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Studies on carbon and DNA preservation in allophanic soils and paleosols on Holocene tephras in New Zealand

A thesis

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of the requirements for the degree

of

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Abstract

The recovery of ancient DNA (aDNA) and palaeoenvironmental DNA (PalEnDNA) from soils and sediments has enabled detailed paleontological and ecological records to be obtained. Because of the superior ability of allophanic soils and Andisols to adsorb considerable soil organic matter (SOM), and to slow the rate of carbon turnover in such soils, I hypothesised that environmental DNA can be preserved in allophanic soils and Andisols together with SOM, and that such DNA may be able to help reveal past environments. I characterised the preserved SOM in stratigraphic successions of buried paleosols on Holocene tephras of known age in northern New Zealand and attempted to extract and analyse the DNA to search for possible PalEnDNA.

Using synchrotron radiation-based carbon near-edge X-ray absorption fine structure (C NEXAFS) spectroscopy, I found the compositions and proportions of carbon functional groups of SOM in allophanic paleosols on precisely-dated Holocene tephras (c. 12,000 to 1718 calendar [cal] years BP) at four sites in northern New Zealand were similar and dominated by carboxylic functional groups, with subordinate amounts of quinonic, aromatic, and aliphatic groups. Differences in clay and allophane contents, stratigraphic position, age, parent-tephra composition (andesitic versus rhyolitic), and mode of soil origin (retardant versus developmental upbuilding pedogenesis) seemed not to affect the structure of SOM over time. The similarity of the SOM in allophanic paleosols of different ages implied its preservation in allophanic soils over a long time, and the presence of quinonic carbon, normally very susceptible to degradation and transformation, shows that the allophanic soils have protected it very effectively. The quinonic carbon in buried horizons is thus indicative of the preservation of biological materials originating from bacteria and plants. The SOM originated through upbuilding pedogenesis: as soil genesis began in a newly-deposited tephra at the soil surface, allophane formed and it sequestered SOM from the modern (surface) organic cycle dominated by inputs from broadleaf-podocarp forest. Ongoing tephra deposition then caused the land surface to rise so that once-surface horizons were buried more deeply and hence became increasingly divorced from the modern organic cycle over time. The SOM adsorbed when the soil horizon was at the land surface was preserved in the buried soils because a fractal pore network of allophane aggregates and nanopores encapsulated and shielded the

'old' or relict SOM (including quinonic carbon) derived from past environments of the Holocene.

To provide fundamental knowledge about the interaction of allophane, DNA, and SOM in soils, I examined the adsorption capacity and adsorption mechanisms of salmon-sperm DNA on pure synthetic allophane and humic acid-rich synthetic allophane. The pure synthetic allophane was able to adsorb up to 34 $\mu g\ mg^{-1}$ of salmon-sperm DNA, but the humic acid-rich synthetic allophane adsorbed only 3.5 $\mu g\ mg^{-1}$ of salmon-sperm DNA. Salmon-sperm DNA was adsorbed chemically through its phosphate group to the aluminol groups of synthetic allophane, and adsorbed chemically through humic acid covering the synthetic allophane spherules, and thus became bound indirectly to synthetic allophane. The chemical adsorption of salmon-sperm DNA on synthetic allophane led to the aggregation of allophane spherules to form nanoaggregates and microaggregates, and ~80% of total adsorbed DNA on allophane was held physically within the interstices (pores) between allophane spherules and nanoaggregates. The encapsulated DNA within the stable allophane-DNA aggregates may not be accessible to enzymes nor microbes, hence enabling DNA protection and preservation in allophanic soils. By implication, organic carbon is therefore likely to be sequestered and protected in allophanic soils (Andisols) in the same way as demonstrated here for DNA – that is, predominantly by encapsulation within a tortuous network of nanopores and submicropores amidst stable nanoaggregates and microaggregates, rather than by chemisorption alone.

A novel two-step method was developed to isolate DNA from allophane, based in part on experiments devised to extract salmon-sperm DNA from synthetic allophane. The two-step method for DNA extraction from allophanic soils is based on (1) chelating DNA and blocking adsorptive sites on allophane using EDTA and phosphate, respectively, and (2) dissolving allophane using acidified ammonium oxalate. The DNA yield from three allophanic paleosols on Holocene tephras was up to 44.5 $\mu g\ g^{-1}$ soil (oven-dry basis). The extracted DNA was then successfully purified via gel electrophoresis followed by a gel purification kit, and the amplifiable and sequenced DNA extracted from a paleosol (which had been at the land surface for around 4000 years between c. 9423 and c. 5526 calendar years BP) on Rotoma tephra contained New Zealand endemic and exotic plants that differed from the European grasses growing

currently on the land surface. The difference in vegetation indicates that the DNA extraction method I (with others) have developed is able to access environmental DNA originating from previous vegetation cover. The DNA extraction method could be used to facilitate the search for possible PalEnDNA in allophanic paleosols for reconstructing the past terrestrial environments as well as to investigate the biodiversity in allophanic soils and the origins of SOM.

Allophanic soils are demonstrably able to protect environmental DNA from degradation for a long period of time, and such DNA is able to reveal past environments. However, the duration over which that environmental DNA can be preserved in allophanic soils needs to be resolved, and additional investigations of gene diversity in allophanic paleosols of different ages using high-throughput sequencing (HTS) are required to determine the taxonomic profiles recovered in paleosols and to rule out contamination of modern DNA.

Acknowledgements

It has been a privilege to undertake the project with my advisors, Prof David Lowe, Prof Louis Schipper, Dr Jock Churchman, and Dr Ray Cursons, and I acknowledge them for their great help and encouragement during the journey of this research. I have been additionally grateful to Prof Lowe and Dr Churchman for providing the opportunity to undertake the research; and their constant support, brilliant research concepts, guidance and enthusiasm are appreciated. I especially thank Prof Schipper and Dr Cursons for their great company and support, and I am grateful to Dr Churchman for his willingness to make time and trips to Waikato University for discussions and to reply to my many e-mails. All of my advisors have been wonderful and they have made great contributions to supervise me and facilitate the consummation of this PhD thesis. We are a good team and I have been very proud to be one of your students.

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Chapter 1 Introduction

1.1. Background and significance of study

Palaeontology, archaeology, and biology have been radically transformed by the study of ancient DNA (aDNA) (e.g. Willerslev et al., 2003; Willerslev and Cooper, 2005; Hofreiter, 2008; Rawlence et al., 2014; see review paper in Appendix D). A new area of application is the recovery of environmental DNA from soils and sediments (Chelomina, 2006; Haile et al., 2007; Boere et al., 2011; Andersen et al., 2012; Pederson et al., 2015) because these materials potentially provide a detailed paleontological and ecological record almost everywhere, irrespective of whether a fossil record exists. Andisols (Soil Survey Staff, 1999) and other allophane-bearing (allophanic hereafter) soils have been reported to stabilise organic carbon and protect organic matter from degradation over a long time (e.g., Parfitt et al., 2002; Parfitt, 2009; Baisden et al., 2010; Chevallier et al., 2010), and therefore they could be potential reservoirs for DNA as well. A key advantage of Andisols and allophanic soils in central North Island, New Zealand, is that the associated tephra (volcanic ash) deposits, which form the soil parent materials, have been precisely-dated using radiocarbon and Bayesian-based age modelling (Lowe et al., 2008, 2013), and the ages can be transferred from site to site using the principles of stratigraphy together with mineralogical and geochemical analysis via tephrochronology (Alloway et al., 2007, 2013; Lowe, 2011). As well as providing ages, the tephra layers provide tie points to connect sequences of tephra-derived buried soils with palynological sequences obtained from lake sediments or peats containing the same tephra layers (Lowe, 1988; Newnham et al., 1989, 2007). Hence the detailed records of past environments and climates obtained from sedimentary records for certain periods of time (e.g., the early or middle Holocene), bounded by tephra layers of known age, provide a chronostratigraphic basis to compare with those records obtained from paleosols (soils of landscapes or environments of the past) dating to the same period (Lowe and Alloway, 2015).

I hypothesize that organic matter and DNA originating from organisms (including animals, insects, microbes, and plants) could be adsorbed and protected within tephra-derived Andisols and buried allophanic soils over a long period of

time, and the organic matter and DNA remain intact in soils after the burial of land surfaces by newly-erupted tephra deposits. The preserved organic matter and genetic materials in paleosols on tephras of known ages may provide a history of terrestrial environments at certain times. If so, buried allophane-rich soils could then be considered a reservoir for terrestrial palaeoenvironmental DNA (PalEnDNA) (Rawlence et al., 2014), potentially markedly facilitating studies involving archaeology, biology, and biological evolution. Therefore, my project, in attempting to provide insight in the search for possible PalEnDNA in buried allophane-rich paleosols, is of significance for developing a new approach to reconstruct past terrestrial environments and climates using the unique New Zealand resources including the well-documented volcanic record and allophane-rich soils (Lowe et al., 2010). Moreover, the methodologies developed in this study to examine organic matter and environmental DNA in allophanic soils could be of great benefit to soil-based research in New Zealand and internationally, for issues such as carbon sequestration and the assessment of biodiversity in deep allophanic soils and Andisols.

1.2. Aim and objectives

The overall aim of this study is to establish a scientific basis for (1) the potential of allophanic soils and Andisols to preserve organic materials including DNA over a long period of time, and (2) the potential of PalEnDNA (if it exists) in paleosols on Holocene tephras to reveal past terrestrial environments in northern New Zealand. I examine the structure of preserved organic matter in stratigraphic successions of paleosols (buried soils) on Holocene tephras to determine the alteration of organic matter with time, to study where and how organic matter and DNA are held on allophane (the dominant clay nanomineral in well-drained tephra-derived soils), and to develop a useful technique to extract DNA efficiently from allophanic soils and paleosols and hence facilitate the search for possible PalEnDNA in such paleosols in New Zealand and elsewhere.

The aim is addressed from two perspectives including (i) soil organic matter and (ii) PalEnDNA. To achieve the aim using an experimental approach, my main objectives are:

(1) To examine the ability of synchrotron radiation-based techniques to characterise organic matter sequestered by some New Zealand allophanic soils and to interpret the interactions between allophane and DNA in soils.

(2) To characterise the functional groups of preserved organic matter and to detect the possible presence of DNA in buried allophane-rich paleosols on Holocene tephras of different ages at four sites (two sites exhibiting developmental upbuilding pedogenesis and two showing retardant upbuilding pedogenesis) in North Island, New Zealand, using synchrotron radiation-based carbon near-edge X-ray absorption fine structure (NEXAFS) spectroscopy, and to find out if (and why) functional groups are persistent in allophanic soils over a long period of time.

(3) To examine adsorption mechanisms of salmon-sperm DNA (well-characterised and commonly used to study physicochemical interactions of DNA with other binding agents and materials) on synthetic allophane, through applications of classical analytical techniques and synchrotron radiation-based phosphorus X-ray absorption near-edge structure (P XANES) spectroscopy and Fourier transform infrared (FTIR) spectroscopy, to evaluate the implications for DNA storage and carbon stabilisation in allophanic soils and Andisols.

(4) To develop an effective method for DNA (including intercellular and extracellular DNA) extraction from allophanic soils and to assess the taxonomic diversity of vegetation recorded in a c. 4000-year-old paleosol formed on Rotoma tephra deposited c. 9400 calendar [cal] years BP at a site near Mt Tarawera in central North Island.

1.3. Thesis structure and chapter outline

My thesis is divided into 7 chapters, including introduction (Chapter 1), literature review (Chapter 2), four manuscripts of my main findings (Chapters 3 to 6), and a synthesis/conclusions (Chapter 7). Chapters 3−6 comprise published or submitted manuscripts in word format as accepted by, or submitted to, a book or journals.

Chapter 1 is a general introduction describing the issues with which I am concerned, my main aim, and the objectives to be undertaken to achieve the aim.

Chapter 2 performs a function as research background and a review of previous work, demonstrating the theoretical basis and derived hypothesis that allophane protects organic carbon and DNA from degradation, and an overview of analyses and facilities to be employed and their advanced efficiency of analytical resolution.

Chapter 3 is a book chapter showing my preliminary results including (1) carbon near-edge X-ray absorption fine structure (C NEXAFS) spectra for clay samples extracted from a New Zealand allophane-rich Andisol profile (Tirau series) and (2) salmon-sperm DNA adsorption isotherm on synthetic allophane.

Chapter 4 is a manuscript that has been submitted to *Quaternary Science Reviews* and documents research that characterises functional groups of the preserved organic matter in paleosols on Holocene tephras over four sites in New Zealand, using synchrotron radiation. This chapter discusses the differences of composition and proportions of functional groups with time, depth, parent-tephra composition (andesitic versus rhyolitic), and explains their origin and preservation using a model of soil genesis (developmental versus retardant upbuilding).

