>0.09</td>
<td>0.03</td>
<td>0.02</td>
<td>0.05</td>
<td>0.25</td>
<td>0.01</td>
<td>0.26</td>
<td>0.10</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>MGSF318 matrix</td>
<td>04.91</td>
<td>46.02</td>
<td>0.25</td>
<td>0.40</td>
<td>0.63</td>
<td>13.34</td>
<td>0.06</td>
<td>0.09</td>
<td>0.02</td>
<td>0.12</td>
<td>0.78</td>
<td>0.43</td>
<td>0.02</td>
<td>0.13</td>
<td>0.13</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0025494.t001
of point analyses taken in an 18 × 20 grid with 10 spectral scans per point. Extant samples were analysed using a Bio-Rad 600–IR system (wavenumber range 4000 to 900 cm⁻¹). Spectra were taken with a 2 cm² aperture and 4 cm⁻¹ resolution; final spectra were an average of 16 scans (20 for natural S. officinalis melanin). All spectra and maps were background subtracted. Organic peak assignments were made using the Bio-Rad KnowItAll Informatics System 8.2 Multi-Technique database. Inorganic peaks were assigned using reference mineral spectra from Russell et al. 1987 [23].

Electron paramagnetic resonance (EPR)

X-band (9 GHz) Electron Spin Resonance spectroscopy was carried out at room temperature on an EMX Spectrometer using a High Q X-band HSW0541 cavity. All sample tubes were pre-fumaced at 400°C and stored in foil in order to avoid organic contamination. Due to the small samples sizes only MGSF318 was analysed with EPR spectra were collected for 125 scans. Power was varied to maintain line shape and to avoid saturation. Spectra were recorded with an attenuation of 24 dB (equivalent to 0.8 mW). All spectra were background subtracted. Magnetic field correction used the Bruker standard Strong Pitch (g = 2.0028) and the measured frequency was recorded by an internal frequency counter in the microwave bridge. g values are calculated by dividing the product of Planck’s constant (h) and the frequency of the incident radiation (v) by the product of the Bohr magneton (μB) with the magnetic induction of the magnetic field at resonance (μBr): g = (hv)/[μBμBr].

Pyrolysis gas chromatography mass spectrometry (Py-GCMS)

Extant feather, fossil and melanin samples were analysed by normal flash pyrolysis GCMS. The samples were pyrolysed using a CDS (Chemical Data Systems) 5200 series pyroprobe pyrolys unit by heating at 600°C for 20 s to fragment macromolecular components. These fragments were then analysed using an Agilent 7890A gas chromatograph fitted with a HP-5 fused column (JW Scientific; 5% diphenyl-dimethylpolysiloxane; 30 m, 0.32 mm i.d., 0.25 μm film thickness) coupled to an Agilent 5975C MSD single quadrupole Mass Spectrometer operated in electron ionization (EI) mode (scanning a range of m/z 25–650 at 1 scan s⁻¹ with a 4 minute solvent delay; ionization energy 70eV). The pyrolysis transfer line and injector port temperatures were set at 350°C, the heated interface at 280°C, the EI source at 230°C and the MS quadrupole at 150°C. Helium was used as the carrier gas and the samples were introduced in split mode in a ratio of 2:1. The oven was programmed from 40°C (held for 4 minutes) to 320°C at 4°C minute⁻¹ and held at this temperature for 5 minutes. Compounds were identified by comparison with spectra from the literature.

Results

SEM/EDS

Elongate mouldic structures 1.20 μm long and 0.26 μm wide (an average of 10 measurements, standard deviations 0.17 and 0.02 respectively) were observed in the extant Marabou stork feathers arranged regularly within fractured surfaces (Fig. 1C). No such structures were observed in the feathers of the extant White-naped Crane (Fig. 1D). Similar structures 1.66 μm long and 0.49 μm wide (an average of 10 measurements, standard deviations 0.16 and 0.07 respectively) were observed in dense patches on the dark areas of both fossil feathers (Fig. 1E–H), no such structures were seen in the light parts of the feathers or the matrix (Fig. 2). EDS analysis showed that the extant feathers are predominantly composed of carbon, oxygen and sulphur, though a significant amount of calcium (14.3 wt%) occurs in the Marabou stork feather but not in the White-naped Crane feather (Table 1). Analysis of the fossil feathers of MGSF317 and MGSF318 showed a much higher proportion of carbon in the dark areas (42.3 wt% and 19.0 wt% respectively) compared to the lighter areas (25.0 wt% and 3.4 wt% respectively) and the matrix (19.9 wt% in and 4.9 wt% respectively), which was mostly composed of silicon and oxygen as would be expected for sediment dominated by silicate minerals (Table 1). The calcium content of the dark area of the feather in MGSF317 is over twice that measured in lighter areas (10.5 wt% and 4.6 wt% respectively) however the opposite pattern was observed in the MGSF318 (Table 1).

FTIR

Figure 3 presents infrared spectra of an extant feather (A), a eumelanin standard (B), a fossil feather (C) and its sedimentary matrix (D). The infrared spectra obtained from the fossil feathers were clearly different from those taken within the surrounding matrix. Because the spectra of both fossil specimens were so similar, only those of MGSF317 are shown in figure 3. The matrix showed only the presence of an inorganic silica band (Fig. 3D), whereas the fossil feathers also showed carboxylic acid, ketone, hydroxyl and potential secondary amine peaks (Fig. 3C, Table 2). The asymmetric C = O stretch and ketone C = O stretch are convolved in a single broad peak. We are also aware of the likelihood that additional C = C aromatic ring vibration signals may be convolved with both this peak and that of the C = O
symmetric stretch [24]. These peaks all occur in the _Sepia officinalis_ melanin spectra and the responsible functional groups are clearly seen in the chemical structure of eumelanin (Scheme 1b [25]). In addition, when the _Sepia officinalis_ melanin spectrum (Fig. 3B) is subtracted from that of the fossil feathers (Fig. 3C) the only remaining peak is the inorganic silica band, i.e. all other peaks except atmospheric CO₂ are accounted for by those of _Sepia officinalis_ eumelanin (Fig 3E). A map of the absorbance of the carboxylic acid C = O symmetric stretch at 1622 cm⁻¹ in MGSF317 (Fig. 4) shows good correlation of infra-red absorbance with visually dark areas of the feather. Melanin peaks were not resolvable in the spectrum of the extant Marabou Stork feather (Fig. 3A), this we attribute to the intense amide peaks of β-keratin overwhelming the signal from the melanin. The fossil feathers showed only extremely weak evidence of these amide peaks, seen as a subtle shoulder on the left of the carboxylic acid/ketone C = O peak, indicating that little IR reactive β-keratin has been preserved in comparison to the pigment. Details of peak assignments are given in Table 2.

**EPR**

The spectrum of the _Sepia officinalis_ melanin showed a clear signal (Fig. 5) with a g value of 2.00421. This value fits well with the σ-semiquinone eumelanin signal identified in previous studies (g value between 2.0044 and 2.0030 [26]). A similar signal was present in the extant Marabou Stork feather (g value of 2.00418) and one was also detectable in the extant White-naped Crane feather (g value of 2.00357) but only in trace amounts, as seen in previous studies [27]. Due to limited sample volumes only the MGSF318 fossil could be analysed with EPR. The spectra from both MGSF318 matrix and feather samples showed an organic free radical signal as well as characteristic manganese hyperfine peaks (not shown). The calculated g value of the fossil feather sharp signal (2.00353) fell within the demonstrated σ-semiquinone

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Bond</th>
<th>Wavenumber range (cm⁻¹)</th>
<th>Intensity</th>
<th>Mode of vibration</th>
<th>MGSF318</th>
<th>MGSF317</th>
<th>Marabou stork feather (black)</th>
<th>White-naped Crane feather</th>
<th>Sepia melanin standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicon compounds (Si-O)</td>
<td>Si-O</td>
<td>1100–1000</td>
<td>Strong</td>
<td>Stretching</td>
<td>—</td>
<td>X</td>
<td>X</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Si-O</td>
<td>990–945</td>
<td>Strong</td>
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<td>Asymmetric Stretching</td>
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<td>2882–2862</td>
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<td>Symmetric stretching</td>
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<td>C = O</td>
<td>1680–1630</td>
<td>Strong</td>
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<td>—</td>
<td>—</td>
<td>X</td>
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doi:10.1371/journal.pone.0025494.t002
g value range characteristic of eumelanin, whereas that of the matrix signal (2.00485) did not.

Py-GCMS Analysis of the natural melanin sample produced a chromatogram containing mostly benzene, benzenenitrile, pyrrole and its methyl derivatives, phenol, phenol-4-methyl, styrene, indole and 3-methylindole (Fig. 6), comparable to previous studies [28,29]. The total ion chromatograms of both fossil feather and matrix samples were dominated by a strong aliphatic signal and a series of benzene derivatives (not shown). Furthermore, comparison of partial m/z 57 and 55 mass chromatograms showed similar distribution patterns of n-alkanes/n-alkenes for both fossil feathers (a range from C9 to C25 with maxima at C12, C17 and C20, (Fig. 7A–B)), which differed from that of their corresponding sedimentary matrices (Fig. 7C–D). The abundances of n-alkanes/n-alkenes were higher in the matrices than the fossil feathers by a factor of 4 for MGSF317 and a factor of 2 for MGSF318. The MGSF318 matrix sample showed a bimodal distribution pattern dominated by peaks at C24 and C19, though the bimodal pattern is less pronounced; here carbon chain lengths again range from C11 to C28 (Fig. 7D). Both the matrix and feather samples of MGSF317 and MGSF318 contained benzene, toluene and styrene though the distribution patterns again varied between the feathers and their respective matrices (not shown).

Discussion

SEM analysis of the *Gansus yumenensis* feathers demonstrates the presence of elongate structures that have been identified as eumelanosomes (Fig. 1E–H) after comparison with analysis of extant feathers (Fig. 1C) and previous studies [8–12]. These structures are confined to the visually darker areas of the fossil feather and were absent from both lighter areas and the matrix.
could be a consequence of a similar process though more experimental work is needed to confirm this. The signal in the matrix samples is most likely to come from other organic material present within the sediment, such as from plant material [31,32]. No eumelanin pyrolysis products were observed in the chromatograms of the fossil feathers, nor indeed in the chromatograms of the extant feathers. In the case of the fossil feathers either the levels are below the detection limits of the instrument, or they are simply not detectable by this method in a specimen of this age. In the extant feathers the chromatograms showed mostly lipid and protein signals which likely overwhelmed any from the breakdown of eumelanin. The EPR data shows the presence of organic free radicals signals in both the MGSF318 matrix and the feather however these have measurably different g-values. The fossil feather has a g-value in accordance with that of the σ-semiquinone signal seen in the natural melanin standard whereas that of the sedimentary matrix does not, consistent with the presence of eumelanin within the fossil and not in the matrix. However we are cautious due to the low separation of these values and believe that on its own this does not constitute enough evidence to unequivocally identify eumelanin derived material within the fossil.

A key issue in this type of investigation is the problem of contamination, either ancient, modern or both. No bacterial hopanoid biomarkers were detected by the Py-GCMS analysis making contamination from modern bacteria unlikely, however the level of organic matter in the samples was too low for contributions from ancient bacterial biofilms to be detected. FTIR analysis revealed no bands characteristic of modern bacteria including CH bending from fatty acids or P-O-C and P-O-P stretching from phospholipids, ribose and phosphate chain pyrophosphate [33]. The dominant organic signals from bacteria are generally protein bands (amides I, II and III, 1655, 1546 and 1240–1235 cm$^{-1}$ respectively) [33,34]; whilst no distinct amide peaks are observed in the fossil feathers we cannot rule out the possibility that they occur in trace amounts due to the observation of a slight shoulder at 1660–1674 cm$^{-1}$. However, amide bands may also be caused by the presence of keratin breakdown products derived from the feathers as recently demonstrated for fossil skin [32]. There are some types of bacteria that produce melanin [35], though we are currently unaware of any that are commonly associated with feathers; there are also some forms of soil fungi that produce melanin [36]. Though we see no evidence of either in the morphological or geochemical analyses we cannot definitively discount the possibility of their presence. Unfortunately due to the small sample sizes the number of destructive geochemical techniques we could perform were limited, this restricted the amount of information that could be gleaned from the sample. More investigation into the decomposition of fungi, bacteria and melanin would be useful and is the subject of future work. Human handling might be another potential source of contamination; however in this study every care was taken to avoid contamination by handling the samples with gloves, storing them in foil packets within a sealed plastic bag and also by taking the samples for EPR and Py-GCMS analysis under a fume hood. There is no chemical evidence of human contamination, no intact protein signals were detected by the FTIR and no cholesterol signals were visible in the Py-GCMS chromatograms. However yet again the most convincing argument against contamination, particularly human contamination, is not only that eumelanic functional groups faithfully replicate the darker areas of the feather, but also that the aliphatic signal is distinctly different in the matrix and the feathers. Our results are also in accordance with recent work demonstrating the use of trace metals, especially organically bound copper, within fossilized soft tissue as a biomarker for eumelanin presence within Gansus yumenensis [37]. The issue of contamination at the time of deposition by melanosomes leaching from decomposing skin has previously been raised [38], but the fact that the feathers are both isolated from any other soft tissue makes this highly unlikely. We note that there are possible transfer mechanisms even for an isolated feather, for example the feather could have been dislodged from a rotting carcass just before burial. However it is much more likely, given the evidence presented above, that the eumelanin was endogenous.