Chapter 5 is a journal article published in *Applied Clay Science* concerning adsorption mechanisms of salmon-sperm DNA on synthetic allophane and the implications for carbon sequestration in allophanic soils and Andisols.

Chapter 6 is a manuscript that has been submitted to *Geoderma* and records the work associated with the development of techniques for DNA extraction and purification from allophane-rich soils, and the genera/species of DNA found in paleosols on Rotoma tephra (c. 9400 cal years BP) at a site near Mt Tarawera (Brett Rd) in central North Island.

Chapter 7 provides a synthesis of the main results described in Chapters 3 to 6 and conclusions from the most important findings.

1.4. Note regarding Marsden Fund

This doctoral project was supported largely by the New Zealand Marsden Fund, administered through the Royal Society of New Zealand, for the project entitled “New views from old soils: testing the reconstruction of environmental and climatic change using genetic signals preserved in buried paleosols” (contract UOW1006 to David J. Lowe [University of Waikato] and G. Jock Churchman and

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Chapter 2 Literature review

In this review, I focus on six topics relating to the studies undertaken towards achieving my main aim: (1) Quaternary volcanism, tephras, and tephra-derived soils and paleosols in North Island, New Zealand; (2) Andisols, allophanic soils, and allophane; (3) carbon storage in Andisols; (4) application of synchrotron radiation to soil carbon chemistry; (5) adsorption of environmental deoxyribonucleic acid (DNA) in Andisols; and (6) ancient DNA (aDNA) and palaeoenvironmental DNA (PalEnDNA). The relevant literature relating to the approaches I used to achieve my objectives is included in chapters 3 to 6 containing the experimental results.

2.1. Quaternary volcanism, tephras, tephra-derived soils and paleosols in central North Island, New Zealand

North Island of New Zealand lies on the leading edge of the Australian Plate and is being obliquely underthrust by the subducting oceanic Pacific Plate, resulting in extensive rhyolitic and andesitic volcanism and tectonic-related features that dominate the geological development of much of the central North Island (Kamp, 1984; Cole et al., 1986; Wilson et al., 2009; Leonard et al., 2010; Graham, 2015) (Fig. 2.1). Most of the tephra deposits derived from eruptions in the late Quaternary (past c. 50,000 calendar [cal] years) in the North Island of New Zealand have been identified by their dominant ferromagnesian mineralogy, their stratigraphic positions, and radiocarbon dates (Lowe, 1988, 1990; Froggatt and Lowe, 1990; Shane, 2000; Lowe et al., 2008) (Fig. 2.2). Many of the most-widespread and younger tephras (<c. 25,000 cal years old) have been precisely-dated using radiocarbon and Bayesian age modelling (Lowe et al., 2013; Vandergoes et al., 2013). The ages of tephras can be transferred from site to site using the principles of stratigraphy together with mineralogical and geochemical analysis of the primary components including volcanic glass, an age-equivalent correlational and dating technique referred to as tephrochronology (Alloway et al., 2007, 2013; Lowe, 2011; Lowe and Alloway, 2015).

The use of tephrochronology, together with palynological data obtained from analyses of lake sediments or peats containing the same tephra layers, has provided important insight into the history of vegetation and climate for the late

Quaternary of New Zealand (e.g. Newnham et al., 1989, 1995, 1999, 2007, 2013; Barrell et al., 2013).

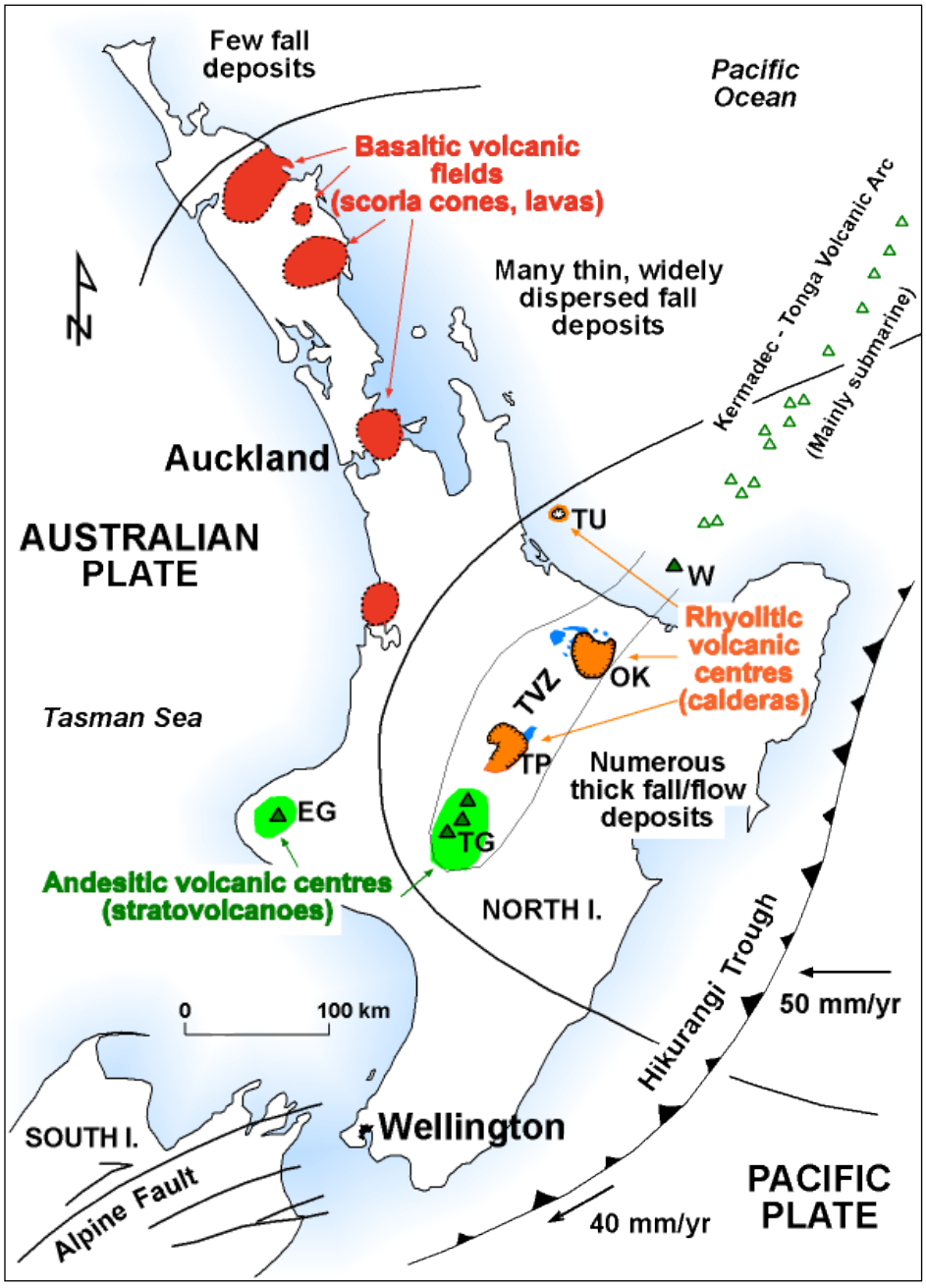

Fig. 2.1 Map showing plate tectonic setting, the main volcanic centres that produced parent materials for many tephra-derived soils, and the general dispersal of tephra on North Island. EG, Egmont/Taranaki volcano; TG, Tongariro Volcanic Centre; TP, Taupo Volcanic Centre; OK, Okataina Volcanic Centre (includes Mt Tarawera and Haroharo volcanic complexes); TU, Tuhua Volcanic Centre (Mayor Island); W, Whakaari (White Is.) (from Lowe and Palmer, 2005).

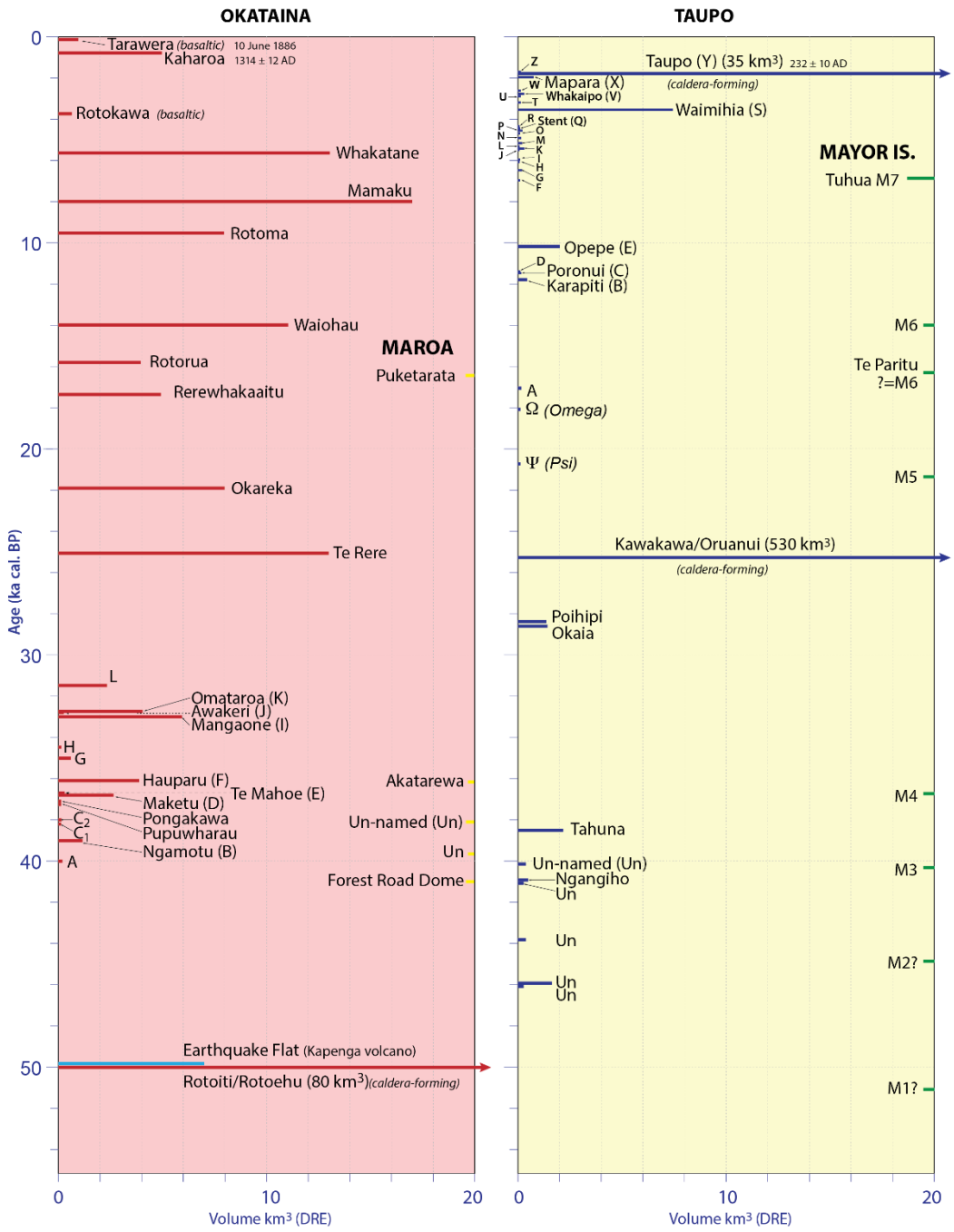

Fig. 2.2 Summary of stratigraphic relationships, ages, and volumes (as non-vesiculated, void-free magma or dense-rock equivalent, DRE) of tephras erupted from Okataina, Taupo, Maroa, Mayor Island (Tuhua), and Kapenga (Earthquake Flat tephra) volcanoes in North Island, New Zealand, since c. 50,000 cal years ago (from Lowe et al., 2015; modified after Lowe, 2011, and based mainly on Wilson et al., 2009; Leonard et al., 2010).

Many of the soils that result from the weathering of tephras are distinct because of their unique properties (see next section 2.2.), and such soils with andic properties or a high glass content have been designated their own order of

Andisols in *Soil Taxonomy* since 1990 (Parfitt and Clayden, 1991; Soil Survey Staff, 1999). (Andisols can also be derived from non-volcanic materials.) The genesis of soils developed on tephras is usually different from the 'classical' topdown processes of pedogenesis that normally occur on rock or other deposits on stable sites, because tephra deposits are added intermittently to the land surface (either as thick layers or incrementally, or both) so that the soil forms by upbuilding pedogenesis, forming a multi-layered or composite Andisol that reflects the interaction of 'competing' geological and pedological processes (Neall, 2006; Lowe, 2010; Lowe and Tonkin, 2010; Lowe et al., 2012; see also Muhs et al., 2004) (Fig. 2.3). The tephra-derived soils (including Andisols) and paleosols (soils of an environment or landscape of the past) in New Zealand, and their nature, genesis, and classification, have been well studied (e.g. Gibbs, 1968, 1970; Lowe and Palmer, 2005; Hewitt, 2010; Lowe, 2010; Lowe and Tonkin, 2010; Lowe et al., 2014) (Fig. 2.4).