Of the methods used in this study, only FTIR was both successful and non-destructive. Both EPR and Py-GCMS required the complete destruction of the samples for analysis, thus precluding the specimens from further future analysis. FTIR was able to identify eumelanic functional groups within the feathers and was also able to map their spatial distribution, clearly
demonstrating their occurrence solely within the feather and not the matrix. Further organic geochemical analyses and studies into actualistic taphonomy must be done to help understand the preservation of organic material within fossil bacteria, this will assist in determining the extent of bacterial activity at the time of deposition.

Conclusion
Current studies of purported melanosomes in fossil soft tissue solely rely upon morphological data to support their conclusions. Morphological evidence alone is insufficient to identify such structures with confidence, as has been found in similar studies of supposed Archean bacteria; "we caution against identifying microstructures as biological in origin without a full morphological and geochemical assessment" [39]. Here, in addition to morphological analysis, three geochemical analytical techniques were applied to G. yumenensis feathers to examine the preservation of any endogenous pigmentation. Structures were identified within the fossil feathers concordant with eumelanosomes comparable to structures seen in extant feathers as well as to previous studies of fossilized soft tissue. Infrared analysis strengthens this conclusion by demonstrating the presence of characteristic eumelanin functional groups within the fossil feather but not in the matrix, and these groups are shown to be spatially resolved with the feather material. EPR analyses proved unable to confidently distinguish characteristic melanin free radical signals within the fossil material despite showing the presence of different organic signals between feather and matrix. Pyrolysis GCMS clearly showed a difference between the organic material present within the fossil feathers as compared to their sedimentary matrices. There was also a clear similarity between the spectra of the two fossil feathers, implying that they may have a characteristic aliphatic signature. The combination of these techniques strongly suggests that there is both morphological and geochemical evidence for the preservation of original endogenous organic material and eumelanin pigment residue within the 105–115 million year old fossil feathers of Gansus yumenensis.

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Author Contributions
Conceived and designed the experiments: RAW HEB BevD. Performed the experiments: HEB NPE. Analyzed the data: HEB RAW BevD. Contributed reagents/materials/analysis tools: DL PLM. Wrote the paper: HEB RAW BevD PLM.

References
Evidence of Eumelanin Preservation in Fossil Birds


Chapter 4

Paper 3: Geochemical analysis of a 25 million year old tadpole and its use to determine the presence of melanin

This chapter contains the following paper which is in preparation to be submitted to the journal *Geochimica et Cosmochimica Acta*

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Abstract

Many exceptionally preserved soft tissue fossils have long been thought to be the product of preservation by bacterial autolithification, based largely on the presence of mineralised spherical or elongate bodies on their surface. This conclusion has recently been challenged by studies of similar fossils citing morphological and geochemical evidence that these structures are more likely to represent fossilised melanosomes, pigment-containing organelles. We studied a 25 million year old fossil tadpole from the Oligocene Enspel Formation in Germany which displays such small spherical bodies on its surface. Pyrolysis gas chromatography mass spectrometry (Py-GCMS) shows evidence of bacterial biomarkers in the matrix but not the tadpole, and infra-red spectrometry shows a difference in chemical inventory. This strongly indicates that not only are the organic remains in the tadpole original to the organism and not the result of external contamination, but also that they are very unlikely to be bacterial. In addition, Py-GCMS also shows the presence of a key plant biomarker in the matrix but not the tadpole, suggesting that, contrary to the previously held notion, such carbonaceous fossils do not develop their black colour from the incorporation of plant material. The tadpole also shows high levels of zinc in its soft-tissues, a metal known to chelate to both eu- and pheomelanin. X-ray absorption near edge structure spectroscopy (XANES) indicates that this zinc is organically bound, and shows significant similarities to the zinc bound in pheomelanin in modern human hair. Bacteria are also known to take up zinc, and though we have no geochemical evidence for bacteria in the tadpole, its presence cannot be ruled out. More work is needed to determine the precise coordination of zinc in the fossil tadpole and compare it to that of zinc in pure eu- and pheomelanin.
4.1 Introduction

Bacterial autolithification as a mechanism for the exceptional preservation of fossil organisms is a well established taphonomic model that has been studied across various taxonomic groups and geological formations [16, 58, 149, 156, 165]. The process is particularly characteristic of the Oligocene Enspel Formation of Germany and was first described by Wuttke [30, 164] after the observation of coccoid or bacillus cell-like structures within fossilised soft tissues. It involves the mineralization of the cells in a bacterial mat that has covered a carcass [14] by the precipitation of either siderite, utilizing iron from the surrounding sediments [52, 165], or apatite, where the phosphorus from the decaying organism has become trapped by the mat [15, 16]. The typically black colour of the preserved soft tissues is thought to derive from the subsequent incorporation of organic matter from plants [52] to form a carbonized film.

In recent years the structures thought to be fossil bacterial cells have been newly identified as fossilised melanosomes, the membrane bound organelles that constrain the synthesis of the chemical pigment melanin. This reinterpretation is based on a growing number of studies citing both morphological [24, 27, 94, 95, 150, 151, 167] and geochemical evidence [6, 59, 96], including a study on the use of copper chelated to eumelanin as a biomarker for its presence [159]. Extant melanosomes exist in both spherical and elongate forms depending on the type of melanin they contain [99], though this is not always the case [99]. Thus far there have been no such studies on fossilised amphibians though some fossil tadpoles from the Enspel Formation have dense layers of sub-micrometer-sized spherical bodies within their soft tissues [149]. In this study we geochemically analyse a fossil tadpole from the same formation to look for biomarkers indicative of the preservation of melanin. Such analyses will not only give great insight into the taphonomy of the organism [67, 68] but can also help to improve our understanding of its original biochemistry.
4.2 Materials and methods

4.2.1 Samples and handling

The fossil tadpole is composed of both part (fs003a) and counterpart (fs003b; Fig. 4.1a-b). The samples were collected in the summer of 2011 from the black shale layers, section S12 of the Enspel Formation by Dr. M. Wuttke, and were wrapped in aluminium foil to reduce contamination.

The tadpole appears to be broken in half with the end of the tail curling round to meet the head (Fig. 4.1a-b). Preservation is not good enough to allow species identification with any confidence, and also makes assignment of growth stage difficult. In addition, though no legs are apparent the tadpole lies on the edge of the block leaving the possibility that either they became disassociated from the main organism or were separated from the main body during collection. As measured from the part, fs003a, the tadpole is 67mm long including soft tissues. On comparison to other fossil tadpoles [110,127] it is likely to be between Gosner stages 36 and 37 [64] due to the size and ossification of the fronto-parietals.

Samples of extant black headed grosbeak chest feathers (Pheucticus melanocephalus) were obtained from partially intact specimens of the Manchester museum, and a blonde human hair sample was donated by a member of staff. These provided modern pheolemelanin references. Zinc standards used for comparison were zinc foil, zinc acetate (Zn(O\textsubscript{2}CCH\textsubscript{3})\textsubscript{2}) and zinc sulphate (ZnSO\textsubscript{4}). Zinc foil was obtained from and additionally used for edge calibration at Diamond Synchrotron Lightsource. Zinc sulphate and acetate were purchased from Sigma Aldrich. Once in the lab all samples were kept in individual aluminium foil packets within sealed plastic bags. Gloves were worn at all times when handling the fossils, and when sampled for Py-GCMS a scalpel was used to scrape off the carbonized film of the tadpole under a fume hood to avoid contamination.
with organics in the surrounding air.

4.2.2 Scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy (EDS)

Samples were analysed using both variable pressure field emission gun scanning electron microscopy (VP-FEG-SEM), a type of environmental SEM, and conventional SEM. Fossil samples were left uncoated, and extant grosbeak feathers were prepared for imaging according to Zhang et al. [167], gold coated and mounted on an aluminium stub. Imaging was done on two machines. Variable pressure and traditional secondary electron images of the tadpole and matrix were taken using a Zeiss Supra40VP SEM at 15keV and 14.5mm working distance, and 1keV and 3.5mm working distance respectively. On the same instrument secondary electron images of fs003b were taken at 1keV and 3.5mm working distance. Additional samples were also analysed using an FEI XL30 instrument. General secondary electron images of associated matrix samples were taken at 15keV and 12.4mm working distance, and secondary electron images of extant grosbeak feathers were taken at 15 keV and 10mm working distance. Standardless EDX spectra of fossil material was collected using an EDAX energy dispersive x-ray spectrometer at 15keV and 12.4mm working distance for 100 seconds. Errors are estimated to be approximately 30% of the reported value. Image features were measured using imageJ [130].

4.2.3 Pyrolysis gas chromatography mass spectrometry (Py-GCMS)

2.4mg of powdered tadpole and matrix samples (fs003a) were analysed using normal flash Py-GCMS. Samples were pyrolysed with a CDS (Chemical Data Systems) 5200 series pyroprobe pyrolysis unit by heating at 600°C for 20 seconds. The fragmented
4.2. MATERIALS AND METHODS

macromolecular components were analysed using an Agilent 7890A gas chromatograph fitted with a HP fused column (J+W Scientific; 5% diphenyl-dimethylpolyolsiloxane; 30m, 0.32mm i.d., 0.25µm film thickness) coupled to an Agilent 5975C MSD single quadrupole mass spectrometer operated in electron ionization (EI) mode (scanning a range of m/z 50-650 at 1 scan s$^{-1}$ with a 4 minute solvent delay; ionization energy 70 eV). The pyrolysis transfer line and the injector port temperatures was set at 350°C, the heated interface at 280°C, the EI source at 230°C and the MS quadrupole at 150°C. The carrier gas was helium and the sample were introduced in split mode at a 2:1 ratio. The oven was programmed from 40°C (held for 3 minutes) to 320°C at 4°C min$^{-1}$ and held at this temperature for 5 minutes. Thermochemolysis of samples was carried out by reaction with 10µl of tetramethylammonium hydroxide solution and then analysed using the same equipment. The scan range was m/z 60-700 with a 10 minute solvent delay. The oven was programmed from 40°C (held for 3 minutes) to 300°C at 4°C min$^{-1}$ and held at this temperature for 15 minutes. Compounds were identified using the NIST database and by comparison with spectra from the literature.

4.2.4 Fourier transform infrared spectroscopy (FTIR)

Spectra were taken in Attenuated Total Reflectance (ATR) mode using a germanium crystal in a Spotlight 400 Perkin Elmer FTIR imaging system (wavenumber range 4000-650cm$^{-1}$). Scans were taken with an aperture of 20µm$^2$ and a 4cm$^{-1}$ resolution, each spectrum is an average of 10 scans and is background subtracted. The ATR crystal was cleaned using isopropanol after each scan to avoid cross contamination. Peaks were identified using the Bio-Rad KnowItAll Informatics System 8.2 Multi-Technique database. Inorganic bands were identified by comparison with spectra from the Rruff mineral database [37].
4.2.5 Synchrotron rapid scanning x-ray fluorescence (SRS-XRF)

SRS-XRF was carried out at wiggler beamline 6-2 of the Stanford Synchrotron Radiation Lightsource (SSRL). Scans were undertaken in ambient conditions with a beam energy of 13.5keV and a flux of $1.5 \times 10^{11}$ photons s$^{-1}$. A 50µm diameter pinhole aperture was used to control beam spot size. Samples were mounted on an x-y motorised stage at a fixed 45° incident angle to the beam, signals were detected using a single element drifted (Vortex) detector fixed at a 90° scattering angle to the beam with a 70mm air path from the sample. Both zinc and calcium were mapped with energy windows 350eV wide to capture their respective characteristic x-ray emission line intensities. Point analyses were taken by locating and driving the mounted sample to a point of interest and collecting a full energy dispersive spectrum for 100 seconds. Spectra were calibrated by comparison with a point analysis from a durango apatite standard of known elemental composition. Errors are estimated to be approximately +/- 40%. Spectra were analysed using PyMCA software version 4.4.1. [140].

4.2.6 X-ray absorbance near edge structure spectroscopy (XANES)

XANES spectra were collected for fs003b at beamline I18 of the Diamond Synchrotron Lightsource. The fossil and extant human hair were analysed under fluorescence mode; the zinc standards in transmission mode. The monochromator was rotated to allow the beam energy to scan through the zinc K edge which was calibrated using a zinc foil standard. The emitted intensity of the zinc Kα line was recorded as a function of energy (µE). A linear combination analysis was carried out in Athena [121].
4.3 Results

4.3.1 SEM and EDS

The tadpole is composed of a carbonaceous layer which has become cracked into a number of interconnected polygonal shapes (Fig. 4.1c-g). The surface of the film is covered in a dense layer of spherical bodies (Fig. 4.1c,d) 0.76μm in diameter +/- 18nm (average of 10 measurements), as well as amorphous filamentous material occasionally entangling diatom skeletons (Fig. 4.1d). This filamentous material also appears on the matrix (Fig. 4.1b), which is a diatomaceous sediment consisting of a dense mat of silicious diatom skeletons (Fig. 4.1f). Also present on both tadpole (Fig. 4.1g) and matrix (Fig. 4.1f) are chains of up to 20 spherical bodies 1.6μm in diameter +/- 18nm (average of 10 measurements).