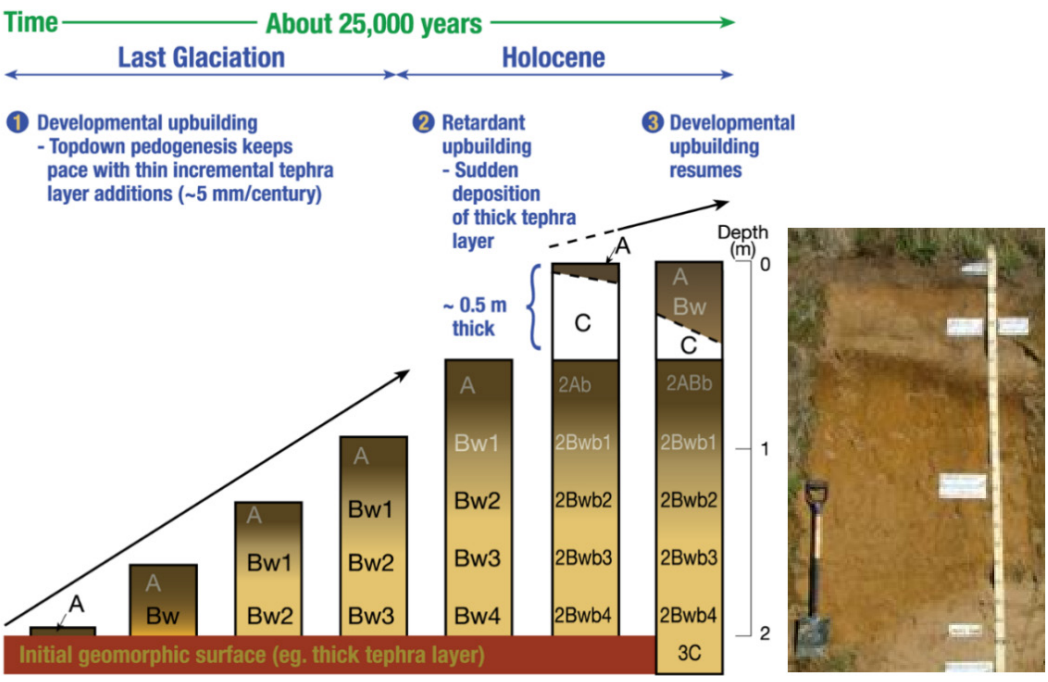

Fig. 2.3 Model of upbuilding pedogenesis in tephra deposits and the formation of a multisequal profile (photo at right) over c. 25,000 years in South Waikato, central North Island. The underlying initial geomorphic surface is marked here in photo at right by Kawakawa Tephra (c. 25,400 cal yrs BP) and horizon 3C. In phase 1, thin, distal tephras accumulate slowly whilst topdown processes imprint weak horizonation features on them as the land surface gradually rises (developmental upbuilding). In phase 2, the sudden deposition of a tephra layer ~0.5 m thick, Taupo ignimbrite here (c. 232 AD), from a particularly powerful eruption, buries the antecedent soil, isolating it from most surface processes so

that topdown processes begin anew on the freshly deposited tephra (retardant upbuilding). In phase 3, incremental tephra deposition on the new soil continues and developmental upbuilding resumes (from McDaniel et al., 2012).

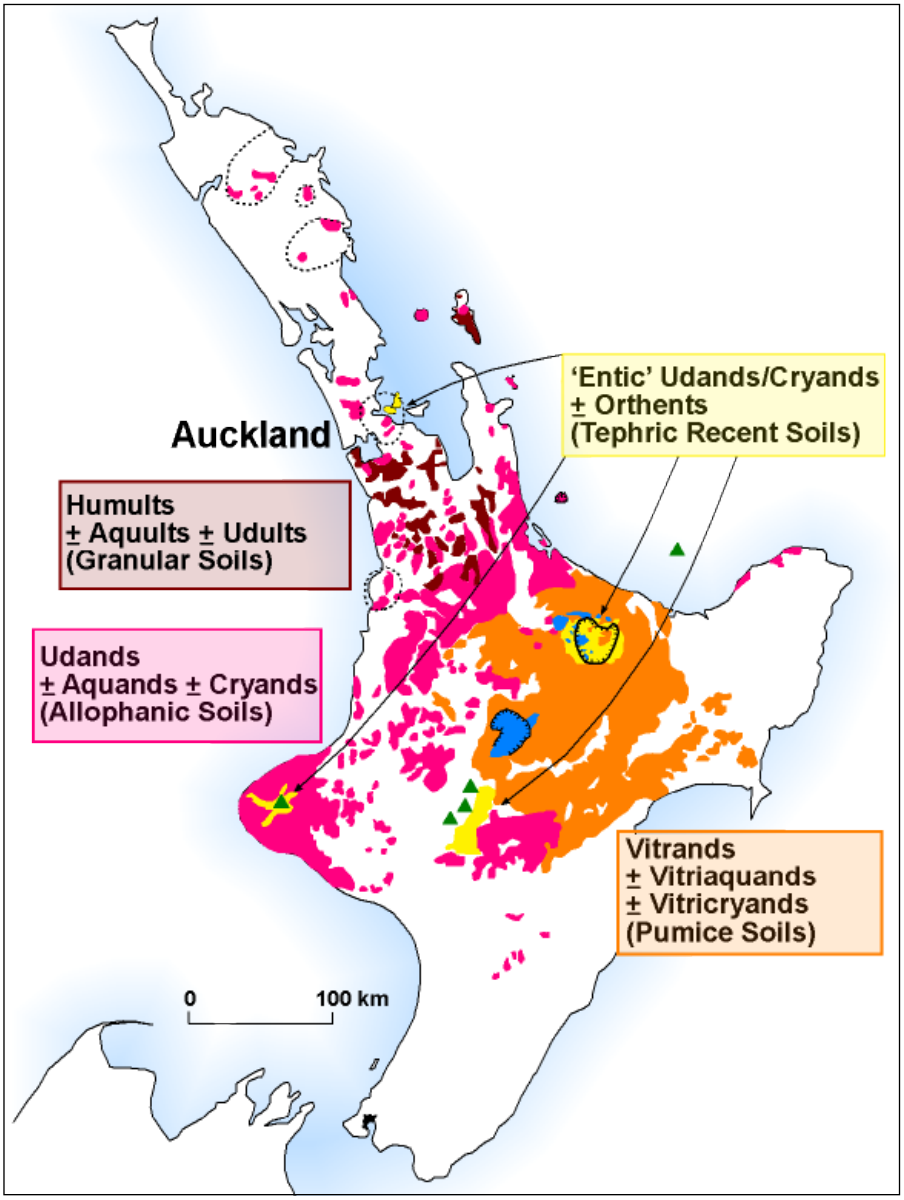

Fig. 2.4 Distribution of four main groupings of tephra-derived soils in North Island (after Kirkpatrick, 1999, 2005, based on Rijkse and Hewitt, 1995; modified from Lowe and Palmer, 2005). Names of suborders and great groups are those of *Soil Taxonomy* (Soil Survey Staff, 1999) with orders of the *New Zealand Soil Classification* in parentheses (Hewitt, 2010).

Because these tephras provide distinctive isochrons in a range of different environments in North Island of New Zealand, the development of secondary minerals can reveal insights into past climates (e.g. Birrell and Pullar, 1973; Parfitt et al., 1983; Stevens and Vucetich, 1985; Hodder et al., 1990; Lowe and

Percival, 1993; Newnham et al., 1999, 2003; Churchman and Lowe, 2012). Therefore, paleosols on tephras of different ages (Fig. 2.5) may provide critical information about past terrestrial environments and climates at certain times (e.g. the early or middle Holocene).

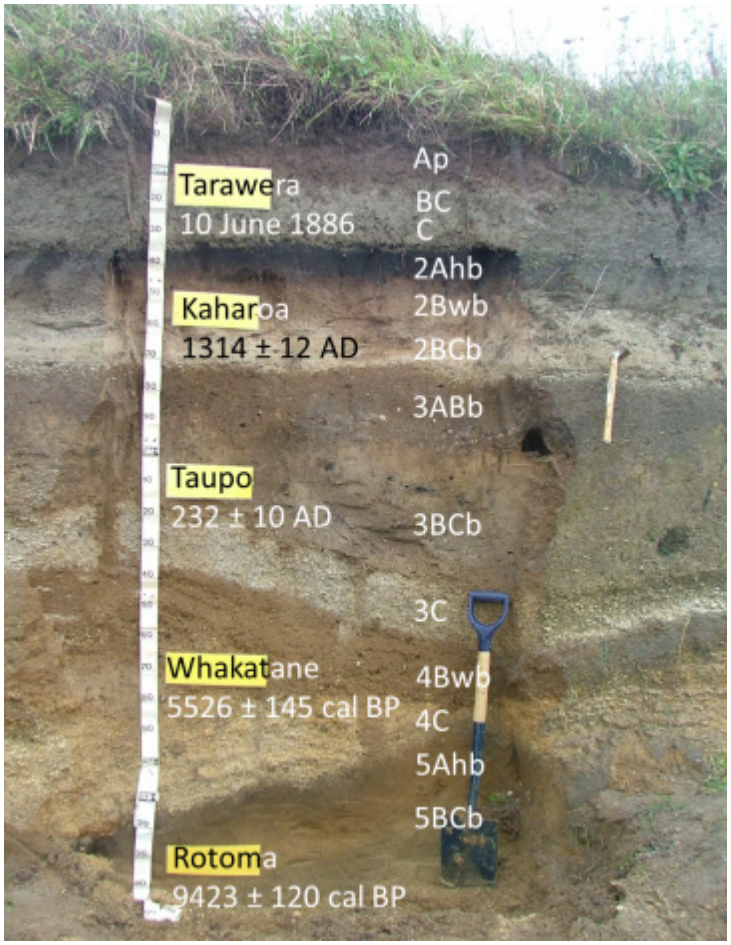

Fig. 2.5 Example of a multi-layered Andisol in New Zealand, showing five distinct Holocene tephra deposits of different ages, and the associated soils (all but Tarawera are now buried) on them (paleosols) (see McDaniel et al., 2012). (Photo: D. J. Lowe)

2.2. Andisols, allophanic soils and allophane

2.2.1. Characteristics of Andisols and allophanic soils

Clay minerals and clay-sized (<2 μm) colloids in soils play important roles to adsorb moisture, nutrient, and soil organic matter (SOM) because clays and associated colloids possess high surface areas and adsorptive capacities. Among

mineral soils, Andisols (an order in *Soil Taxonomy* as noted earlier) or Andosols (an equivalent order in *World Reference Base*) (McDaniel et al., 2012) are dominated by nanocrystalline clay minerals (nanominerals) including allophane and ferrihydrite, which are typically derived from the synthesis of the products of weathering of fragmental volcanic deposits (especially glass) referred to as unconsolidated pyroclastic deposits or tephra (Churchman and Lowe, 2012; McDaniel et al., 2012). Imogolite, which is paracrystalline, may also occur with the nanominerals as can considerable amounts of Al- and Fe-humus complexes where Al is limited (Percival et al., 2000; Churchman and Lowe, 2012; McDaniel et al., 2012). Because of the occurrence of these nanocrystalline clay minerals and organo-clay complexes, Andisols are usually highly porous and 'light' with low bulk densities and high porosity and they have an inclination to adsorb SOM so that more than ~1.8% of soil organic carbon is sequestered in Andisols, which represent about 1% of the ice-free land surface (Allbrook, 1985; Parfitt, 1990; Eswaran et al., 1993; Soil Survey Staff, 1999; Dahlgren et al., 2004; Goh, 2004; McDaniel et al., 2012; Matus et al., 2014). Moreover, the metal-humus complexes also strongly bind to phosphate (high P retention and fixation) in soils, leading to a P deficiency of microorganisms (Ugolini and Dahlgren, 2002); however, humic and fulvic acids are known to block (or hamper) P adsorption capacity in Chilean Andisols (Mora and Canales, 1995).

2.2.2. Nature of allophane

Allophane is a secondary nanocrystalline mineral that forms essentially via four mechanisms: (1) dissolution of Al from volcanic glass, (2) hydrolysis of the Al into solution to form gibbsitic sheets, (3) dissolution of Si from volcanic glass (via breaking of Si−O bonds) to form monosilicic acids in solution, and (4) reaction between the gibbsitic sheets and monosilicic acids to generate allophane (Fig. 2.6) (Hiradate and Wada, 2005; Churchman and Lowe, 2012; McDaniel et al., 2012). Allophane has a formula in the range $(1\text{-}2)SiO_2 \cdot Al_2O_3 \cdot (2\text{-}3)H_2O$).

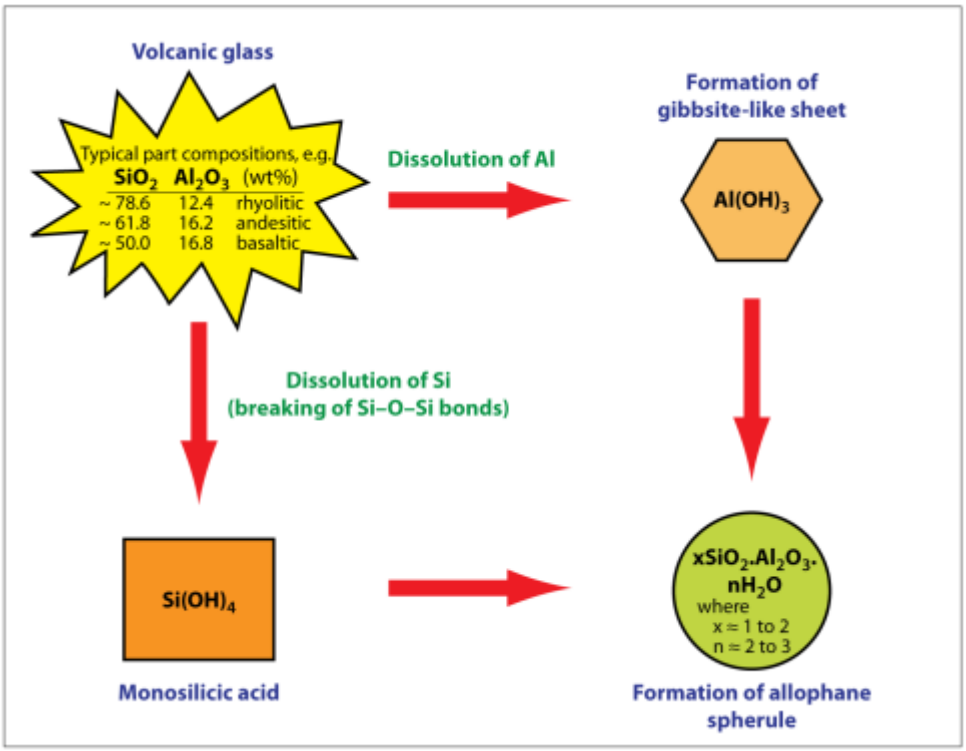

Fig. 2.6 Volcanic glass compositions and their dissolution to release Al and Si to synthesise as allophane (from McDaniel et al., 2012; after Hiradate and Wada, 2005).

Allophane comprises tiny hollow spherules that are 3.5 to 5 nm in diameter (Fig. 2.7) , and therefore these have extremely large surface areas − up to ~1125 $m^2\ g^{-1}$ (Henmi and Wada, 1976; Parfitt et al., 1980; Allbrook, 1983; Parfitt, 1990; Ohashi et al., 2002; Parfitt, 2009). The broken-bond defects, also called perforations, on the surface of allophane spherules are approximately 0.3 to 0.7 nm in diameter (Abidin et al., 2007; Creton et al., 2008). The protonation and deprotonation of aluminol groups exposed at the defects give rise to the variable (pH-dependent) charge of allophane, and therefore results in adsorption of anions (Clark and McBride, 1984; Parfitt, 1989) and cations (Yuan et al., 2002). The interaction between allophane and negatively-charged humic substances is illustrated below in equation (1) (from Yuan and Theng, 2012).

$$[\text{allophane–Al}(H_2O)(OH)]^0 + [R.COO]^{n-} \Rightarrow [\text{allophane–Al}(H_2O)(R.COO)]^{(n-1)-} + OH^- \quad (1)$$

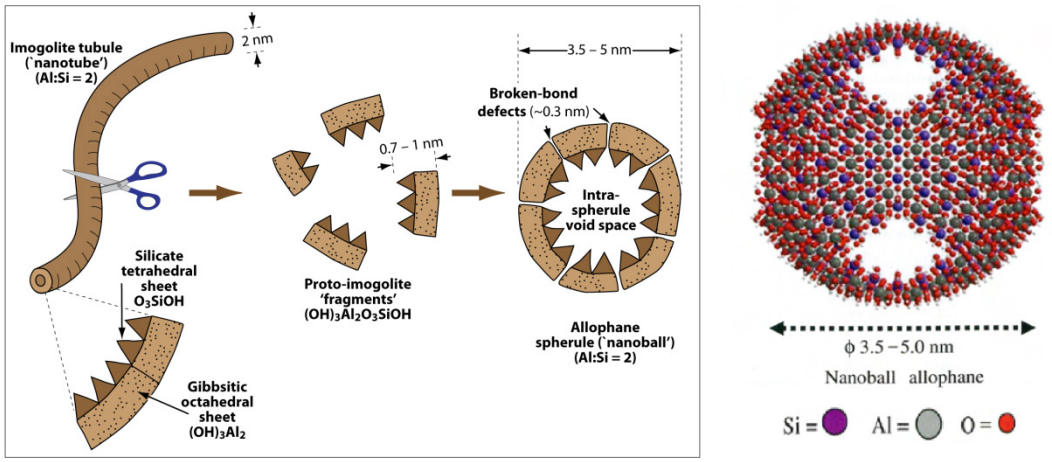

Fig. 2.7 Left: nanoscale dimensions and composition of imogolite tubules and allophane spherules (from McDaniel et al., 2012). Right: the atomic structure of and spherical morphology of a hollow allophane 'nanoball' (from Theng and Yuan, 2008).

Unlike long-range ordered clay minerals, allophane behaves as gels during drying with an irreversible shrinkage that leads to the loss of specific surface area (Allbrook, 1992; Gray and Allbrook, 2002; Woignier et al., 2007). The gel-like allophane spherules have surficial moisture films on their surface, allowing them to remain discrete (Allbrook, 1985), and allophane spherules tend to cluster to form sub-rounded stable aggregates up to about 100 nm in diameter (Calabi-Floody et al., 2011). The pores between the aggregates provide space for the storage and protection of carbon in allophanic soils (Strong et al., 2004; Chevallier et al., 2010; Calabi-Floody et al., 2011).

2.3. Carbon storage in allophanic soils and Andisols

2.3.1. Adsorption mechanisms of carbon

As noted above, it is the broken-bond defects on the surface of allophane spherules which comprise aluminol groups that give rise to the variable (pH-dependent) charge of allophane, and the point of zero charge (PZC) of allophane ranges from pH 7.9 (for Al/Si 2.0 allophane) to 6.7 (for Al/Si 1.6 allophane) (Harsh, 2012). Therefore, allophane presents positive charges in acid soils (pH <7), resulting in an anion exchange capacity (Clark and McBride, 1984; Parfitt, 1989). Allophane is able to adsorb humic substances chemically through their

carboxylic functional groups or deprotonated groups (Theng et al., 2005; Theng and Yuan, 2008; Yuan and Theng, 2012).

"Aggregate hierarchy" is a concept referring to a range of aggregates of various sizes and it has been considered to be essential in any discussion of the nature and genesis and stability of soil structure (Dexter, 1988; Oades and Waters, 1991; Tisdall, 1991; Golchin et al., 1994; Six et al., 2000a, 2000b). The sizes of soil aggregates influence the distribution of SOM (Lehmann et al., 2008): variable functional groups of organic matter can be found at different locations of soil aggregates. Six et al. (2000b) showed that the macroaggregates (>250 μm) sequestered ~1.65 times more carbon than microaggregates (<250 μm), and the microaggregates are bound together into macroaggregates to provide yet more porosity for possible carbon storage.

2.3.2. Stabilisation and protection of carbon

The molecular structure of SOM itself does not control the life-time of SOM (Schmidt et al., 2011). The key process in the protection of SOM and stabilisation of soil organic carbon (SOC) in soil microaggregates is evidentally strongly related to a porous structure and the structure of aggregate packing (McCarthy et al., 2008). Through experimental approaches, Mikutta et al. (2005) also suggested that the capacity of soil to protect organic matter against decomposition is mainly controlled by the content of poorly crystalline minerals (i.e. nanominerals). The size and aggregation of allophane spherules accord with the essential factors of carbon stabilisation and protection. As a result, the porous and adsorptive allophane soils are strongly associated with SOC (Buurman et al., 2007; Parfitt, 2009; Calabi-Floody et al., 2011, 2014). Stable allophane-humus complexes can be formed. SOC stabilisation and protection in allophanic soils occur via the formation of clay-humus complexes and aggregates (Huygens et al., 2005). Chevallier et al. (2010) showed a positive correlation between allophane content and SOC content, and the bioavailability of SOC significantly decreased with the size of allophane aggregates and with the extent of what they termed the fractal cluster, or fractal pore structure. The work of Calabi-Floody et al. (2011) also illustrated that ~12% of carbon within aggregates of natural allophane spherules resisted intensive peroxide treatment. The occlusion and protection of SOC against degradation in allophanic soils have been attributed to (1) poor

accessibility of SOC to microbes and enzymes because the pore networks within allophane aggregates are tortuous (such pore networks were called "nanolabyrinths" by Chevallier et al., 2010), and hence the diffusion pathway for enzymes is constrained, and (2) the behaviour of enzymes is restricted by allophane because of the surface charge and Al toxicity of allophane (Lützow et al., 2006; Matus et al., 2014). Therefore, carbon turnover in Andisols, as measured by bomb-derived ^{14}C, is much slower than that in other mineral soil orders (Parfitt et al., 2002; Parfitt, 2009; Baisden et al., 2010) (Fig. 2.8).

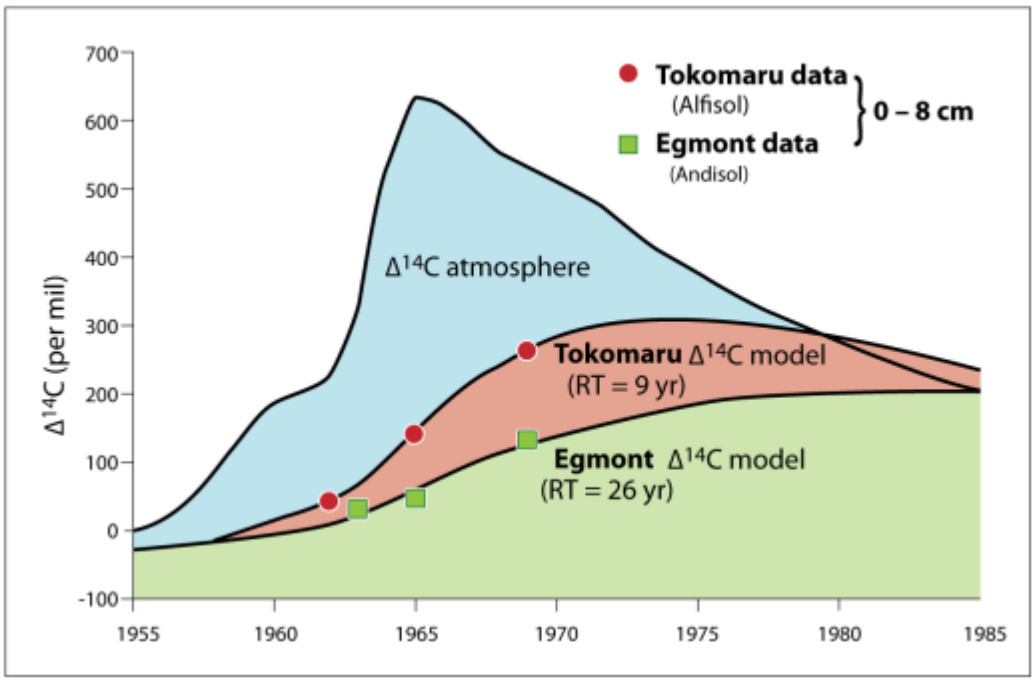

Fig. 2.8 Model showing slower rates of incorporation and turnover of organic C using bomb-derived ^{14}C in an allophanic soil (Egmont series) compared with those of a soil without appreciable allophane (Tokomaru series) under similar climate and land use in New Zealand. From Churchman and Lowe (2012) and based on Baisden et al. (2010) and modified diagram from Parfitt (2009). RT, residence time.