EDX analysis shows the matrix to be predominantly composed of silicon and oxygen whereas the tadpole is mostly carbon and oxygen with some silicon, there is also more sulphur present in the tadpole than the matrix (Table 4.1) as noted in previous studies [110, 111]. Pheomelanosomes examined for comparison were found within fractured barbules of an extant grosbeak feather (Fig. 4.1h). They are spherical, 0.3μm in diameter +/- 18nm (average of 10 measurements) and entangled within the filamentous material within the centre of the barbules.

4.3.2 Py-GCMS

Pyrolysis of both tadpole and matrix samples (fs003a) produced a series of \textit{n}-alkane/\textit{n}-alkene doublets of chain lengths C$_8$ to C$_{31}$ (matrix) and C$_8$ to C$_{29}$ (tadpole; Fig. 4.2), indicating the presence of an aliphatic polymer [67, 69, 141]. The distribution of these groups is different in the tadpole and matrix. Maxima in the matrix occur
Figure 4.1: Photographic images of (a) the part (fs003a) and (b) counterpart (fs003b) of the fossil tadpole from the Upper Oligocene sediments of the Enspel Formation. The dashed line in (a) represents the tadpole. Square indicates the area scanned using XRF, shown in Fig. 4.5. Secondary electron images show the surface of the tadpole (fs003b) as a cracked carbonized film (c, g) covered in microscopic spherical bodies approximately 0.76 µm in average diameter (c-d). The diatomaceous matrix (fs003b) shows the presence of organic filaments (e) and chains of spherical bodies approximately 1.6 µm in average diameter (f), these also appear on the tadpole (white arrows in g). (h) shows the pheomelanosomes (white arrows) of average diameter 0.3 µm present in the ginger feather barbules of a black headed grosbeak. Scale bars represent 2 cm (a-b), 10 µm (c, e-g) and 2 µm (d, h).
4.3. RESULTS

Table 4.1: Elemental composition of fs003a and fs003b, from EDX and SRS-XRF analysis

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<th>Tadpole fs003b (wt. %)</th>
<th>Matrix fs003a (wt. %)</th>
<th>Matrix fs003b (wt. %)</th>
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<td>Cu</td>
<td>3.09</td>
<td>5.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRS-XRF</td>
<td>Zn</td>
<td>3.67</td>
<td>29.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

at C<sub>10</sub> and C<sub>23</sub>, and in the tadpole at C<sub>11</sub>, C<sub>17</sub> and C<sub>21</sub> (Fig. 4.2c, d). The Compounds present in both matrix and tadpole include benzene, di- and trimethyl benzene, 1,ethyl-3,methyl benzene, propenyl benzene, ethyl phenol, trimethyl-di- and tetrahydronaphthalene, dimethyl napthalene and methylated alcohols. Additional compounds found only in the matrix are methyl phenol, and phytol; those only in the tadpole are methyl indene, napthalene, methyl-dihydronapthalene, methyl napthalene and benzyl alcohols. Studying the partial m/z 191 chromatograms shows the presence of a series of hopanes within the matrix but none in the tadpole (Fig. 4.2a,b, insets). Analysis after thermochemolysis reveals a pattern of n-alkanoic acid methyl esters with a distinctly different distribution pattern in matrix compared to the tadpole (Fig. 4.3). In the matrix the chain lengths range from C<sub>8</sub> to C<sub>28</sub>, with maxima at C<sub>16</sub> and C<sub>22</sub>, whereas in the tadpole the chain lengths range from C<sub>14</sub> to C<sub>18</sub> with the maxima at C<sub>14</sub>, C<sub>16</sub> and C<sub>18</sub>. Additional compounds present in the tadpole after such treatment
are 1,3,5-trimethylhexahydro-s-triazine and oleic acid. In all cases abundances of organic moieties are higher in the matrix than in the tadpole.

### 4.3.3 FTIR

There are some areas of the matrix (Fig. 4.4d) that show a similar absorption pattern to the tadpole (Fig. 4.4a-b) and some that show a different one (Fig. 4.4d). In all cases the spectra are dominated by an absorption band at approximately 1100 cm\(^{-1}\) from quartz. It seems that the mineral composition is slightly different in the tadpole and matrix due to the slight shift in this peak. There is absorption generally between 1800 and 700 cm\(^{-1}\) in both tadpole and matrix as well as a wide band at approximately 3200-3400 cm\(^{-1}\) from alcohol OH stretching groups.

The identification of peaks in the region 1800 and 700 cm\(^{-1}\) is somewhat difficult to interpret with confidence due to the likely presence of inorganic mineral absorption bands, many of which occur in this area and most likely convolve with organic bands. Nevertheless we can with reasonable confidence identify some of the major peaks as a C=O stretch of an ester or ketone at 1750 cm\(^{-1}\), the amide NH bending group at 1550 cm\(^{-1}\), the CN amide stretch at 1410 cm\(^{-1}\), and the alcohol CO stretch at 1200 cm\(^{-1}\), all of which occur in both tadpole and matrix (Fig. 4.4a, b, d), though not in all of the matrix (Fig. 4.4c). The tadpole shows an additional alcohol OH deformation group at 1350 cm\(^{-1}\) that does not appear in the matrix, and also what is most likely an alkane CH symmetric stretching band at 2650-2950 cm\(^{-1}\), probably convolved with other unidentified peaks as it is quite broad. There is one other prominent peak that occurs in both tadpole and matrix at 1650 cm\(^{-1}\) which may represent the C=O amide I stretch, though this is inconclusive as an inorganic pyrite peak occurs at the same place the presence of other amino acid groups means it is likely to be present. Spectra b on the tadpole (Fig. 4.4b) shows two relatively broad peaks at approximately 1350
4.3. RESULTS

Figure 4.2: Py-GCMS total ion current and $m/z$ 55+57 chromatograms of the tadpole and matrix (fs003a), insets (not to scale) show the $m/z$ 191 mass chromatograms of the dotted areas revealing the presence and distribution of hopane groups. Brackets represent $n$-alkane/$n$-alkene doublets, # contamination, p phytol, c cyclohexadiene, i methylindene, ba benzyl alcohol; bx benzene derivative, dhx dihydronaphthalene derivative, thx tetrahydronaphthalene derivative, nx naphthalene derivative and px phenol derivative, where x represents the number of carbon atoms in the alkyl group. Abundances are not to scale.
Figure 4.3: Py-GCMS Total ion current chromatograms of the tadpole and matrix (fs003a) after reaction with TMAH. Insets (not to scale) are the m/z 74 mass chromatograms showing the distribution of fatty acid moieties (measured as methyl esters) represented by filled circles with numbers indicating the carbon chain length. Brackets represent n-alkane/n-alkene doublets. bx benzene derivative, nhx tetra hydronaphthalene derivative, nx naphthalene derivative (where x represents the number of carbon atoms in the alkyl group. pa 2-propenoic acid, 3-(4-methoxyphenyl)-methylester, t 1,3,5-Trimethylhexahydro-s-triazine, oa oleic acid methylester, # contamination, open circles indicate unidentified compounds. Abundances are not to scale.
4.3. RESULTS

Figure 4.4: FTIR spectra from fs003b and a eumelanin standard, with a photograph indicating where spectra were sampled. The scale bar represents 2cm. The grey band represents inorganic silicon groups from mineral components, the peak appears slightly shifted in the tadpole compared to the matrix (dotted line). All spectra also appear to have a broad OH band from alcohols, shown in the dashed box, and there is evidence of both major melanin C=O groups in the tadpole (grey lines).
and 1550 cm\(^{-1}\) which agree well with the two major C=O ketone and carboxylic acid peaks of the eumelanin standard (Fig. 4.4e). Absorption at 2364 and 2329 cm\(^{-1}\) is attributable to atmospheric carbon dioxide. Full peak assignments are given in Table A.1 of the appendix.

### 4.3.4 SRS-XRF

When mapped, calcium is predominantly associated with a specific area on the tadpole (Fig. 4.5d), the rest of the soft-tissue is strongly associated with zinc (Fig. 4.5b), no other elements associate with the tadpole in such a way but are present all over the sample. Point analyses reveal that there is more zinc and calcium in the tadpole compared to the matrix (Table 4.1).

![Image](image.png)

Figure 4.5: Comparative photographic (a) and SRS-XRF images of fs003b (b-d). Scale bars represent 1cm. In b-d white indicates high elemental levels and black indicates low levels.

### 4.3.5 XANES

The XANES spectra of the fs003b tadpole is very similar to those of the blonde human hair and the organic zinc acetate standard, and dissimilar from both the inorganic standards, the zinc foil and zinc sulphate (Fig. 4.6a). The tadpole spectra also shows no similarity to other inorganic zinc minerals including sphalerite (ZnS), willemite (Zn\(_2\)SiO\(_4\)), zincite (ZnO) or franklinite (ZnFe\(_2\)O\(_4\)) \[125\][154]. Both tadpole and hair
show a bifurcated peak though the pattern of dominance is switched between the samples. Linear combination analysis shows that of the standards analysed, the tadpole XANES spectrum most resembles the organic samples (Table A.2, Fig. A.1 in the Appendix). In order to see better separation of peaks for determining oxidation state the first derivative of the normalised absorbance was used (Fig. 4.6b). The tadpole occurs very close to the blonde hair and close to the zinc acetate indicating a +2 oxidation state. The similarity to zinc acetate also indicates that both the tadpole and blonde hair are light element bound and tetrahedrally coordinated. However the fact that they are not the same indicates a slight difference in coordination chemistry.

Figure 4.6: XANES spectra of zinc standards, blonde human hair and fs003b at the Zn K edge shown as the normalised absorption (a) and the first derivative (b). The distinct similarities in the spectra of fs003b, the human hair and zinc acetate indicate that the zinc in the fossil tadpole is organic.
4.4 Discussion

4.4.1 Physical structure

The cracked nature of the carbonaceous film of the tadpole, presence of small spherical bodies on its surface, and abundance of carbon and sulphur as seen in EDS are all found in other studies of such samples [111, 149]. As they observe, the spherical bodies are fully comparable in both size and form to coccoid bacteria, however they are also of a similar form to pheomelanosomes in extant tissues (Fig. 4.1h). The presence of the pigment pheomelanin, contained within pheomelanosomes, has recently been demonstrated in amphibian skin [161], however its presence does not seem to be common. The reliance on the structure of fossilised melanosomes to denote original colour has also been shown to be unreliable due to diagenesis. Recent work [108] has demonstrated that the shape of melanosomes can change dramatically when exposed to high temperatures and pressures and therefore should not be taken as a definitive indicator of the type of melanin present. Indeed, though eumelanin is found in the modern world to occur in elongate melanosomes, a recent geochemical study identifying eumelanin in a Jurassic cephalopod found their ink sacks were covered in spherical bodies [96].

4.4.2 Organic geochemistry

The presence of sulphur in other fossil tadpoles has been cited as evidence of organosulphur compounds which potentially aid the preservation of the organism by sulphurization [109]. Sulphur may also be indicative of the presence of pheomelanin, as the sulphur containing amino acid cysteine is one of the key triggers for its formation [120] p. 134-152] [126] p. 26-28], however we find no evidence of this in our samples. There is also no evidence of eu- or pheomelanin breakdown products in the Py-GCMS chromatograms [40, 91], which show the presence of generic organic matter, including
series of benzene and naphthalene compounds. Though they do show distinctly different distribution patterns of \( n \)-alkane/\( n \)-alkene doublets and fatty acid methyl esters. Therefore there is no evidence of the movement of organics from the matrix into the tadpole and therefore those of the tadpole are most likely endogenous to the original organism. This is strengthened by the detection of phytol, indicative of plant material, in the matrix and not the tadpole. This runs counter to previous studies where this has been suggested as the mechanism for the darkening of the carbonaceous film in such samples [52]. The fact that a series of bacterial hopane compounds was detected in the matrix but not in the tadpole (Fig. 4.2) is suggestive but not conclusive of the absence of bacteria in the tadpole, it shows only the absence of aerobic bacteria. Indeed there is evidence of bacterial chain structures on both matrix and tadpole (Fig. 4.1f, g) which are clearly not picked up by the analysis, indicating that they are either aerobic, present at abundances below detection limits or not preserved well enough for their organic compounds to be detected. The FTIR data suggests the presence of amino acids (C=O, NH and CN groups) within tadpole and matrix. Given that the organic material of the tadpole is most likely to be endogenous this suggests the amino acids are from the original tadpole tissues, whereas in the matrix the signal is most likely from bacteria. One area of the tadpole does however seem to show both C=O ketone and carboxylic acid peaks seen in eumelanin (Fig. 4.4), the C=O carboxylic acid group at 1350 cm\(^{-1}\) in particular is only seen in the tadpole. This is suggestive but not conclusive of the presence of melanin, due to the likely presence of inorganic mineral peaks that co-occur with many of the organic peaks. Despite the fact that FTIR is useful in identifying melanin it has been shown to be less so in distinguishing between eumelanin and pheomelanin [99].
4.4.3 Zinc

The presence of zinc in fossilised tadpoles has been noted before [111] but this is the first time it has been shown to be organic Zn(II) and to occur throughout the soft tissues. Zn(II), Cu(II), Mg(II), Fe(III) and Ca(II) are the most common metals present within both eu- and pheomelanin [99]. In solutions of mild acidic or neutral pH Cu(II) and Zn(II) have a higher binding affinity to eumelanin than Mg(II) and Ca(II) [98]. The melanin can act like a metal ion sink during diagenesis, leading to the build up of these metals where melanin was present and allowing them to be used as a melanin biomarker [159]. Zinc is known to naturally accumulate in red human hair and levels of zinc commonly exceed those of copper in eu-and pheomelanin extracted from human hair [91]. Another reason for higher zinc levels in the tadpole may be the presence of matrix metalloproteinases. These enzymes are interstitial collagenases [17] and members of the metzincin group of proteins, so named for, among other things, their dependence on the Zn(II) ion [117]. These enzymes were first described after their study in tadpoles [66] and are an essential part of the process of metamorphosis they undergo to obtain an adult body. The tadpoles’ dependence on this protein may have increased the amount of zinc present within the tissues before death which was subsequently chelated to the pheomelanin and thereby retained in the soft tissues.