2.4. Application of synchrotron radiation to soil carbon chemistry

In studies of mineralogy and environmental sciences, soil chemistry has relied on chemical analysis and spectroscopic approaches. The use of microscopy, including transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM), and other spectroscopic methods including infrared (IR), nuclear magnetic resonance (NMR), and X-ray diffraction

(XRD), has improved the understanding of speciation and distribution of compounds and their interactions with other environmental components. A synchrotron is an accelerator built to accelerate charged particles moving rapidly in a curved path by a magnetic field, hence generating electromagnetic radiation called synchrotron radiation (Schulze and Bertsch, 1999; Sparks and Ginder-Vogel, 2012) (Fig. 2.9). The intensity and brilliance of synchrotron light can be across the range of the electromagnetic spectrum from infrared to X-rays, and synchrotron light is much brighter than any other conventional infrared and X-ray generators. Synchrotron radiation of different energies allows surface chemistry, crystalline structures, transformations, and advanced molecular structures of compounds to be studied at micro- or nanometre scales (Schulze and Bertsch, 1999; Brown et al., 2006a, 2006b).

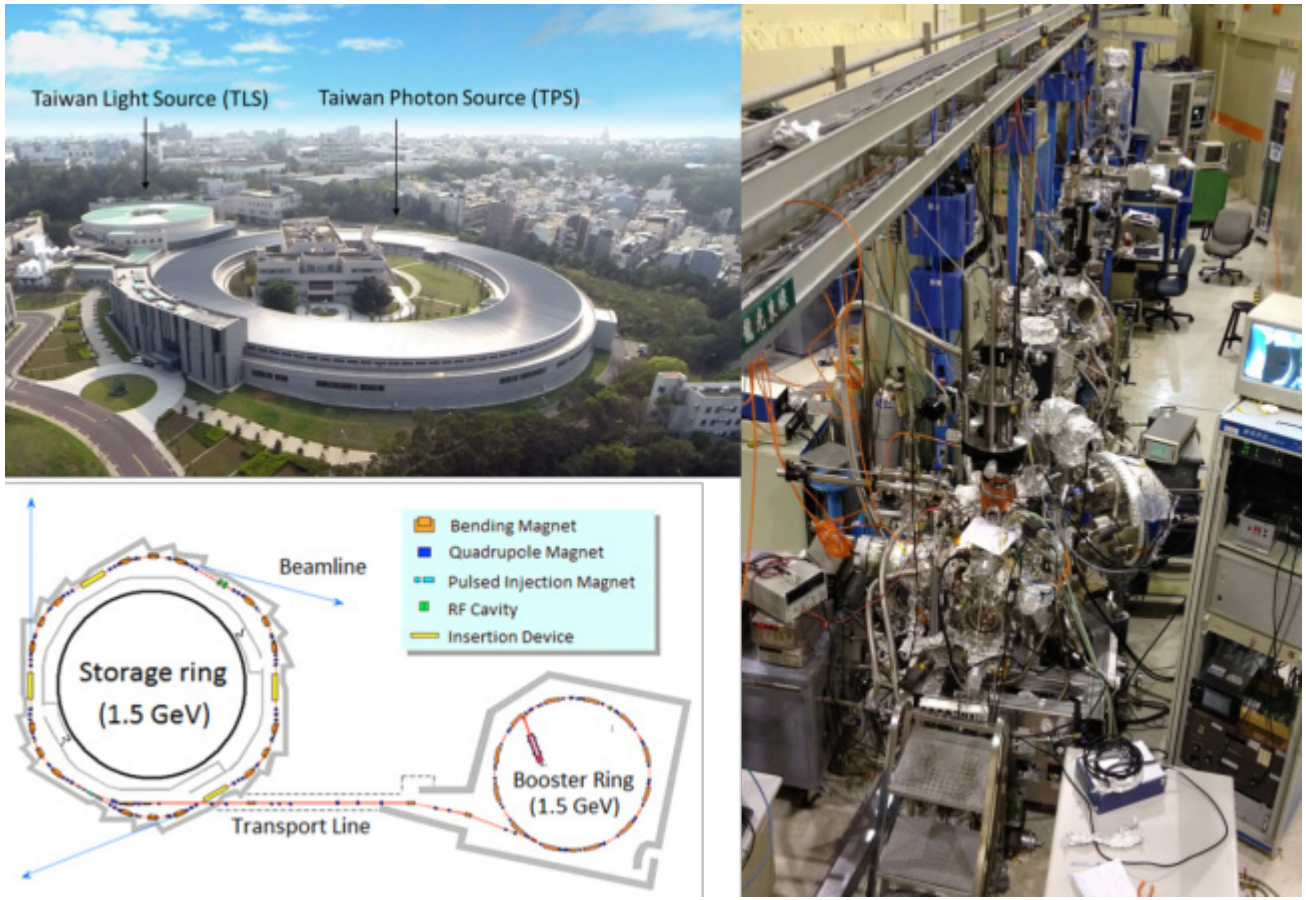

Fig. 2.9 Photos of Taiwan Light Source (TLS) and Taiwan Photon Source (TPS) at National Synchrotron Radiation Research Center (NSRRC), Hsinchu, Taiwan (left top; after NSRRC, www.nsrrc.org.tw); schematic of synchrotron radiation X-ray source (left bottom; after NSRRC, www.nsrr.org.tw); a photo of beamline 24A inside TLS (right; photo by Y.-T. Huang).

Two of the advantages of synchrotron radiation are that the X-ray beam is tunable and spatially-resolved mapping can be accomplished once the beam is focused (Schulze and Bertsch, 1999; Holman, 2010), and so synchrotron radiation-based infrared spectromicroscopy (SR-FTIR) and X-ray absorption spectroscopy (XAS) have been widely adopted to analyse biogeochemical materials or, for example, environmental pollutants (Ade et al., 1992; Hesterberg et al., 1999; Hesterberg, 2010; Holman, 2010; Selim Reza et al., 2012). For instance, the preserved organic residues on embryonic bones of an early Jurassic dinosaur were successfully examined and located spectrophotometrically using SR-FTIR rather than by extracting organics by decalcifying bone samples and analysing the extract (Reisz et al., 2013).

The use of carbon near-edge X-ray absorption fine structure (C NEXAFS) spectroscopy is of interest in my study because such spectroscopy has solved the technical problems which traditional analytical methods, NMR and FTIR, have encountered in the examination of the nature of SOM (Smernik and Oades, 2002; Lehmann and Solomon, 2010; Schmidt et al., 2011). The excited phase of a carbon electron is characteristic of the structure of carbon functional groups, and therefore the X-ray absorption energies of carbon in different functional groups vary and can be correlated to specific carbon forms (Braun et al., 2005; Lehmann and Solomon, 2010). As well, the concentrations of SOM are always low compared with those of biological samples and are not easy to be analysed via spectroscopy, but the high energy of synchrotron radiation facilities improves the detection limits with less noise and provides better spectral data than conventional spectroscopic techniques.

C NEXAFS, in conjunction with scanning transmission X-ray microscopy (STXM), has been widely used over the past decade or more to map functional groups of SOM at nano-scales (Lehmann, 2005; Solomon et al., 2005, 2009, 2012b; Wan et al., 2007; Sedlmair et al., 2012). With the use of C NEXAFS and STXM, it has been shown that variable functional groups were identified at distinct locations of mineral aggregates (Lehmann et al., 2008). However, the compositions of organic functional groups of whole soils from temperate and tropical forests with different vegetation and mineralogy were remarkably similar. Other studies also displayed variability of organic functional groups at locations within individual soil aggregates (Schumacher et al., 2005; Wan et al., 2007;

Solomon et al., 2012a). Solomon et al. (2012b) mapped functional groups over many soil organo-mineral assemblages and black carbon and found that the spectra collected from the inner region of black carbon were dominated by quinonic carbon that could have originated mainly from bacteria and plants (Collins and Jones, 1981; Karp, 2013); however, the resonances of quinonic carbon were either weak or absent in the spectra collected from the intermediate or outer regions. Moreover, Kleber et al. (2011) examined the functional groups of preserved organic matter sequestered by soils of different ages (107 versus 680 years) and found that the stabilised and old SOM is not necessary chemically recalcitrant.

2.5. Adsorption of environmental DNA in soils

A DNA strand is about 20 Å (2 nm) in diameter, which comprises heterocyclic bases and a pentose-phosphodiester backbone (Fig. 2.10) (Fujii et al., 2003; Brown and Poon, 2004). Ogram et al. (1988) studied DNA adsorption on different soils and lake sediment and fitted the sorption data with the Freundlich equation to obtain the affinity coefficient and DNA adsorption capacity of soils, and later DNA was found to have strong associations with some clay minerals including montmorillonite and allophane, and humics, in soils (Cai et al., 2006b, 2007; Saeki et al., 2010, 2011).

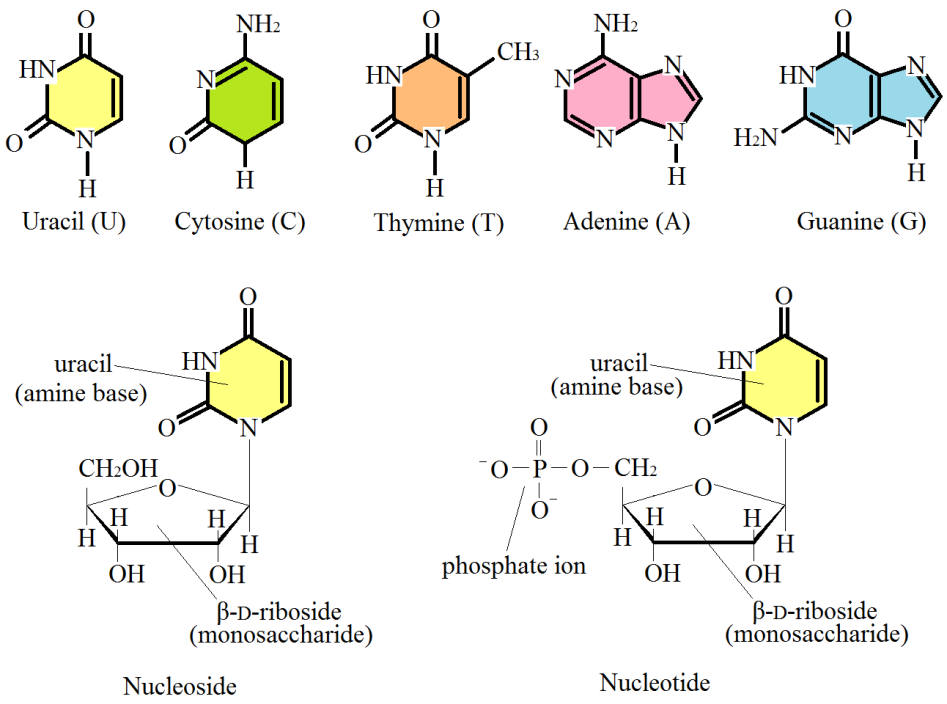

Fig. 2.10 Five heterocyclic aromatic amine bases most common to nucleic acids and the basic structures of DNA fragments (after Brown and Poon, 2004).

Clay minerals possess adsorptive sites for organic and inorganic matter and also deoxyribonucleic acid (DNA) (Lorenz and Wackernagel, 1987, 1994; Cai et al., 2006a; Yu et al., 2013). The persistence of DNA in soil is thought to have resulted from "adhesion" of DNA to soil particles (Paget and Simonet, 1994). Therefore, the extracellular DNA in soils can be protected against nuclease degradation and heavy metal damage through adsorption on soil colloids or particles, and on modified clays (Pietramellara et al., 2009; Hou et al., 2014a, 2014b), similar to the concepts of carbon stabilisation and protection in soils. Moreover, Demanèche et al. (2001) indicated that the adsorption of nucleases on clays induced the immobilisation of nucleases and thus protection of the extracellular DNA. Such environmental DNA in soils potentially provides information about biological communities including microbes, fungi, insects, and plants (Ogram et al., 1988; Pietramellara et al., 2009; Yoccoz et al., 2012) in different soil systems and also enables the genetic study of non-culturable bacteria in soils (Taberlet et al., 2012).

The capacities of natural and synthetic allophane to adsorb DNA have been studied (Saeki et al., 2010; Kawachi et al., 2013; Matsuura et al., 2013), and the strong association between allophane and DNA could be ascribable to the

active sites of allophane spherules as well as the phosphate groups of DNA molecules (Parfitt, 1989; Harsh, 2012). Allophane in natural soils also strongly adsorbs organic matter which is expected to increase the negative charge on allophane (Harsh, 2012), presumably leading to a mutual exclusion between organo-allophane and negatively-charged DNA. However, Saeki et al. (2011) showed that DNA has a high affinity for humic acids, and therefore DNA in allophanic soils is likely to bind to humic substances covering allophane spherules and thus bind indirectly to allophane.

No one has yet discussed the potential of allophane to protect DNA from degradation (as far as I am aware). However, Greaves and Wilson (1969) found that the adsorption of DNA by montmorillonite was largely dependent on the pH of the system, and they later suggested that the ribonucleic acid (RNA) adsorbed in the central zones of montmorillonite may undergo less attack by microbes or enzymes, but that RNA sorbed on the surfaces of individual montmorillonite particles is not protected (Greaves and Wilson, 1970). Their results helped to catalyse my research interest in that DNA may be physically protected and preserved in the tiny interstices or spaces (pores) within and between allophane nanoaggregates (Fig. 2.11). Therefore, the preserved DNA in allophanic soils may not be mobile, even though recent research showed the potential for DNA to leach downwards in non-frozen soils (Pääbo et al., 2004; Haile et al., 2007).

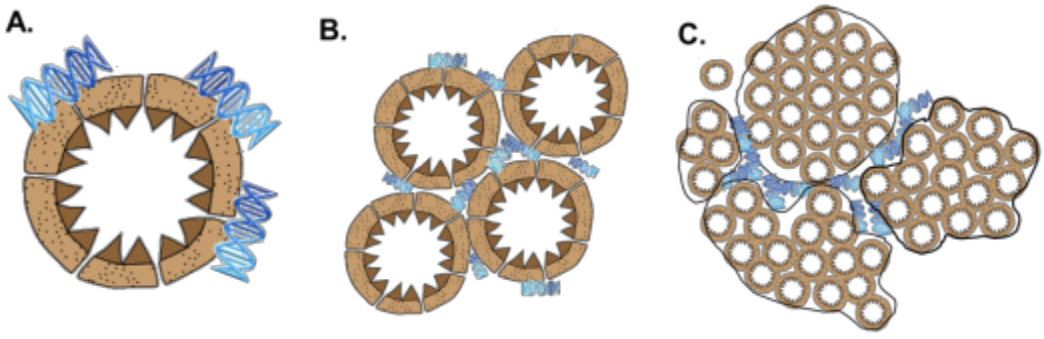

Fig. 2.11 Hypothesized chemical and physical adsorption of DNA to allophane: (A) chemical adsorption at the broken-bond defects (perforations) on the surface of an allophane spherule, (B) physical adsorption between a cluster of allophane spherules (i.e. in the inter-spherule spaces), and (C) physical adsorption in pores between clusters of allophane nanoaggregates (i.e. in the inter-nanoaggregate spaces). The cluster of nanoagregates in (C) is termed a microaggregate.

2.6. Ancient DNA (aDNA) and palaeoenvironmental DNA (PalEnDNA)

When an organism dies, degradation of its DNA by extracellular nucleases occurs. Under 'normal' conditions (reasonable salt concentrations, neutral pH, and a temperature of 15 °C), it would take ~100,000 years for hydrolysis to destroy all DNA (Lindahl, 1993; Hofreiter et al., 2001). However, under some circumstances, such as desiccation, low temperature, low oxidant, high salt concentrations, or with specific clays, nucleases can be destroyed or inactivated before all DNA fragments are degraded (Demanèche et al., 2001; Hofreiter et al., 2001), leading to only slow decay of DNA and hence DNA preservation. Because of the preservation of biological material in such environments for a long time period, the use of ancient DNA (aDNA) has become an increasingly useful tool for reconstructing past environments and Quaternary sciences (e.g., Willerslev and Cooper, 2005; Jørgensen et al., 2012; Rizzi et al., 2012; Rawlence et al., 2014).

Many of the aDNA studies have used historical, archaeological, and fossil remains, such as bone, teeth, leaves, and eggshell (Gugerli et al., 2005; Oskam et al., 2010; Rawlence et al., 2014). However, aDNA has also been extracted from disseminated genetic materials within palaeoenvironmental samples, including coprolites, sediments, and soils (Willerslev et al., 2003; Haile et al., 2007; Poinar et al., 2009). Such aDNA has been defined as palaeoenvironmental DNA (PalEnDNA) in studies by the research group of which I am part (Rawlence et al., 2014; see Appendix D) (Fig. 2.12). Moreover, many technical pitfalls need to be avoided to allow scientists to go back in time without interference from modern contaminations (Hofreiter et al., 2001; Rawlence et al., 2014). Regarding the slow carbon turnover in allophanic soils and DNA adsorption capacity of allophane, buried allophane-rich soils derived from tephra deposits (e.g., paleosols on well-dated tephras in the North Island, New Zealand: Pullar et al., 1973; Lowe and Palmer, 2005; Lowe, 2010) potentially represent a valuable source for storing a wide variety of PalEnDNA which may be used for reconstructing past environments or climates of the Holocene or earlier periods. However, soil properties sometimes impede DNA extraction from soil samples (Li et al., 2011; Miao et al., 2014; Young et al., 2014), particularly from Andisols, many of which are naturally dominated by andic materials (see section 2.2.) (Takada-Hoshino and Matsumoto, 2004; Herrera and Cockell, 2007; Rai et al., 2010). The difficulty of DNA extraction from Andisols could be attributed to the association of DNA with

allophane and Fe- and Al-humus complexes and also the substantial presence of humic substances in Andisols and allophanic soils (Saeki et al., 2010; McDaniel et al., 2012). Therefore, effective methods for DNA extraction from Andisols are essential to examine the possible presence of PalEnDNA in allophane-rich paleosols.

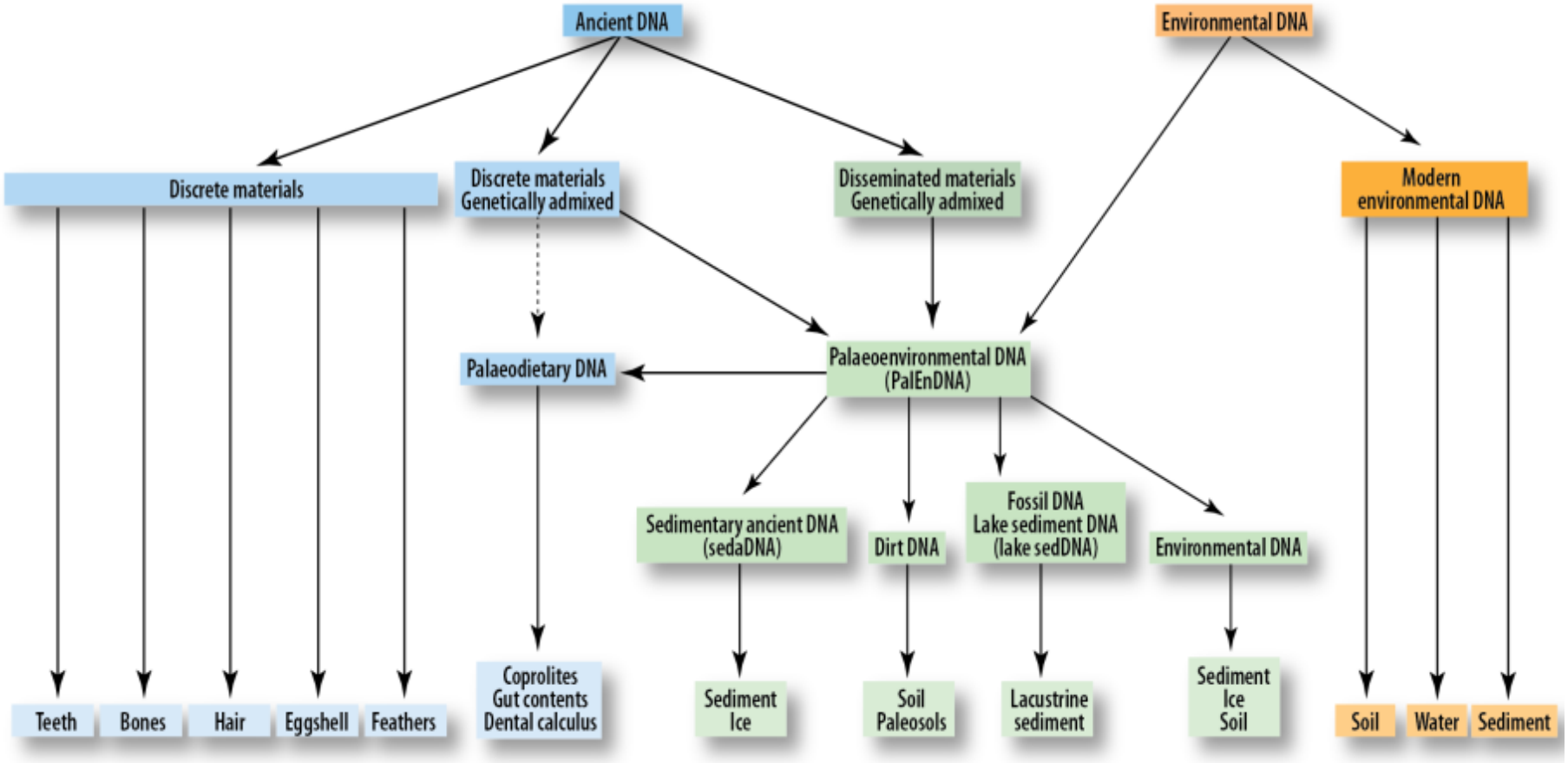

Fig. 2.12 Hierarchy and relationships of the main descriptive terms and sampling materials for PalEnDNA research in comparison with terms used for modern environmental DNA research and aDNA research focusing on discrete samples (from Rawlence et al., 2014; see review paper in Appendix D).

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Chapter 3 Carbon storage and DNA adsorption in allophanic soils and paleosols: preliminary findings

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Chapter 17
Carbon Storage and DNA Adsorption in Allophanic Soils and Paleosols

Yu-Tuan Huang, David J. Lowe, G. Jock Churchman, Louis A. Schipper, Nicolas J. Rawlence, and Alan Cooper

Abstract Andisols and andic paleosols dominated by the nanocrystalline mineral allophane sequester large amounts of carbon (C), attributable mainly to its chemical bonding with charged hydroxyl groups on the surface of allophane together with its physical protection in nanopores within and between allophane nanoaggregates. C near-edge X-ray absorption fine structure (NEXAFS) spectra for a New Zealand Andisol (Tirau series) showed that the organic matter (OM) mainly comprises quinonic, aromatic, aliphatic, and carboxylic C. In different buried horizons from several other Andisols, C contents varied but the C species were similar, attributable to pedogenic processes operating during developmental upbuilding, downward leaching, or both. The presence of OM in natural allophanic soils weakened the adsorption of DNA on clay; an adsorption isotherm experiment involving humic acid (HA) showed that HA-free synthetic allophane adsorbed seven times more DNA than HA-rich synthetic allophane. Phosphorus X-ray absorption near-edge structure (XANES) spectra for salmon-sperm DNA and DNA-clay complexes indicated that DNA was bound to the allophane clay

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through the phosphate group, but it is not clear if DNA was chemically bound to the surface of the allophane or to OM, or both. We plan more experiments to investigate interactions among DNA, allophane (natural and synthetic), and OM. Because DNA shows a high affinity to allophane, we are studying the potential to reconstruct late Quaternary palaeoenvironments by attempting to extract and characterise ancient DNA from allophanic paleosols.

Keywords Andisols • Allophane • Carbon sequestration • C NEXAFS • P XANES • Ancient DNA

Introduction

Carbon Storage in Soils

Soils are the largest reservoir of terrestrial organic carbon (Batjes 1996; Blanco-Canqui and Lal 2004), and soil organic matter (SOM) has become subjected to many diverse investigations because C is an important contributor to soil quality and because of its role as a potential store of CO_2. Nanocrystalline clay minerals including allophane and ferrihydrite have a greater affinity than crystalline clay minerals for organic functional groups, hence soil organic carbon (SOC), due to ligand exchange with their charged hydroxyl groups (Basile-Doelsch et al. 2005; Kleber et al. 2005; Churchman and Lowe 2012). Soils developed mainly on free-draining, explosively erupted, fragmental volcanic material (collectively, 'tephra'), typically classed as Andisols, 'hold' more C than other mineral soils (Batjes 1996; McDaniel et al. 2012) because of their constituent allophane (Chevallier et al. 2010; Calabi-Floody et al. 2011). Andisols occupy ~1 % of the world's land area, but contain 5 % of global C (Dahlgren et al. 2004; McDaniel et al. 2012). Sequestration of organic materials in soils is an important research area, and we are specifically interested in DNA adsorption and storage by allophanic soils because preserved DNA or ancient DNA (aDNA) from buried paleosols could in turn provide a way of reconstructing past environments.