More work needs to be done to characterise the precise coordination environment of the zinc in both the tadpole sample and natural pheomelanin. Whilst the XANES spectra indicate that the zinc coordination is similar to that in blonde human hair, it is also very similar to that of zinc bound by bacterial enzymes [112][116]. The zinc in the human hair is also not a pure sample, there are likely to be contributions from both eu- and pheomelanin bound zinc as well as that in proteins. A comparison needs to be made to zinc bound to pure eu- and pheomelanin. It is possible that the build up of zinc within the soft tissues may have had a biocidal effect and therefore prevented bacterial
4.5 Conclusions

- The fossil tadpole is covered in small spherical bodies consistent in size and form with both coccoid bacteria and pheomelanosomes.

- The organic material in the tadpole is different from that in the matrix and is therefore most likely original to the organism and not a result of transfer from the matrix. This rules out the possibility that the carbonaceous layer of the tadpole is a result of the incorporation of plant material.

- Py-GCMS indicates the presence of bacterial biomarkers in the matrix but not the tadpole, though this does not rule out the possibility of there being bacteria on the tadpole.

- FTIR shows possible melanin groups in the tadpole but not the matrix, though this method is not reliable for distinguishing between eu-and pheomelanin.

- SRS-XRF and XANES analysis shows that the soft tissues of the tadpole are high in organic Zn(II). More work needs to be done to determine the precise chemical coordination of the zinc and compare it to that in pure pheomelanin.

Acknowledgements

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Chapter 5

Paper 4: Geochemical evidence of the seasonality, affinity and pigmentation of *Solenopora jurassica*

This chapter contains the following paper which is in preparation to be submitted to the journal *Geochimica et Cosmochimica Acta*

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5.1. INTRODUCTION

Abstract

The fossil calcareous alga *Solenopora jurassica* is known for its distinctive pink and white banding. Though currently widely accepted as an alga there is some debate over its taxonomic affinities, with some arguing that it is a chaetetid sponge. The banding pattern may be seasonal, but there has so far been no conclusive evidence of this. Recent work has however demonstrated the presence of an organic boron-containing pink/red pigment. Here we present new geochemical evidence of seasonality, pigmentation and affinity. Seasonal growth is shown by differences in calcite density, and increased Mg/Ca molar ratios and chlorine levels indicate higher temperatures during the time the white bands were deposited than the pink. Pyrolysis gas chromatography mass spectrometry and infrared spectroscopy (Py-GCMS) show the presence of tetramethyl pyrrole, protein moieties and carboxylic acid groups, indicative of the presence of the red algal pigment phycoerythrin. This supports the identification of *S. jurassica* as an alga as the pigment is only known to occur in cyanobacteria and algae. No other taxonomically indicative biomarkers were identified. Attempts to map boron using x-ray photoelectron spectroscopy were also unsuccessful.

5.1 Introduction

*Solenopora jurassica*, popularly known as the ‘beetroot stone’ is particularly striking for its pink and white banding. Originally classified by Dybowski in 1877 as a chaetetid sponge, it has since been described and accepted as a fossil calcareous alga [19, 71], though there is still some debate over this assignment [122]. Cellular structure is clearly identifiable, being elongate cells radiating from the inner core of the thallus to its exterior, indicating the direction of growth [71]. The visible bands have been shown to correspond to the degree of cellular preservation [19, 71, 163]. Pink
bands are composed of poorly preserved cells, with detail obliterated by extensive re-
crystallization [71], whereas white bands are composed of relatively well preserved
cells. The reason for this preservational difference and its relationship to the visible
banding pattern is currently unclear. Various mechanisms have been proposed, often
invoking the idea that they are most likely seasonal growth bands, however thus far no
conclusive evidence of this has been seen.

Harland and Torrens [71] proposed that the pigment had been leached out of the
white bands due to differences in porosity created by the difference in cellular preser-
vation. The pink band was thought to be less porous due to the recrystallisation of
the cellular structure creating a more densely packed material than the well ordered
structure of the white band. A mechanism for the difference in cellular preservation
was outlined by Wright [163], in which he argued that it is dependent on the levels of
magnesium in the cell wall calcite. Extant calcareous algae have been shown to have
different amounts of magnesium in their cell walls dependent on growth rate [89].
During periods of slower growth the magnesium uptake by the cells for photosynthesis
and respiration is low and the preferred Mg/Ca ratio in the calcite is easily maintained
by diffusion. When growth is faster the diffusion of magnesium is not fast enough to
replace the amounts used by the cells and so the ratio cannot be maintained. This leads
to more magnesium being stored in the calcite during periods of slower growth than
periods of faster growth. Wright proposed that this occurred in the tissues of S. juras-
sica, and that the pink bands retained pigment because they contained higher levels of
magnesium in life and therefore during diagenesis the conversion of high magnesium
calcite to low magnesium calcite caused extensive degradation in cellular structure.
For the species studied by Kolesar [89] (Calliarthron tubercolsum, a calcareous al-
gae) the growth rate was higher in the winter months (lower temperatures) than in the
summer months (higher temperatures). However Wright [163] predicts that the thicker
pink bands in S. jurassica were deposited in the summer months and the white bands
in the winter months, indicating that higher magnesium is deposited in bands of faster
growth, contradicting the results of Kolesar [89]. Despite electron microprobe analysis
no evidence of significantly different levels of magnesium was found [163].

Until very recently nothing was known about the pigment present within the pink
bands of *S. jurassica*, other than it had been shown to be organic [48] and suggested
to be a porphyrin, preserved due to the rapid burial of the algae by sediments [71].
Wolkenstein et al. [160] have isolated novel boron containing organic pigments, borolithochromes,
that when concentrated exhibits a bright red/pink colour. The pigment is composed of
a highly condensed aromatic system with the boron bound to phenolic moiety ligands,
with no known modern analogue.

The aims of this work are to use biomarker analysis to try and resolve the affinity of
*S. jurassica* and investigate the preservation of the organic pigments. To use elemental
analysis to determine whether the previous mechanism for banding by magnesium
levels in cell wall calcite is valid, whether the boron in the borolithochrome can be
mapped, and whether there is any evidence of seasonality.

5.2 Materials and methods

5.2.1 Sample and handling

The sample used in this study (sample number UOM-232; Fig. 5.1) is part of the
collections of the University of Manchester. It was collected from the Middle and
Upper Bathonian White Limestone Formation of Foss Cross quarry near Chedworth
in Gloucestershire. The depositional environment has been described as moderate to
high energy shoaling with occasional storms [71]. Precise information as to the exact
sampling location has been lost, however based on the work of previous authors on
similar fossils [19,71,163] we can confidently assign it to *S. jurassica*. The thick
section cut for analysis by infrared and chromatography techniques was taken from the interior of the specimen and the outer edges were sanded down. The drill tip and then the whole section was rinsed in dichloromethane to minimize the chances of contamination from handling of the sample during collection and display. This section was then ground to a powder for analysis. Subsequently the main block and all sections taken were kept in foil packets and sealed in plastic bags to avoid further contamination.

5.2.2 Electron microprobe analysis (EMA)

A section was cut and placed into the bottom of a circular aluminium mount and ground so that the surface was flush with the top of the mount (Fig. A.2, appendix). Half of it was then etched with 1% HCl for 5 minutes, thoroughly rinsed with deionized water and air dried, after which it was glued into the aluminium mount. The sample was carbon coated and silver dag applied to one edge to draw current from the surface. A Cameca SX 100 microprobe in Wavelength Dispersive Spectrometry (WDS) mode was used to detect and map elements on the surface of the sample. K, S, Ca and Cl were analysed using a pentaerythritol (PET) detector, P, Si and Mg using a thalium acid pthalate (TAP) detector, and Zn, Cu, Fe and Mn using a lithium fluoride detector (LIF). All elements measured were k\(\alpha\). 400\(\mu\)m\(^2\) maps of each element were taken of 0.5\(\mu\)m\(^2\) resolution, as well as 30 point analyses in the pink bands, 30 on the cell walls and 30 on the cell vacuoles of the white band to determine abundance. The electron beam was of accelerating voltage 15kV and current of 20nA, excitation volume was approximately 1\(\mu\)m\(^3\).
5.2. MATERIALS AND METHODS

5.2.3 X-ray photoelectron spectroscopy (XPS)

The XPS spectra were recorded using a Kratos Axis Ultra spectrometer employing a monochromated Al Kα x-ray source and an analyser pass energy of 80eV for survey scans and 20eV for elemental scans, resulting in a total energy resolution of ca. 1.2-1.4eV or 0.6-0.7eV respectively. Uniform charge neutralisation of the photoemitting surface was achieved by exposing the surface to low energy electrons in a magnetic immersion lens system (Kratos Ltd.). The system base pressure was 1x10^{-9}mBar. Spectra were analysed by first subtracting a Shirley background and then obtaining accurate peak positions by fitting peaks using a mixed Gaussian/Lorenzian (30/70) line shape. Quantification of surface atom % was achieved using a derived analyser transmission function and Scofield theoretical elemental cross-sections. During fitting, spin orbit split components were constrained to have identical line width, elemental spin orbit energy separations and theoretical spin orbital area ratios. All photoelectron binding energies (BE) are referenced to C1s peaks set at 285eV BE. The analyser was calibrated using elemental references; Au 4f7/2 (83.98eV BE), Ag3d5/2 (368.26eV BE) and Cu2p3/2 (932.67eV BE).

5.2.4 Extraction and decalcification

100g of powdered bulk sample was extracted with dichloromethane:methanol (DCM : MeOH, 2:1, v/v) for 24 hours using Soxhlet equipment. Total lipid extracts (TLE) were recovered and concentrated by using rotary evaporation. One aliquot was blown down under a flow of N₂ and 10µl of internal standard (54 n-alkane/hexadecanol) was added. The TLE was then blown down under N₂ and derivatized to convert acid groups to their corresponding methyl esters by adding 100µl BF₃ in MeOH and heating at 70°C for 1 hour. The residue was then dissolved in DCM:MeOH (1:1, v/v), this was passed through an activated Al₂O₃ column and eluted with 4 column volumes of DCM:MeOH.
(1:1, v/v) and all elutants were collected. Further derivatization was carried out by reaction with 50µl bis(trimethylsilyl) trifluoroacetamide (BSTFA) and heating at 70°C for 1 hour. The TLE was analysed by GCMS and the residue was air dried.

Bulk and residue powder samples were reacted with 10% HCl to remove calcium carbonate and thereby concentrate organic material. After no further reaction was observed with the acid the samples was washed in deionized water, centrifuged to remove the water, and then freeze dried. Approximately 99% of the mass was lost in the process [46]. Bulk and residue samples were then analysed by Py-GCMS.

5.2.5 Pyrolysis gas chromatography mass Spectrometry (Py-GCMS) and gas chromatography mass Spectrometry (GCMS)

2mg of the sample was analysed using normal flash Py-GCMS. Samples were pyrolysed with a CDS (Chemical Data Systems) 5200 series pyroprobe pyrolysis unit by heating at 600°C for 20 seconds. The fragmented macromolecular components were analysed using an Agilent 7890A gas chromatograph fitted with a HP fused column (J+W Scientific; 5% diphenyl-dimethylpolyolsiloxane; 30m, 0.32mm i.d., 0.25µm film thickness) coupled to an Agilent 5975C MSD single quadrupole Mass Spectrometer operated in electron ionization (EI) mode (scanning a range of \( m/z \) 45-650 at 1 scan s\(^{-1}\) with a 4 minute solvent delay; ionization energy 70 eV). The pyrolysis transfer line and the injector port temperatures was set at 350°C, the heated interface at 280°C, the EI source at 230°C and the MS quadrupole at 150°C. The carrier gas was helium and the sample were introduced in split mode at a 2:1 ratio. The oven was programmed from 40°C (held for 4 minutes) to 300°C at 4°C min\(^{-1}\) and held at this temperature for 5 minutes. Thermochemolysis of samples was carried out by adding 10µl of tetramethylammonium hydroxide solution to the samples, leaving them for 5 minutes, and then analysing them using the same equipment. The scan range was \( m/z \) 60-650 with
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a 15 minute solvent delay. The oven was programmed from 40°C (held for 4 minutes) to 320°C at 4°C min\(^{-1}\) and held at this temperature for 5 minutes. Compounds were identified using the NIST database and by comparison with spectra from the literature.

GCMS was performed using the same equipment. The MS scanned from \(m/z\) 50-650 at 2.7 scans s\(^{-1}\). The GC was equipped with an Agilent 7683B auto sampler and a programmable temperature variation (PTV) inlet. The samples were dissolved in hexane prior to injection, injected using pulsed split-less injection (1µl; inlet pressure of 25psi for 0.25min). The samples were injected at 70°C and the oven was programmed to 130°C at 20°C min\(^{-1}\) and then at 4°C min\(^{-1}\) to 300°C at which it was held isothermally for 25 min.

5.2.6 Fourier Transform Infrared Spectroscopy (FTIR)

Samples were analysed using a Bio-Rad 6000-IR system (wavenumber range 4000 to 900cm\(^{-1}\)). Spectra were taken with a 2cm\(^2\) aperture and 4cm\(^{-1}\) resolution; final spectra were an average of 16 scans. All spectra and maps were background subtracted. Organic peak assignments were made using the Bio-Rad KnowItAll Informatics system 8.2 Multi-Technique database, inorganic peaks by reference values \[128\].

5.2.7 Synchrotron Rapid Scanning X-ray Fluorescence (SRS-XRF)

SRS-XRF was carried out at wiggler beamline 6-2 of the Stanford Synchrotron Radiation Lightsource (SSRL). Scans were undertaken in ambient conditions with beam energies of 13.5keV (hard x-rays, flux approximately \(10^{11}\) photons s\(^{-1}\)) and 3.15keV (soft x-rays, flux approximately \(2.44\times10^{11}\) photons s\(^{-1}\)). A 100µm diameter pinhole aperture was used to control beam spot size. Samples were mounted on an x-y motorised stage and signals were detected using a single element drifted (Vortex) detector. For hard x-rays the stage was at a fixed 45° incident angle to the beam and the detector
was fixed at a 90° scattering angle to the beam. The incident beam had a 70mm air path to the sample, whilst the detector maintained contact with the polythene window and thus gave zero path in air for the fluoresced signal. For soft x-ray scans the samples were mounted inside a metal case, the top sealed in polythene and the case filled with helium, creating a lower density atmosphere to reduce absorption of both incident and fluoresced x-rays. The stage was at a fixed 45° incident angle to the beam and the detector was fixed at a 64° scattering angle to the beam with a 15mm air path from the sample. Point analyses were taken by locating and driving the mounted sample to a point of interest and collecting a full energy dispersive spectrum for 100 seconds. Spectra were calibrated by comparison with a point analysis for a durango apatite standard of known elemental composition. Detection limits are approximately 1ppm for higher atomic weight elements such as arsenic, and several weight percent for lighter elements. Spectra were analysed using PyMCA software version 4.4.1 [140].

5.2.8 2D x-ray radiography

The radiographic images were taken on the Nikon Metrology 225/320kV Custom Bay system of the Henry Moseley X-ray Imaging Facility (HMXIF) with the 225 kV source and a molybdenum target using a voltage of 100kV and a current of 100µA. The x-ray beam was not filtered. A Perkin Elmer 2000 x 2000 pixels 16-bit amorphous silicon flat panel detector was used to obtain the images. The acquisition software was Nikon Metrology proprietary software InspectX version XT 2.2 service pack 5.5.

5.2.9 X-ray Diffraction (XRD)

Scans were run using a fixed incidence angle of 2.5°, with a step size of 0.02 degrees at a speed of 2s step\(^{-1}\). A Goebel mirror attachment allowed a parallel x-ray beam, and a scintillation detector with a Soller slit assembly was used. Peak assignment was
achieved using Eva 14.0 software to compare the measured data with standards from the ICDD Powder Diffraction File.

5.3 Results

5.3.1 Light microscopy

As noted in previous studies [71, 163], the striking pink and white banding displayed on the sample (Fig. 5.1h) correlates with the degree of cellular preservation as visible in a thin section of the sample.

Figure 5.1: (a) Photograph of a thick section of UOM-232. A denotes the section subsequently analysed by 2D x-ray radiography, and B the area mapped by XRF. (b-g) Light microscopy images of a thin section of UOM-232 showing the pink and white bands at different magnifications. The difference in cellular preservation between the two bands can be clearly seen under the light microscope, with the cellular structure clearly discernable in the white bands but not the pink ones.

The white bands are composed of relatively better preserved cells (Fig. 5.1p, e-g) than the pink bands (Fig. 5.1p-d), which have been reported to have lost their definition
by means of extensive recrystallisation [19,163]. The thin section was taken in vertical cross section of the sample (same plane as Fig. 5.1a), and the cells in the white bands appear for the most part to be longitudinal, the long axis indicating the direction of growth (Fig. 5.1f), usually radially outwards from the centre [71]. Horizontal growth (Fig. 5.1g) and vertical growth can be seen occurring in the same cross section (Fig. 5.1b, e). This may indicate a response to a sediment infil and/or covering of the thallus, as seen in Fig. 1b and e. Average length of cells is 264µm and diameter is 64µm (+/- 18 nm, average of 10 measurements). The average width of the white band is also significantly smaller (3.83mm) than the pink band (9.38mm) (+/- 18nm, average of 45 measurements, p=0.0001, Mann Whitney U test).

5.3.2 EMA

Figure 5.2: Electron Microprobe images of UOM-232. The Mg and Si is concentrated in the cell walls and highlights the much better cellular preservation in the white bands compared to the pink ones.

Electron mapping indicates that Mg and Si are bound within the cell walls (Fig. 5.2), and thereby shows that there is better cellular preservation in the white band than the pink band. Patterns of abundance for other elements are less clear, but point analyses (Table 5.1) indicate that there is significantly more P, Si, S, Mg and Ca in the cell walls of the white band than in the pink band (p<0.001 Mann Whitney U test), and significantly more Mg and Fe in the white band cell vacuole than in the pink band.
5.3. **RESULTS**

Table 5.1: Elemental abundances in pink and white bands indicated by Electron Microprobe analysis. BDL indicates a value is below the detection limit for that element.

<table>
<thead>
<tr>
<th></th>
<th>White band vacuole (ppm)</th>
<th>White band cell wall (ppm)</th>
<th>Pink band (ppm)</th>
<th>Significance between white band cell vacuole and pink band</th>
<th>Significance between white band cell wall and pink band</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>3290</td>
<td>3030</td>
<td>3360.00</td>
<td>&gt;0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Si</td>
<td>74.0</td>
<td>1860</td>
<td>107</td>
<td>&gt;0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>S</td>
<td>834</td>
<td>1780</td>
<td>879</td>
<td>&gt;0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mg</td>
<td>1240</td>
<td>2870</td>
<td>1950</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ca</td>
<td>409000</td>
<td>382000</td>
<td>409000</td>
<td>&gt;0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mn</td>
<td>135</td>
<td>146</td>
<td>180</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td>Fe</td>
<td>889</td>
<td>2460</td>
<td>1240</td>
<td>&lt;0.01</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Cu</td>
<td>72.4</td>
<td>67.1</td>
<td>98.3</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td>Zn</td>
<td>96.9</td>
<td>95.9</td>
<td>64.3</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>K</td>
<td>22.0</td>
<td>138</td>
<td>13.0</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td>Cl</td>
<td>48.3</td>
<td>131</td>
<td>54.7</td>
<td>BDL</td>
<td>BDL</td>
</tr>
</tbody>
</table>

(p<0.01 Mann Whitney U test). The cellular preservation in the pink band was too poor to differentiate between cell wall and vacuole. Mg/Ca molar ratios were calculated for the white band cell wall (12.39mmol mol$^{-1}$) and the pink band (7.86mmol mol$^{-1}$).

5.3.3 **XPS**

Wide scans (Fig. 5.3a, d) show the presence of many different elements in both bands, the highest in abundance being Ca, C and O, due to the sample being predominantly composed of calcite (Table 5.2). In both bands fine scans of the P 2p peak at 130eV (Fig. 5.3b, e) shows the presence of a phosphate peak at 133.5eV. The P 2s peak should occur at approximately 188eV, however due to the presence of phosphate this will induce a shift and make it appear from 190-191eV, exactly the same binding energy range at which we expect to find the borate B 1s peak. Higher resolution scans
Figure 5.3: X-ray Photoelectron spectra of the pink and white bands of UOM-232. (a) and (d) show wide energy range scans, (b) and (e) scans of 220-70eV, and (c) and (f) high resolution scans of the Cl 2p, P 2s and S 2p peaks. Due to the presence of phosphate the phosphorus peak 2s peak occurs in the same range as the boron peak (190-191eV) meaning boron could not be resolved.
Table 5.2: Quantification data of elements present in UOM-232 shown by x-ray photoelectron spectroscopy

<table>
<thead>
<tr>
<th>Element and orbital</th>
<th>% conc. pink band</th>
<th>% conc. white band</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na 1s</td>
<td>0.22</td>
<td>0.18</td>
</tr>
<tr>
<td>Zn 2p</td>
<td>0.11</td>
<td>0.15</td>
</tr>
<tr>
<td>F 1s</td>
<td>0.17</td>
<td>0.26</td>
</tr>
<tr>
<td>O 1s</td>
<td>37.81</td>
<td>33.76</td>
</tr>
<tr>
<td>N 1s</td>
<td>1.41</td>
<td>1.85</td>
</tr>
<tr>
<td>Ca 2p</td>
<td>11.31</td>
<td>8.32</td>
</tr>
<tr>
<td>C 1s</td>
<td>46.10</td>
<td>51.32</td>
</tr>
<tr>
<td>Si 2p</td>
<td>1.16</td>
<td>1.62</td>
</tr>
<tr>
<td>Cl 2p</td>
<td>0.41</td>
<td>1.32</td>
</tr>
<tr>
<td>S 2p</td>
<td>0.48</td>
<td>0.66</td>
</tr>
<tr>
<td>Al 2p</td>
<td>0.60</td>
<td>0.34</td>
</tr>
<tr>
<td>P 2p</td>
<td>0.21</td>
<td>0.23</td>
</tr>
</tbody>
</table>

(Fig. 5.3c, f) were no more successful at separating 2 peaks in this region, we cannot therefore identify boron within the sample using XPS.

5.3.4 Py-GCMS and GCMS

The bulk sample Py-GCMS Total Ion Current (TIC) chromatogram is dominated by an \(n\)-alkane/\(n\)-alkene doublet pattern ranging from C\(_9\) to C\(_{25}\) with the maxima at C\(_{19}\) (Fig. 5.4). The GCMS TIC of the TLE is similarly dominated by \(n\)-alkanes, though of a different distribution pattern. The range is from C\(_{17}\) to C\(_{29}\), and the maxima is at C\(_{21}\). The removal of such a signal from the bulk material leaves the \(n\)-alkane/\(n\)-alkene pattern for the residue (shown by Py-GCMS) not only reduced in relative abundance but also skewed in distribution to the lower carbon chain lengths, C\(_8\) to C\(_{23}\), with the maximum at C\(_{15}\). The other products detected in the residue are mostly benzene and naphthalene derivatives. No odd/even predominance pattern is observed, indicating that the signals are unlikely to be from higher terrestrial plant waxes [43]. In addition, the loss of the majority of the aliphatic signal after extraction, as indicated by the \(n\)-alkane abundance
in the TLE demonstrates that these moieties are not part of a macromolecular complex, such as those that dominate in plant material [67].

Py-GCMS following TMAH enhanced thermochemolysis shows the main differences after extraction of the bulk material to be a lack of $n$-alkane/$n$-alkenes (Fig. 5.5). The signals detected in the bulk and residue are otherwise very similar, the only difference being the lack of biphenyl and trifluoromethylbenzoic acid, pentadecylester in the residue. This indicates that the majority of the material in UOM-232 is non-extractable. The presence of protein is indicated by the detection of phenol-(dimethylamino) and the amino acid phenylalanine. The fatty acid distribution is unchanged after extraction, the distribution ranges from $C_8$ to $C_{18}$, with maxima at $C_{16}$ and $C_{18}$ (Fig. 5.5 insets). This pattern is very dissimilar to that of fossil leaves [41] again indicating that there is no higher plant material present in the sample. The extract was colourless and the residue brown upon reaction with HCl, indicating that the pigment responsible for the pink colour bands was not extracted. No bacterial hopane groups were detected.