Allophane and Carbon Sequestration

Allophane comprises tiny (3.5–5 nm in diameter), Al-rich nanocrystalline hollow spherules ($(1–2)SiO_2 \cdot Al_2O_3 \cdot (2–3)H_2O$) (Fig. 17.1a) with extremely large surface areas (up to 1,500 $m^2\ g^{-1}$) (Parfitt 2009; Calabi-Floody et al. 2011). Broken-bond defects in the spherules, also called perforations, are ~0.3 nm in diameter (Fig. 17.1a) and it is the protonation and deprotonation of $(OH)Al(H_2O)$ groups exposed at these sites that give rise to allophane's variable (pH-dependent) charge (Parfitt et al. 1977; Yuan and Theng 2012), and thus its anion exchange capacity. The adsorption of negatively charged humic substances on allophane through ligand exchange is

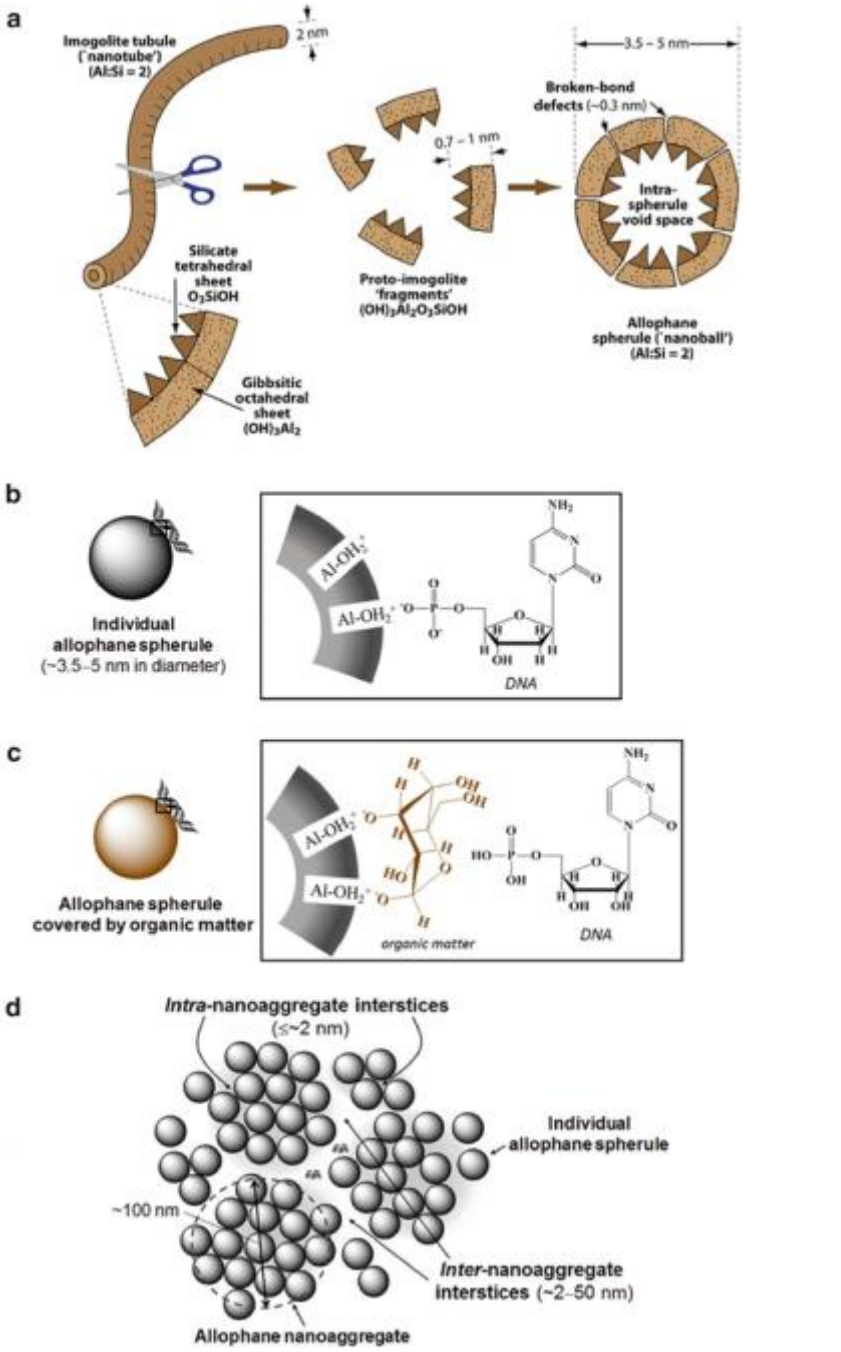

Fig. 17.1 Illustration of (**a**) nanoscale dimensions and composition of imogolite tubules and allophane spherules (from McDaniel et al. 2012), (**b**) direct chemical adsorption of DNA on allophane, (**c**) indirect chemical adsorption of DNA on organic matter-rich allophane, and (**d**) physical adsorption of DNA in the interstices of allophane nanoaggregates

represented by Eq. (17.1) (from Yuan and Theng 2012). $[R.COO]^{n-}$ denotes a humic molecule containing *n* carboxylate functional groups.

$$\left[\text{allophane}-\text{Al}\left(\text{H}_2\text{O}\right)\left(\text{OH}\right)\right]^0 + \left[\text{R.COO}\right]^{n-} \Rightarrow \left[\text{allophane}-\text{Al}\left(\text{H}_2\text{O}\right)\left(\text{COO.R}\right)\right]^{(n-1)-} + \text{OH} \quad (17.1)$$

These high specific surface areas and surface properties help govern the strong association between allophane and SOM (Fig. 17.1b, c) (Buurman et al. 2007; Calabi-Floody et al. 2011; Yuan and Theng 2012). In addition, allophane spherules tend to form nanoaggregates up to about 100 nm in diameter (Fig. 17.1d) (Chevallier et al. 2010; Calabi-Floody et al. 2011). We suggest that the interstices (nanopores) both within and between such nanoaggregates (Fig. 17.1d) provide a haven for SOM so that a portion of SOC is poorly accessible to microbes or enzymes and thus protected. Calabi-Floody et al. (2011) showed that significant SOC, which resisted the treatment of hydrogen peroxide, was strongly held by allophane and imogolite in Andisols, a finding consistent with studies of the physical protection and stabilisation of SOM within larger micro- and macro-aggregates (Elliott 1986; Strong et al. 2004). It is possible that SOC is protected against the attack of enzymes in the narrowest interstices, such as in nanoaggregates, because the diffusion pathway for the passage of enzymes is constrained (McCarthy et al. 2008; Chevallier et al. 2010).

DNA Adsorption on Allophane

The adsorption of DNA on soil particles plays a major role in protecting extracellular DNA from degradation in soils (Paget and Simonet 1994), and the interactions between DNA and common clay minerals have been investigated using FTIR spectroscopy (Cai et al. 2006a). Allophane adsorbs more DNA than other minerals in soils (Saeki et al. 2010; Harsh 2012), and may be capable of preserving DNA as well. Greaves and Wilson (1970) suggested that nucleic acids in the centre of expandable montmorillonite crystal structures may be protected from attack by microbial enzymes, and hence we hypothesise that aDNA may also be physically protected and preserved within intra- and inter-nanoaggregate interstices of allophane in Andisols and andic paleosols. However, Cai et al. (2006b) revealed that DNA is more tightly adsorbed by organic matter-free clay than by clay containing organic matter, indicating that the abundant SOM in allophanic soils might hamper DNA adsorption on allophane. SOM is expected to increase the negative charge on minerals (Eq. 17.1), leading to a mutual exclusion between SOM-rich allophane and negatively-charged phosphate groups of DNA. In contrast to Cai et al. (2006b), Saeki et al. (2011) showed that humic acid had a high affinity for DNA, hence the influence of SOM on DNA (and potentially aDNA) adsorption in Andisol is still debatable.

A DNA strand comprises heterocyclic bases and a pentose-phosphodiester backbone, and it was suggested that DNA binds to clays through the phosphate group (Cai et al. 2006a) but there has been no direct evidence. We propose three main mechanisms for DNA adsorption on allophane: (1) direct chemical adsorption on the organic matter-free and protonated surface of allophane, (2) indirect chemical adsorption/dissolution on/into the SOM-rich allophane, and (3) physical adsorption in interstices/nanopores of allophane nanoaggregates (Fig. 17.1b–d). A clearer understanding of DNA adsorption on allophanic materials would help inform ways to extract preserved DNA or aDNA from buried paleosols.

Materials and Methods

Application of Synchrotron Radiation

Traditionally SOM was extracted chemically from soils, leading to possible artifacts of the treatment (e.g. see Schmidt et al. 2011; Churchman and Lowe 2012). Synchrotron radiation has been developed for several decades for studying materials (Sutton et al. 2006) including the chemical composition of soil components such as 'resistant' C (Wan et al. 2007; Lehmann and Solomon 2010). It allows the analysis of C at low concentrations and within bulk soil samples. C near-edge X-ray absorption fine structure analysis (NEXAFS) enables examination of speciation and structures of SOM (Lehmann 2005; Lehmann et al. 2007). Phosphorus X-ray absorption near-edge structure (XANES) reveals the configuration for phosphorus bonding to Fe(III)- or Al(III)-rich minerals (Kizewski et al. 2011).

Carbon NEXAFS

We used the Beamline 24A1 at the National Synchrotron Radiation Research Center (NSRRC), Hsinchu, Taiwan, for our C NEXAFS study of the C compositions of SOM associated with allophane through the electron yield mode. Contaminant C contributions from the beamline have been eliminated by a golden mesh anterior to the sample chamber.

Allophanic materials were collected in northern New Zealand from an Andisol of the Tirau series (a medial, mesic Typic Hapludand: Bakker et al. 1996) at Tapapa and from buried andic paleosols at two sites at Lake Rerewhakaaitu (near Mt Tarawera) and one near Lake Rotoaira (adjoining Tongariro National Park). Clay fractions (<2 μm) were pressed into indium foil to enable them to be conductive, and analysis was accomplished under 10^{-9} torr using a chemical pump.

DNA Adsorption Studies

Salmon-sperm DNA from Sigma-Aldrich Co. LLC that ranged from 0 to 300 μg was added to the natural clays from all sites and to a 1 mL suspension of pure synthetic allophane (synthesized following the method of Ohashi et al. 2002), and the mixture was gently rotated at room temperature for 3 h. DNA in the supernatant was analyzed at 260 and 280 nm spectrophotometrically.

Phosphorus XANES

P XANES spectra were obtained for pressed pellets of clays from Tirau soil, pure salmon-sperm DNA, and salmon-sperm DNA-clay complexes, at the Beamline 16A1 at NSRRC.

Results and Discussion

Carbon NEXAFS

The clay fraction of the upper subsoil of the Tirau soil contains about 10 % total C, and C NEXAFS spectra showed its SOM comprises quinonic, aromatic, aliphatic, and carboxylic C (Fig. 17.2a). Although C contents varied between horizons and sites, the compositions of SOM were similar in all horizons of the andic paleosols (data not shown). We attribute this compositional similarity to either (1) the downward leaching of dissolved SOM through the profiles vertically, effectively homogenising the composition of organic matter in each horizon, or (2) integrative developmental upbuilding pedogenesis whereby the profiles have formed by incremental additions of thin distal tephra over the past ~25,000 years that have interacted with the soil organic cycle whilst temporarily at the land surface, the land/soil surface slowly rising at an average rate of ~5 mm per century (Lowe et al. 2012; McDaniel et al. 2012), or both (1) and (2).

DNA adsorption studies

There was a negative relationship between total C and DNA uptake by all soil samples. However, at pH 6.5, humic-acid-free synthetic allophane adsorbed salmon-sperm DNA at the rate of up to 26 μg/mg allophane, which is about seven times more than that for humic-acid-rich allophane (Fig. 17.3). Hence humic acid (or SOM) occupied the active sites on allophane spherules, limiting their capacity for DNA adsorption.