### 5.3.5 FTIR

There are few notable differences between the bulk material and the residue after extraction, only the removal of alkane CH groups between 2900 and 3050 cm$^{-1}$, as well as the apparent disappearance of the convolved alkane CH and carboxylic acid CO group (Fig. 5.6). The presence of a broad convolved peak at approximately 1300 cm$^{-1}$ makes the identification of individual peaks difficult, however in combination with others there is good evidence in both spectra of carboxylic acid, alcohol and alkene groups, and the presence of proteins as evidenced by the amine NH and NH$_2$ groups. The large peak at 1000 cm$^{-1}$ is inorganic silica. Full peak assignments are given in table A.3 of the appendix.
Figure 5.4: Py-GCMS total ion current and m/z 55+57 chromatograms of the bulk, Total Lipid Extract (TLE) and Residue of UOM-232. The insets are not to scale. Black brackets represent n-alkane/n-alkene doublets (numbers indicate carbon chain length), # contamination, ac acetophenone, bp biphenyl, f fluorene, in indane, phn phenanthrene/anthracene; and bx benzene and nx naphthalene derivatives, where x represents the number of carbon atoms in the alkyl group. A black outline indicates the presence of a double bond.
Figure 5.5: Py-GCMS Total ion current chromatograms of the bulk and residue of UOM-232 after thermochemolysis, insets are (not to scale) are the m/z 74 mass chromatograms. Fatty acid moieties (measured as methyl esters) are represented by filled circles and n-alkane/n-alkene doublets by black brackets, numbers indicate carbon chain length; an alkane nitrile, bp biphenyl, f fluorene, fb trifluoromethylbenzoic acid pentadecylester, pha phenol-(dimethylamino), phe phenylalanine 4-amino-N-t-butyloxycarbonyl-t-butylester, phn phenthrene, pi phenol 4 4’-(1-methylethylindene)bis(2-methyl), py pyrrole, 2,3,4,5-tetramethyl; and bx benzene, ix indene, iox indole, nx naphthalene, ndx dihydronapthalene, nhx tetrahydronapthalene and phx phenol derivatives, where x represents the number of carbon atoms in the alkyl group.
5.3. RESULTS

Figure 5.6: FTIR spectra of the bulk and residue of UOM-232. The major peak at approx. 1000 cm\(^{-1}\) is inorganic silica. Major components of both spectra include oxygen OH, alkene CH and C=C and NH and NH\(_2\) amine groups. Signal at approx. 2300 cm\(^{-1}\) is atmospheric CO\(_2\). The major difference between the two is the lack of CH alkane/alkene groups in the residue, consistent with their removal during extraction.

5.3.6 SRS-XRF

SRS-XRF mapping shows the white bands to be higher in Cl than the pink bands (Fig. 5.7). Other elements do not appear to show such significant banding (Fig. A.3, A.4 appendix). Point analyses support this, showing higher levels of Cl in the white band (Table 5.3).

5.3.7 2D x-ray radiography

2D x-ray radiographs of UOM-232 show that the white bands on the samples correspond to areas of relatively lower density calcite than the pink bands (Fig 5.8). The outer layer despite being visibly white, looks to be of a similar density to the pink bands.
Figure 5.7: XRF map of Cl in UOM-232 with corresponding photograph in longitudinal section clearly showing the enrichment of Cl in the white bands possibly due to increased evaporation as a consequence of higher temperatures.

<table>
<thead>
<tr>
<th>Element</th>
<th>White band (ppm)</th>
<th>Pink band (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>162000</td>
<td>163900</td>
</tr>
<tr>
<td>Ti</td>
<td>20.4</td>
<td>43.1</td>
</tr>
<tr>
<td>Mn</td>
<td>58.4</td>
<td>61.5</td>
</tr>
<tr>
<td>Fe</td>
<td>411</td>
<td>438</td>
</tr>
<tr>
<td>Ni</td>
<td>1.92</td>
<td>2.20</td>
</tr>
<tr>
<td>Cu</td>
<td>2.47</td>
<td>2.05</td>
</tr>
<tr>
<td>Zn</td>
<td>3.56</td>
<td>4.07</td>
</tr>
<tr>
<td>As</td>
<td>0.65</td>
<td>0.70</td>
</tr>
<tr>
<td>Br</td>
<td>0.004</td>
<td>0.00</td>
</tr>
<tr>
<td>Si</td>
<td>570</td>
<td>315</td>
</tr>
<tr>
<td>P</td>
<td>76.2</td>
<td>178</td>
</tr>
<tr>
<td>S</td>
<td>362</td>
<td>434</td>
</tr>
<tr>
<td>Cl</td>
<td>421</td>
<td>305</td>
</tr>
</tbody>
</table>
5.4 DISCUSSION

Figure 5.8: (a) 2D x-ray radiograph of UOM-232 showing differences in calcite density in the different visible bands (b). The letters indicate the same bands on both images. The white bands are of a lower density calcite than the pink bands, indicating that the bands are due to seasonal growth cycles, with one white and one pink band representing one year.

5.3.8 XRD

There appears to be no minerological difference between the white and pink bands. Both are composed predominantly of calcite with minimal quantities of ankerite and quartz (Fig. 5.9).

5.4 Discussion

5.4.1 Seasonality of the banding pattern

X-ray radiography is a commonly used technique to observe seasonally determined density banding in fossil and modern corals (see Barnes and Lough [7] for a review). It was first noted by Knutson [88] who observed that such analysis of vertical cross sections of corals showed that there were regularly alternating bands of different density calcite, one high and one low density band representing one year’s growth. This...
change in density was interpreted to be as a result of changing environmental conditions. However, a number of different variables are known to affect coral growth so it is difficult to narrow down the precise cause. The pattern has since been noted and confirmed in over 1000 different extant species [155], and also in some fossil species [76,85]. The 2D x-ray radiograph of sample UOM-232 clearly shows the same pattern of regularly alternating bands of low and high density (Fig. 5.8), the pink bands being of a higher density than the white bands. Despite the fact that this technique has so far been applied to corals and not calcareous algae, their similarities in depositing a calcium carbonate framework implies that it could possibly be applicable to both. The largest survey of density banding in extant coral found that the high density bands were associated with relatively higher water temperatures [155], but there are many examples of species that show the opposite pattern [36,85,86]. There is also considerable variation in the size of growth bands within individuals of the same species and
well banded specimens were found where there was little seasonal variation in water temperature \[155\].

The relative temperature differences of the depositional environments at the time the different bands were formed can be determined by the Mg/Ca ratio. This is a commonly used palaeotemperature proxy dependent upon the increased uptake of magnesium into skeletal calcite elements at higher temperatures \[93\]. Whilst the ratio can be used to provide absolute temperatures, this relies on well constrained knowledge of sea water composition at the time and can also depend on other factors such as salinity and pH \[49, 63\]. In this case therefore Mg/Ca ratios indicate relative temperature differences, isotopic analysis would help to determine temperature changes more accurately. The higher Mg/Ca ratio in the white bands indicates that they were deposited during higher temperatures than the pink bands. Though this ratio is determined from an average of 30 point analyses on each band, only 2 bands are compared. More bands must be analysed to confirm this pattern.

The differential abundance of trace metals within the bands is another indication of seasonality. In UOM-232 chlorine shows higher abundances in the white bands than the pink bands (Fig. 5.7), this pattern is also indicated by both XRF (Table 5.3) and XPS (Table 5.2) point analyses, however these are both based on the comparison of only 2 points and thus cannot be statistically supported. In more temperate and tropical regions salinity can be used as an indicator of the level of precipitation and evaporation. Increased precipitation leads to decreased salinity and increased evaporation to higher salinity. Given that there is some evidence that the white bands were deposited in higher temperatures we can infer that the increased levels of chlorine in these bands are likely the result of increased ocean salinity due to elevated evaporation.
5.4.2 Mechanism of differential cellular preservation

The cellular structure (Fig. 5.1) is consistent with previous observations [19, 71, 163] that the visible banding on the sample corresponds to a difference in cellular preservation, with the white bands containing significantly better preserved cells than the pink bands. The current mechanism proposed for this differential preservation predicts that there will have been higher magnesium levels in the calcite of the pink bands, which causes loss of cellular detail as it reverts to lower magnesium calcite over time. In UOM-232 however, the magnesium levels are significantly higher in the white bands than the pink bands (Table 5.1), in both the cell walls and vacuoles. As the bands are mineralogically the same (Fig. 5.9), it would not be unreasonable to assume that during diagenesis magnesium levels across the bands if anything would have equalized. But given that the magnesium seems to occur preferentially within the cell walls (Fig. 5.2) it may be that their degradation in the pink band led to a significant loss of magnesium over time. Therefore two possibilities remain, either the magnesium levels as seen in the bands of the fossil are proportionally the same as they were in life, or they are opposite and are higher in the fossilized white bands as the magnesium is preferentially bound to elements of the cell wall. If the former is the case then the mechanism for cellular preservation is nullified, either way more work needs to be done to determine how the magnesium is bound in both bands of the fossil and in modern analogues to determine which is correct. Other problems with the model arise from a probable misinterpretation of Kolesar’s [89] work on magnesium retention in coralline algae calcite by Wright [163]. Kolesar demonstrated that magnesium levels were higher during times of slower growth, whereas Wright infers that magnesium levels were higher in the pink bands, which are consistently thicker than white bands and would therefore seem to indicate faster growth. This may be accountable for if the bands are not the product of seasonal growth, though as discussed in the previous section there is good
evidence for seasonality. This supports the idea that the magnesium levels during life were actually higher in the thinner white bands, and therefore would not have been responsible for the increased cellular degradation seen in the pink bands. We therefore find no evidence to support the current mechanism for the difference in cellular preservation.

5.4.3 Pigment preservation

The presence of boron within *S. jurassica* could not be validated using XPS even at high resolution, indicating that it probably occurs at the ppm level. The presence of phosphate imposed a shift in the binding energy of the associated phosphorus, causing it to occur at the same point that elemental boron would be expected (Fig. 5.3). Boron would therefore not be detectable unless present at significantly higher levels than phosphorus.

The TLE of the UOM-232 bulk material was colourless and contained only alkane/alkene moieties, indicating that the organic pigment was retained in the kerogen (Fig. 5.4), which was brown after calcite removal. This was also seen in the infrared analysis (Fig. 5.6). Py-GCMS analysis of both bulk and kerogen material after thermochemolysis (Fig. 5.5) revealed the presence of protein moieties (dimethylamino phenol and the amino acid phenylalanine) and tetapyrrrole methane. Infrared analysis also indicates the presence of protein (NH, NH$_2$) and carboxylic acid (COOH) groups, though some of these peaks are convolved making identification difficult. Whilst these compounds invidiudally may be found in other pigments and organic compounds, it is the combination seen here that is consistent with the presence of the red pigment phycoerythrin.

Phycoerythrin is a pigment that occurs attached to protein and is therefore known as a phycobiliprotein. It is found in cyanobacteria and certain algae [148] and is a
photosynthetically active light harvesting complex composed of a backbone of open chain tetraoyrroles bonded to various functional groups including carboxylic acids [26, 50]. Whilst phenylalanine is a component amino acid of the phycoerythrin-protein complex it is not the most abundant [53]. Alanine for example is present at higher levels, however the MS range employed in this study was not low enough to detect it. No other amino acids were detected. Proteomic analysis should be used to determine the amino acid inventory of *S. jurassica* and see whether it is different in the different bands.

Py-GCMS analysis reveals no biomarkers indicative of bacteria (hopanes), algae (steranes) [118, p. 361] or sponges (24-isopropylcholestane [101]), however the presence of the pigment phycoerythrin is taxonomically indicative. It only occurs in cyanobacteria and algae, and given that there is no evidence in this study or any previous ones of the presence of bacteria the identification as an algae is far more likely.

### 5.5 Conclusions

The presence of density banding demonstrates that the bands seen in *S. jurassica* are seasonal growth bands. Higher Mg/Ca molar ratios in white bands than pink bands indicates higher temperatures at the time of their deposition. This is supported by the presence of higher levels of chlorine in the white bands, most likely originating from increased evaporation due to the higher temperatures. The identification of Pyrrole tetramethyl, protein moieties and carboxylic acid groups by Py-GCMS and FTIR are together suggestive of the presence of the red algal pigment phycoerythrin. Though individually they are present in other pigments and organic compounds. The likely presence of phycoerythrin indicates that *S. jurassica* is an alga and not a sponge. Phycoerythrin occurs only in cyanobacteria and algae, and there is no evidence to suggest
the sample is bacterial. Boron, from the previously identified borolithochrome pigment, could not be determined as it likely occurs at the ppm level. There is no other biomarker evidence of either algae, bacteria or sponges. The mechanism of differential cellular preservation by differences in magnesium uptake is not supported. Further work should be done to determine whether there are patterns in pH in the bands, as it has been shown in modern calcareous Coralline algae that the combination of high UV radiation levels and atmospheric CO$_2$ led to the inhibition of growth, calcification and caused pigment degradation in certain species \[56\]. Isotopic analysis needs to be carried out as well as the determination of Sr/Ca molar ratios to determine the specific temperatures and salinities present in each band. In addition proteomics analysis would help to determine the amino acids present in *S. jurassica* and see whether they covary with the bands, and artificial maturation of extant red calcareous algae would help to understand the degradation pathways of the phycoerythrin pigment.

Further work should also be done to use isotopic analysis to more accurately determine temperature and salinity changes between the bands. Though there is no independent geochemical proxy for salinity it can be studied by the combination of elemental ratios and isotopic analysis. Oxygen isotopes levels are influenced by a combination of temperature and salinity, therefore the calibration of isotopic analyses with Mg/Ca or Sr/Ca ratios can be used to determine the level of salinity.

**Acknowledgements**

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Chapter 6

Paper 5: A time step analysis of organic material in fossil stromatolites

This chapter contains the following paper which is in preparation to be submitted to the journal *Organic Geochemistry*

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Abstract

Bacterial mats have frequently been proposed as a key agent in the promotion of exceptional preservation in the fossil record. If this is the case however it causes inherent problems in deciphering whether microstructural and geochemical evidence from the analysis of such fossils comes from the organism itself or from the fossilised bacterial mat. This study analyses seven stromatolites of different ages to assess how reliable microstructural, biomarker and general organic geochemical evidence is at determining the presence of bacteria in the fossil record. Two samples contained bacterial hopanes and only one both hopanes and bacterial microstructures. All but the two Precambrian samples however have very similar \( n \)-alkane/\( n \)-alkene distribution patterns, and all seven samples have identical fatty acid distribution patterns. This indicates that all samples are of a common origin which is most likely bacterial given the presence of hopanes in some of the samples. Therefore the presence of bacterial microstructures and/or hopanes is not a generally reliable method of determining the presence of bacteria in the fossil record, and more attention should be paid to their fatty acid distribution pattern.

6.1 Introduction

Bacterial mats have frequently been proposed as vital agents in the exceptional preservation of fossils, the process variously being referred to as ‘bacterial autolithification’ [164], ‘death masks’ [58] and ‘microbial masonry’ [119]. The mechanisms proposed for this mode of preservation include the increased ability to bind to metal ions [38], and the subsequent sharp increase in phosphorus aiding the phosphatisation of soft tissues [156]. All such mechanisms generally invoke two common themes, the isolation of the organism from the external environment, and the hastening of mineral
precipitation. This form of preservation leads to problems in analysing the fossilised organism, namely that if bacterial mats are involved, separating out which microstructures and geochemical signals are from the mat and from the organism itself can be difficult. Geochemical biomarkers can go some way to solving this problem, and there are many biomarkers that are indicative of different taxa, including several different types of bacteria [80, 139, 145, 146]. Not all groups have specific biomarkers however, meaning that the absence of currently known bacterial biomarkers does not confirm the absence of bacteria. Microstructural analysis presents another problem when bacterial bodies are the same size and structure as the morphological features of interest in the preserved organism. This problem has surfaced in the recent work on the preservation of melanosomes in fossil feathers. Many studies claim to be able to determine original colour of the feathers to some extent based on the presence of different types of melanosomes, pigment containing organelles [24, 27, 95, 151, 167]. However melanosomes are the same size and shape as certain bacterial bodies [2, p. 103] [103, p. 67-69], thereby making the distinction very difficult.

In order to examine how reliable bacterial biomarkers and microstructures are overall in determining the presence of bacteria in the fossil record across geological time, this study analyses stromatolites of different ages. Though stromatolites have a history of conflicting definitions [20, 132], they are now generally defined as “macroscopically layered authigenic microbial sediments with or without interlayered abiogenic precipitates” [123]. These microbial communities can be very complex, though they usually have a significant cyanobacterial component, leading to their previously used but erroneous label as blue-green algal mats [12]. Stromatolites are generally identified, at least initially, by looking for common structural traits, such as fine layering [114]. Many indeed are diagnosed purely on this basis with little or no geochemical analysis [20] meaning we cannot often be sure that these specimens are bacterial in origin.

There are two main aims to this study. Firstly to perform a timestep analysis of
6.2 Materials and methods

6.2.1 Samples and preparation

Samples were obtained which had been identified as stromatolites. BHI-7125, 7126, 7137b, 7128 and 7129 are from the collections of the Black Hills Geological Research Institute; UOM-ra41a and UOM-255 are from the Manchester Museum (Fig 6.1). BHI-7125 was collected at Glenwood Springs, Colorado and is from the Coffee Pot Member of the Upper Devonian Dyer Dolomite Formation. Depositional environment was tidal flat muds, stromatolites are interpreted to be formed by the trapping of sediments by blue-green algae [22]. BHI-7126 was sampled at John Green Ranch of Newell, South Dakota, and is from the Lower Oligocene (Chadronian) White River Formation. BHI-7127b was collected from the red desert area of the Eocene Green River Formation, and BHI-7128 from the Early Proterozoic (2.2-2.4 Ga) Biwabik Formation of the Mesabi Iron Range of St. Louis County, Minnesota. BHI-7129 is identified as the Archean (2.58 Ga) Hadrophycus immanus, from the Nash Fork shear zone of the Medicine Bow mountains in Wyoming. Sample UOM-255 was collected from the fossilised tidal flats of the Late Jurassic (Kimmeridgian; 161-145 Ma) shallow-water Jura Carbonate platform (Chevenez-Combe Ronde dinosaur tracksite, Canton Jura, northwest Switzerland, and UOM-ra41a from the Late Jurassic Purbeck Formation of the Dorset coast, UK. To remove any external contamination, the sections cut for analysis
had their outer surfaces ground off and were rinsed in dichloromethane (DCM). Small pieces were cut and etched in 1% HCl (5 minutes) for analysis under scanning electron microscopy (SEM). Samples for organic analysis were ground to a fine powder and decarbonated by reaction with 10% HCl.

6.2.2 Pyrolysis gas chromatography mass spectrometry (Py-GCMS)

2mg of each sample was analysed using normal flash Py-GCMS. Samples were pyrolysed with a CDS (Chemical Data Systems) 5200 series pyroprobe pyrolysis unit by
heating at 600°C for 20 seconds. The fragmented macromolecular components were analysed using an Agilent 7890A gas chromatograph fitted with a HP fused column (J+W Scientific; 5% diphenyl-dimethylpolyolsiloxane; 30m, 0.32mm i.d., 0.25µm film thickness) coupled to an Agilent 5975C MSD single quadrupole Mass Spectrometer operated in electron ionization (EI) mode (scanning a range of m/z 45-650 at 1 scan s\(^{-1}\) with a 4 minute solvent delay; ionization energy 70eV). The pyrolysis transfer line and the injector port temperature was set at 350°C, the heated interface at 280°C, the EI source at 230°C and the MS quadrupole at 150°C. The carrier gas was helium and the samples were introduced in split mode at a 2:1 ratio. The oven was programmed from 40°C (held for 4 minutes) to 300°C at 4°C min\(^{-1}\) and held at this temperature for 5 minutes. Thermochemolysis of samples was carried out by adding 10µl of tetramethylammonium hydroxide solution to the samples, leaving them for 5 minutes, and then analysing them using the same equipment. The scan range was m/z 60-650 with a 15 minute solvent delay. The oven was programmed from 40°C (held for 4 minutes) to 320°C at 4°C min\(^{-1}\) and held at this temperature for 5 minutes. Compounds were identified using the NIST database and by comparison with spectra from the literature.

6.2.3 Scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy (EDS)

Uncoated samples were analysed by variable pressure field emission gun scanning electron microscopy (VP-FEG-SEM) using an FEI XL30 instrument. General secondary electron images of matrix samples were taken at 15keV and 12.6mm working distance. EDS spectra of fossil material was collected using an EDAX energy dispersive x-ray spectrometer at 15keV and 12.6mm working distance for 100 seconds. Microstructures were measured using imageJ [130].
6.3 Results

6.3.1 SEM and EDS

Only UOM-ra41a showed structures that could be potentially interpreted as bacterial. These were dense mats of spherical microstructures (Fig. 6.2) approximately 0.5 µm in diameter (+/- 18 nm; average of 10 measurements). EDS (Fig. 6.2) shows these bodies to be predominantly composed of Fe and O, but also contain Si, Cl, Ca, Ti, Mg and Al. The lack of S indicates that these bodies are not fragmented pyrite frambois.

No other samples were found to contain any such microscopic bodies.

Figure 6.2: SEM images of microscopic bodies in UOM-ra41a and their EDS spectrum. The size and shape of the bodies are consistent with their being bacteria, though the EDS spectrum shows that the bodies are predominantly composed of Fe and O, the lack of S indicates that they are not pyrite frambois.
6.3. RESULTS

6.3.2 Py-GCMS

In general there is no consistent pattern of organic material loss over time, indeed the Oligocene sample (BHI 7126) contained less than one of the Late Jurassic samples (UOM-ra41a, Fig. 6.3). It appears that location and conditions of deposition may be just as, if not more, important to organic preservation than time. The only moieties common to all samples are straight chained $n$-alkane/$n$-alkene doublets, which with the exception of the Precambrian samples show some clear similarities (Fig. 6.4). The Oligocene to Devonian samples have a carbon chain length range from 8 to 17 (UOM-255), 21 (BHI-7125), 22 (BHI 7126 and UOM-ra41a) and 24 (BHI-7127b); maxima occur at 13 (UOM-ra41a and UOM-255), 16 (BHI-7127b and 7125) and 17 (7126). The two Precambrian samples (BHI-7128 and 7129) show low abundances of $n$-alkane/$n$-alkene moieties as well as a pronounced unresolved complex mixture (UCM) (Figs. 6.3, 6.4). $n$-alkane/$n$-alkene carbon chain length in BHI-7128 ranges from 8 to 22, whereas in BHI-7129 the range is only from 21-24; maxima are difficult to determine due to the presence of a large UCM. BHI-7129 only contains $n$-alkane/$n$-alkene moieties, whereas all others also contain benzene and napthalene derivatives. Other moieties the samples show include indene, dihydroindenone, di- and tetrahydronapthalene, biphenyl and fuorene derivatives. Phenanthrene and/or anthrene, as well as dimethoxy thiophene and styrene. There seems to be no consistent pattern of moiety occurrence over time.

After reaction with TMAH more moieties were seen in all samples, with fatty acids (measured as methyl esters), benzoic acid, napthalene and phenol derivatives being the most common (Fig. 6.5), but also including benzene 1,1’-(methylethylidene)bis(methoxy), dimethyl amino phenol, indene, methyl isoindole-dione, benzene and dihydronapthalene derivatives. There again seems to be no consistent observable patterns seen in the moieties over time. The distribution pattern of fatty acid methyl esters however is
Figure 6.3: Py-GCMS total ion current chromatograms of the stromatolite samples. Brackets represent \( n \)-alkane/\( n \)-alkene doublets with the numbers indicating the carbon chain length, # contamination, th thiophene, s styrene, bx benzene, ix indene, iox dihydroindenone, nx naphthalene, ndx dihydronaphthalene, nhx tetrahydronaphthalene derivative, bpx biphenyl, fx fluorene and phnx phenanthrene derivatives, where \( x \) represents the number of carbon atoms in the alkyl group and a box represents the presence of a double bond. Abundances are not to scale.
6.3. RESULTS

Figure 6.4: Py-GCMS m/z 55+57 chromatograms of the stromatolite samples. Brackets represent \( n \)-alkane/\( n \)-alkene doublets and numbers indicate the carbon chain length. Abundances are not to scale.
Figure 6.5: Py-GCMS Total ion current chromatograms of stromatolite samples after reaction with TMAH. Filled circles represent fatty acids (measured as methyl esters), and brackets represent \(n\)-alkane/\(n\)-alkene doublets, numbers indicate carbon chain length. \(ba\) benzoic acid methyl ester, \(bpa\) diamine biphenyl, \(bo\) trimethoxy benzene, \(bi\) benzene, \(1,1'-(\text{methylene})\)bis(methoxy), \(is\) methyl isoindole-dione, \(pa\) dimethyl amino phenol, \(pm\) phenol,4,4’-(1-methylene)bis(methy), \(fa\) fluoroanthrene. \(bx\) benzene, \(ix\) indene, \(nx\) napthalene, \(ndx\) dihydronapthalene, \(nhx\) tetrahydronapthalene, \(phx\) phenol and \(phnx\) phenanthrene derivatives where \(x\) represents the number of carbon atoms in the alkyl group. Abundances are not to scale.
Figure 6.6: Py-GCMS m/z 74 chromatograms of the stromatolite samples after reaction with TMAH. Filled circles represent fatty acids (measured as methyl esters), numbers indicate carbon chain length. Abundances are not to scale.
markedly similar in all samples analysed (Fig. 6.6), each one shows a range in carbon chain length from 8 to 18, with a maxima at 8, 9, 16 and 18. The only sample that varies slightly from the pattern is BHI-7128, where the C_8 and C_9 fatty acids are at a relatively lower abundance than in other samples. There is also some evidence of the presence of hopane groups in the m/z 191 chromatogram of the bulk sample (Fig. 6.7), however they are present at such low concentrations as to make their identification and determination of carbon chain length difficult.

6.4 Discussion

The chromatograms of the bulk material (Fig. 6.3) and that reacted with TMAH (Fig. 6.5) show the presence of a variety of moieties characteristic of general organic matter preservation, such as benzene and napthalene and their derivatives, but no biomarkers specific to certain types of bacteria. BHI-7125 however contains a thiophene moiety, which suggests that it may have been preserved by sulphurization, though this would require more work to confirm. The m/z 191 bulk chromatogram (Fig. 6.7) does show potential hopane groups in only 2 samples, BHI 7127b and UOM-ra41a, however they are present at such low concentrations that their interpretation is difficult. UOM-ra41a is the only sample to display both potential bacterial microstructure (Fig. 6.2), this in
combination with the macroscopic laminated structure is a clear indication of a bac-
terial origin. The microstructures do not show a strong carbon component, however
neither do they have a sulphur peak, indicating that the structures are not disso-
ciated pyrite framboids. There remains the possibility that the remaining samples are
not bacterial in origin, however their similarity in both \( n\)-alkane/\( n\)-alkene and FAME
distribution patterns (Figs. 6.4, 6.6) is at odds with this interpretation and implies a
common origin.

The fact that no odd over even predominance of long carbon chain length \( n\)-alkane/\( n\)-
alkene moieties (\( C_{25}-C_{30} \)) is observed indicates that the organic material in the sam-
plies is not derived from higher plant life \[57, 82\]. The FAME distribution is also
unlike those or either fossil plants or animals \[68, 69, 141, 142\]. The only main differ-
ence observed in the \( n\)-alkane/\( n\)-alkene distribution is the presence of a UCM in both
precambrian samples (BHI-7128 and 7129) (Fig. 6.4). The presence of a UCM is
generally a common feature of biodegraded oils \[152\], and occurs due to a build up of
biodegradation resistant T-shaped molecules (linear chains connected at branch points)
that accumulate over time in sediments \[65\]. The presence of a UCM is an indicator
that these samples contain more mature organic material \[1\]. The FAME distributions
are so similar that all samples show exactly the same carbon chain length range and
maxima (Fig. 6.6). This pattern is maintained regardless of age, unlike the \( n\)-alkane/\( n\)-
alkene distribution. These features indicate that all samples are all of a common origin,
which, given that hopane groups were found in two samples is likely to be bacterial.

That bacterial biomarkers and associated bacterial bodies were not found in the
youngest sample suggests that their preservation is significantly dependent not only on
burial time but also on environmental conditions. There is the possibility that there
are bacterial bodies present in all samples that our analyses were unable to detect,
however this does not preclude the fact that in this case the patterns of alkane/alkene
and FAME distribution were more consistently useful in the identification of bacteria.
The FAME pattern in particular was the only one that was exactly the same in all samples regardless of age.

6.5 Conclusions

In seven fossil stromatolites of different ages only two showed bacterial hopane moieties, and of those only one contained both hopanes and bacterial microstructures. The most reliable evidence for the presence of bacteria was the fatty acid distribution which was identical for all samples, indicating that all fossils were indeed of the same type and supporting the contention that they are all stromatolites. The \( n \)-alkane/\( n \)-alkene signal was also consistent for all samples except those from the Precambrian, where it was dominated by a UCM suggestive of mature organic matter. This suggests that whilst the presence of bacterial biomarkers and microstructural fossils are indicators of bacteria, they are unreliable indicators for such in most fossils and ancient sediments, likely because of preservational biases.

This study shows that care must be taken when trying to eliminate the possibility of fossilised bacterial contamination of preserved soft tissues, as their presence cannot be reliably determined by the presence of bacterial microstructures and biomarkers. The analysis of the distribution pattern of fatty acids however, could potentially be used as a more reliable indicator of the presence of bacteria.

Acknowledgements

The authors wish to thank Peter Larson, Amanda Edwards and Daniel Marty for access to samples.
Chapter 7

Conclusions and future work

7.1 Conclusions

The main aims of this thesis were to use geochemical techniques to study the preservation of melanin and other pigments in the fossil record, and to extend this study to other taxonomic groups than those studied previously. To discuss the relative usefulness of the various geochemical analyses used in the study of melanin in fossils, and to determine how useful biomarkers are at differentiating between bacterial and melanosome bodies in fossils.

Evidence in this thesis showed that:

• There is consistent and reproducible geochemical evidence of melanin within both bird (Chapter 3) and amphibian (Chapter 4) fossils of different ages. However, without using techniques such as alkaline hydrogen peroxide oxidation it is difficult to differentiate between eu- and pheomelanin. Melanosome structure can help in this regard but is not conclusive.

• There is geochemical evidence of the red algal pigment phycoerythrin in the Jurassic fossil Solenopora jurassica suggesting that it is a calcareous alga, as
well as evidence that its visible pink and white banding is a seasonal pattern (Chapter 5).

- To be confident of the identification of melanin in the fossil record a combined morphological and geochemical approach should be taken (Chapter 2). Multiple geochemical techniques will make any study even more robust. Non-destructive chemical mapping can be achieved in large sample using SRS-XRF, and in medium to small sample using ATR-FTIR and EDS. If destructive analysis is permitted then alkaline hydrogen peroxide oxidation is the most reliable technique for distinguishing between eu- and pheomelanin.

- Hopane groups are not reliable biomarkers for the presence of bacteria in fossil stromatolites, and therefore should not be relied upon as indicators of such in other fossils. The fatty acid distribution however was identical in all fossil stromatolites, regardless of age, and different from those patterns seen in fossil plants and animals. This geochemical pattern therefore potentially represents a useful new biomarker for bacteria in the fossil record, which occurs regardless of the presence or absence of bacterial bodies (Chapter 6).

### 7.2 Future work

Further work in this area should cover the following:

- Extending the study of fossil melanin to even more taxonomic groups such as mammals.

- Determining how the age of a fossil determines the geochemical remains of melanin that can be detected.
7.2. FUTURE WORK

- Determining the coordination environment of zinc within eu- and pheomelanin so that it may possibly be used to distinguish between them in fossil material.

- Performing artificial maturation experiments on the red algal pigment phycoerythrin to determine what chemical products we would expect to indicate its presence in fossils.

- Using isotopic analysis on *Solenopora jurassica* to separate the effects of temperature and salinity in the seasonal changes represented by its banding pattern.

- The use of more techniques to study of fossil pigments, including matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS), and time stepped pyrolysis fourier transform infrared spectrometry (Py-FTIR).
Bibliography


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[80] L. L. Jahnke, T. Embaye, J. Hope, K. A. Turk, M. Van Zuilen, D. J. Des Marais,


Appendix A

A.1 Geochemical analysis of a 25 million year old tadpole and its use to determine the presence of melanin

Figure A.1: Linear combination Analysis for fs003b.
Table A.1: Table of organic absorption band assignments for tadpole and matrix (fs003b). X indicates the presence of a group, - its absence, and ? that it may be present but cannot be verified. All Bio rad source data is from the Bio-Rad KnowItAll Informatics System 8.2 Multi-Technique database. AS - asymmetric, S - symmetric, Def - deformation, Comb - combination, Over - overtone

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<th>Functional group</th>
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<th>Intensity</th>
<th>Vibration</th>
<th>Tadpole</th>
<th>Matrix</th>
<th>Eumelatonin standard</th>
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<td>2936-2916</td>
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<td>X</td>
<td>?</td>
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<td></td>
<td>CH</td>
<td>2863-2843</td>
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<td>3350-3200</td>
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<td>Stretch</td>
<td>X</td>
<td>X</td>
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<tr>
<td></td>
<td>OH</td>
<td>1390-1330</td>
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<td>Def</td>
<td>?</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>CO</td>
<td>1260-1180</td>
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<td>Stretch</td>
<td>X</td>
<td>X</td>
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<td>Bio rad Alcohol (R-OH)</td>
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<td>X</td>
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<tr>
<td></td>
<td>OH</td>
<td>1450-1330</td>
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<td>Def</td>
<td>?</td>
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<td></td>
<td>CO</td>
<td>1100-1000</td>
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<td>Stretch</td>
<td>?</td>
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<td>X</td>
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<td>Bio rad Amides (R-CO-NH-C)</td>
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<td>3320-3270</td>
<td>Medium</td>
<td>Stretch</td>
<td>?</td>
<td>?</td>
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<tr>
<td></td>
<td>C=O</td>
<td>1680-1630</td>
<td>Strong</td>
<td>Stretch</td>
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<td>1570-1515</td>
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<td>Stretch</td>
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<td></td>
<td></td>
<td>CN</td>
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<td>Stretch</td>
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<td></td>
<td></td>
<td>NO</td>
<td>1393-1365</td>
<td>Medium Strong</td>
<td>Stretch</td>
<td>?</td>
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<td>Stretch</td>
<td>?</td>
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<td></td>
<td>CH</td>
<td>3040-3010</td>
<td>Medium</td>
<td>S</td>
<td>?</td>
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<td>C=C</td>
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<td>CH</td>
<td>895-885</td>
<td>Strong</td>
<td>Def</td>
<td>?</td>
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<td>Ketone (C-(C=O)-C-OH)</td>
<td>C=O</td>
<td>16401540</td>
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<td>Stretch</td>
<td>X</td>
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<td>Strong</td>
<td>AS stretch</td>
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<td></td>
<td>C=O</td>
<td>14201400</td>
<td>Strong</td>
<td>S stretch</td>
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Table A.2: Results of a combinatorial analysis comparing the XANES of FS003b to standards containing zinc and a modern blonde human hair

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<th>Standards</th>
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<th>ZnSO₄</th>
<th>Zn acetate</th>
<th>Blonde human hair</th>
<th>Chi squared</th>
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<td>fs003b</td>
<td>-0.045</td>
<td>0 (+/-0.018)</td>
<td>0.656</td>
<td>0.389</td>
<td>0.07455</td>
</tr>
<tr>
<td>(+/-0.0067)</td>
<td>(+/-0.050)</td>
<td>(+/-0.041)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A.2 Geochemical evidence of the seasonality, affinity and pigmentation of *Solenopora jurassica*

Table A.3: Table of organic absorption band assignments for UOM-232. X indicates the presence of a group, - its absence, and ? that it may be present but cannot be verified. All Bio rad source data is from the Bio-Rad KnowItAll Informatics System 8.2 Multi-Technique database. AS - asymmetric, S - symmetric, Skel - skeletal

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Bond</th>
<th>Wavenumber range (cm⁻¹)</th>
<th>Intensity</th>
<th>Vibration</th>
<th>Bulk + HCl</th>
<th>Residue + HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkanes (C-CH₃)(₃)</td>
<td>CH</td>
<td>2972-2952</td>
<td>Strong</td>
<td>AS stretch</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>CH</td>
<td>2882-2862</td>
<td>Strong</td>
<td>AS stretch</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CH</td>
<td>1475-1435</td>
<td>Strong</td>
<td>S stretch</td>
<td>X</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>CH</td>
<td>1395-1385</td>
<td>Medium</td>
<td>AS stretch</td>
<td>X</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>CH</td>
<td>1370-1365</td>
<td>Medium</td>
<td>Deformation</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>1255-1245</td>
<td>Medium</td>
<td>Deformation</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

Continued on next page
<table>
<thead>
<tr>
<th>Functional group</th>
<th>Bond</th>
<th>Wavenumber range (cm⁻¹)</th>
<th>Intensity</th>
<th>Vibration</th>
<th>Bulk + HCl</th>
<th>Residue + HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alcohols (Ph-OH)</strong></td>
<td>OH</td>
<td>3350-3200</td>
<td>Variable</td>
<td>Stretch</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>OH</td>
<td>1390-1330</td>
<td>Medium</td>
<td>Deformation</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>1260-1180</td>
<td>Strong</td>
<td>Stretch</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><strong>Amines ((R)₃C-NH₂)</strong></td>
<td>NH</td>
<td>3400-3332</td>
<td>Medium</td>
<td>AS stretch</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>NH</td>
<td>3328-3250</td>
<td>Medium</td>
<td>S stretch</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>NH₂</td>
<td>1650-1590</td>
<td>Medium - strong</td>
<td>Deformation</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><strong>Alkenes (RCH=CHR cis)</strong></td>
<td>CN</td>
<td>1240-1170</td>
<td>Weak - strong</td>
<td>Stretch</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>CN</td>
<td>1038-1022</td>
<td>Weak</td>
<td>Stretch</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>NH</td>
<td>850-750</td>
<td>Strong</td>
<td>Wagging</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><strong>Carboxylic acids (COOH)</strong></td>
<td>OH</td>
<td>3100-2900</td>
<td>Variable</td>
<td>Stretch</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>C=O</td>
<td>1670-1650</td>
<td>Strong</td>
<td>Stretch</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>OH</td>
<td>1440-1395</td>
<td>Weak</td>
<td>Deformation</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>1320-1211</td>
<td>Strong</td>
<td>Stretch</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OH</td>
<td>960-875</td>
<td>Medium</td>
<td>Deformation</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>
Figure A.2: Electron microprobe maps of the pink and white bands of *S. jurassica*
Figure A.3: Synchrotron rapid scanning x-ray fluorescence maps of the 'lighter' elements in UOM-232 with a photographic reference of the area scanned.
Figure A.4: Synchrotron rapid scanning x-ray fluorescence maps of the 'heavier' elements in UOM-232 with a photographic reference of the area scanned.