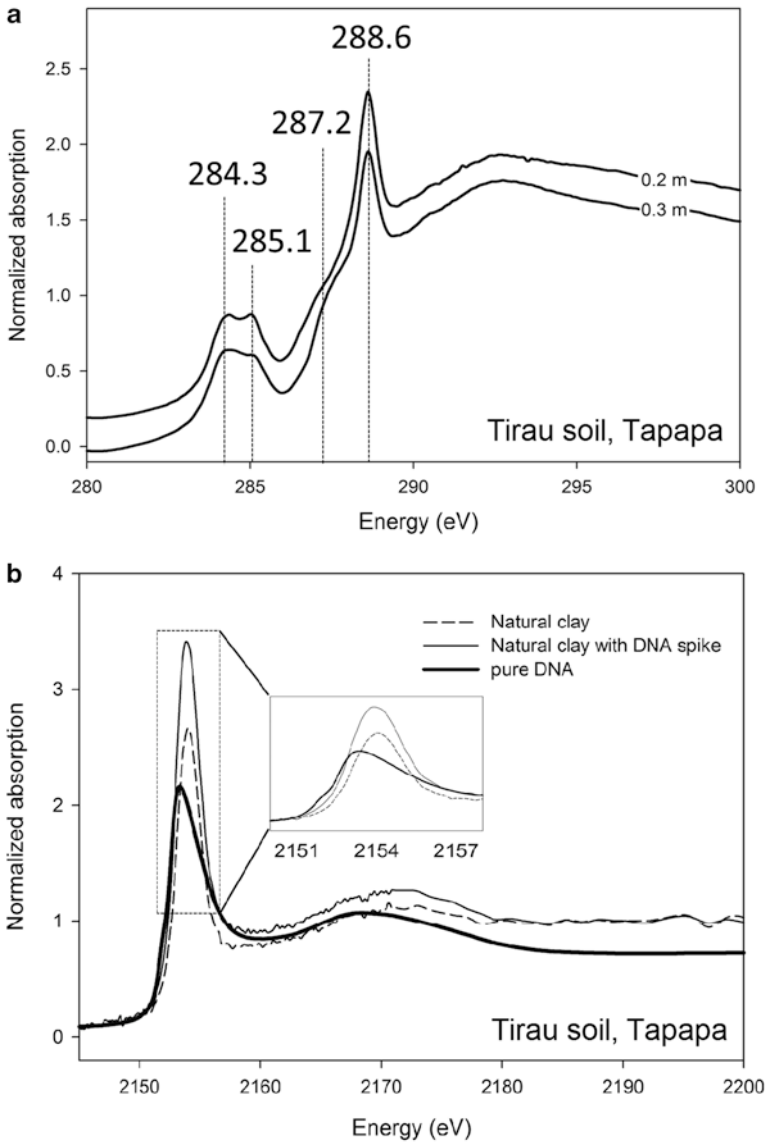

Fig. 17.2 Normalized (**a**) C NEXAFS spectra for organic matter in clays from the upper subsoil (~20–40 cm depth) of the Tirau soil, an Andisol at Tapapa in New Zealand (37° 59′ 41″ S, 175° 52′ 59″ E: Lowe et al. 2012), and (**b**) phosphorus XANES spectra for salmon-sperm DNA, for natural allophanic clay from the Tirau soil at Tapapa, and for allophanic clay with a salmon-sperm DNA spike. Peaks at 284.3, 285.1, 287.2, and 288.6 eV represent quinonic, aromatic, aliphatic, and carboxylic C, respectively

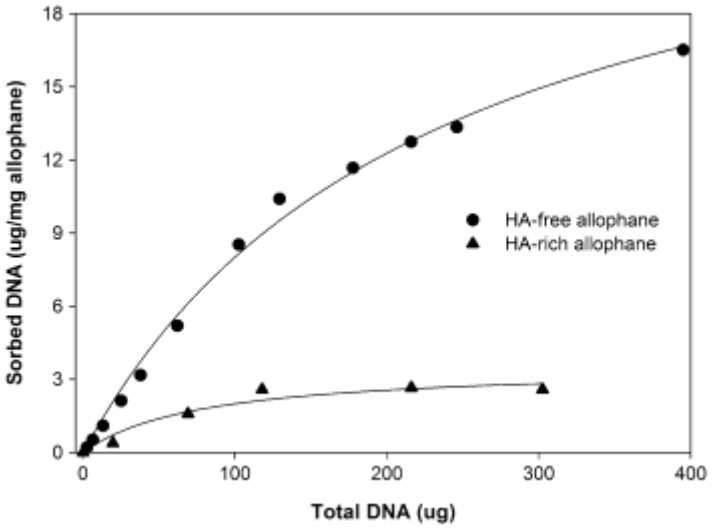

Fig. 17.3 Salmon-sperm DNA adsorption isotherm for humic-acid (HA)-free and for HA-rich synthetic allophane. This experiment was based on work by Saeki et al. (2010), and DNA in the supernatant was measured by UV spectrophotometry at 260 and 280 nm

Phosphorus XANES

The spectra showed that the absorption edge (absorption energy, the sharply rising peak of the spectrum) of phosphorus for pure DNA was 2,153 eV, and that binding of DNA and clay had induced a shift of the absorption edge to 2,514 eV (Fig. 17.2b). This shift indicates that the phosphate group of DNA has changed chemically after binding to natural clay. However, it is unclear whether DNA was bound to clay or to SOM, which is apparently pervasive in clay fractions from andic materials, and further experiments are needed to evaluate how DNA reacts to SOM and to organic-matter-free allophane. An improved understanding of DNA adsorption in Andisols should indicate how we can best potentially extract aDNA from allophanic paleosols that date back through time.

Conclusions

1. C NEXAFS spectra for an Andisol showed that OM comprises quinonic, aromatic, aliphatic, and carboxylic C. In other Andisols, C contents varied but C species were similar, attributable to pedogenic processes operating during developmental upbuilding, downward leaching, or both.
2. The presence of OM in natural allophanic soils weakened adsorption of DNA on clay: HA-free synthetic allophane adsorbed seven times more DNA than HA-rich synthetic allophane.

3. P XANES spectra for salmon-sperm DNA and DNA-clay complexes showed that DNA was bound to allophane clay through the phosphate group, either to the surface of allophane or to OM, or both.

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Chapter 4 Origin and preservation of soil organic matter in clay fractions of allophanic paleosols on Holocene tephras in New Zealand characterised using C NEXAFS spectroscopy

This manuscript has been submitted to *Quaternary Science Reviews*:

Huang, Y.-T., Lowe, D. J., Churchman, G. J., Schipper, L. A., Chen, T.-Y., Rawlence, N. J., Cooper, A. Origin and preservation of soil organic matter in clay fractions of allophanic paleosols on Holocene tephras in New Zealand characterised using C NEXAFS spectroscopy.

Abstract

We examined the soil organic matter (SOM)[1] sequestered by clays in allophanic paleosols (buried soils) on tephrochronologically dated Holocene tephra sequences (aged from ca 12,000 to 1718 calendar years BP) at four sites in North Island, New Zealand, using carbon near-edge X-ray absorption fine structure (C NEXAFS) spectroscopy. Our aim was to characterise the persistent carbon and determine if and how it derived from past environments. This is the first application by C NEXAFS to analyse SOM in sequences of dated paleosols. Clay-bound SOM extracted from all the paleosols was dominated by carboxylic functional groups with subordinate amounts of quinonic, aromatic, and aliphatic groups. All samples exhibited similar compositions despite differences in clay and allophane contents, stratigraphic position (depth of burial), age, parent-tephra composition (andesitic versus rhyolitic), current vegetation, and mode of soil origin (retardant versus developmental upbuilding pedogenesis). We found that the SOM preserved by clays in allophane-rich paleosols, which formed under native podocarp-broadleaf forest for most of the Holocene, does not comprise specific types of functional groups which could be indicative of resistant carbon molecules in soils. Instead, the preserved SOM contains very general functional groups that are likely to be relict from past Holocene environments. We infer that the SOM originated at the land surface during upbuilding pedogenesis. As soil genesis and hydrolysis-dominated weathering began in a newly-deposited tephra uppermost in the soil profile, allophane formed and SOM was sequestered on spherules and within aggregates. Ongoing tephra deposition then caused the land surface to rise so that once-surface horizons were buried more deeply and hence became increasingly isolated from inputs by the modern (surficial) organic cycle and near-surface processes over time, and thus preserved. The presence of quinonic carbon, especially sensitive to oxygen and light and a potential paleopedological biomarker, is indicative of the exceptionally strong protection of SOM by clays in allophanic paleosols, attributable both to a tortuous pore network amidst allophane nano- and microaggregates that encapsulates and shields the "old" or relict SOM from degradation, and to rapid burial by successive tephra-fall deposits. The clay-associated SOM (as expressed by the carbon functional groups) in the paleosols at each of the four study sites thus likely derived from soil processes operating from early to late Holocene and has not been modified by modern surface processes or diagenesis after burial. Analyses involving plant

DNA and pyrolysis gas chromatography-mass spectrometry of such SOM could help ascertain its origins more specifically.

Keywords:

Paleopedology, allophane, tephras, SOM, carbon functional groups, quinonic carbon, synchrotron radiation, C NEXAFS, 3D tomography, TXM, tephrochronology, upbuilding pedogenesis, relict properties, paleosol, geosol, Andisol, multisequal soil, soil stratigraphy, physical adsorption, Holocene, New Zealand

[1]*Abbreviations:*

asl, above sea level; AP, Ashton Dairies Pit; BP, before present ('present' is 1950 in the radiocarbon [^{14}C] timescale); BR, Brett Rd; cal., calendar or calibrated; C NEXAFS, carbon near-edge X-ray absorption fine structure; DNA, deoxyribonucleic acid; DI, deionized; FTIR, Fourier transform infrared; micro-CT (μCT), micro-computed tomography; NMR; nuclear magnetic resonance; NSRRC, National Synchrotron Radiation Research Center; Ok: Okareka tephra; pyrolysis-GC/MS, pyrolysis gas chromatography-mass spectrometry; Rk, Rerewhakaaitu tephra; Rr, Rotorua tephra; SOC, soil organic carbon; SOM, soil organic matter; Te, Te Rere tephra; TXM, transmission X-ray microscopy; yr, year(s).

4.1. Introduction

Carbon storage in soils has received much interest in recent decades because soils contain the largest pool of terrestrial organic carbon and hence are relevant in the uptake of greenhouse gas emissions and budgets. Consequently, many studies have focussed on the mechanisms influencing soil organic carbon (SOC) stabilization and loss in various soil orders (e.g. Lützow et al., 2006; Olson et al., 2014). The secondary clay minerals in soils are the most reactive inorganic components and not only help to retain moisture and nutrients but also form aggregates in strong association with SOC (e.g. Mikutta et al., 2005; Churchman and Lowe, 2012). Moreover, the retention of soil organic matter (SOM) in small pores within soil aggregates has been proven to slow down carbon turnover or decomposition rates (e.g. Six et al., 2000; Strong et al., 2004).

Among secondary clay minerals, the nanocrystalline minerals ferrihydrite and allophane possess a high surface area, variable charge, and gel-like properties (e.g. Woignier et al., 2007; Theng and Yuan, 2008; Parfitt, 2009), enabling allophanic soils derived from volcanic ash (tephra), which include Andisols, to adsorb much organic matter and stabilize SOC (McDaniel et al., 2012). Allophane (and, less commonly, paracrystalline imogolite) is an aluminosilicate that provides hydroxyl groups able to participate in ligand exchange with functional groups of SOM (Yuan et al., 2000; Theng et al., 2005; Hernández et al., 2012), and the aggregation of allophane spherules brings about fractal porous networks to retain and sequester considerable amounts of SOM which is spatially protected against degradation in Andisols (Chevallier et al., 2010; Calabi-Floody et al., 2011, 2015; Matus et al., 2014).

4.1.1. Aim of this study

The slow rate of carbon turnover in allophanic soils or Andisols, and the abundance of Andisols and buried allophanic paleosols (soils of past environments or landscapes) in central North Island, New Zealand (Lowe and Palmer, 2005), provide the opportunity to characterise the nature of stable SOM preserved in clay fractions of allophane-rich paleosols on Holocene tephras. Our objectives were to detect the presence and determine the origin of biological material from past environments associated with the paleosols, to further

understand carbon sequestration in Andisols and allophanic paleosols, and to evaluate a potential new tool – carbon near-edge X-ray absorption fine structure (C NEXAFS) spectroscopy – for soil evolution studies and paleopedology. From four sites in North Island, we sampled a series of buried paleosols on Holocene tephra layers, their ages known via tephrochronology. Such paleosols with stratigraphic significance are called 'geosols' in the North American Stratigraphic Code (North American Commission on Stratigraphic Nomenclature, 2005). Each paleosol represented from ca 1100 to ca 12,000 calendar (cal.) years of pedogenesis (soil formation) at the land surface. We analysed extracted organo-clay complexes using synchrotron-based C NEXAFS spectroscopy to ascertain the alteration of functional groups (also known as carbon speciation) of SOM in the allophane-rich paleosols with time and with differing modes of upbuilding pedogenesis (described below). We also used high-resolution X-ray tomography to examine the internal structure of materials (e.g. Cnudde and Boone, 2013), and synchrotron radiation to generate microtomographic images of natural allophane aggregates from one of our sites to help envisage, and hence explain, the preservation of SOM within them.

Our study of carbon functional groups using C *K*-edge (the energy to create a vacancy in the *K*-shell of C atoms) NEXAFS is the first to be undertaken on sequences of buried allophanic paleosols formed on dated Holocene tephra deposits. One of the few previous applications of the technique to "non-modern" soil materials includes that of Kleber et al. (2011), who studied organo-mineral associations isolated from three different soil orders, namely Oxisols, Alfisols, Inceptisols (Soil Survey Staff, 2014a) with known carbon turnover times of 107, 175, and 680 years, respectively. Another study carried out by Zhou et al. (2014) examined the C functional groups of SOM in soils dated from 50 to 2,000 years old using advanced solid-state nuclear magnetic resonance (NMR) spectroscopy.

Before we present our methods and findings in detail, we outline the process of upbuilding pedogenesis associated with tephra deposits and Holocene environments of North Island, and then provide a brief introduction to spectroscopic techniques used to analyse SOM and the advantages conferred by C NEXAFS spectroscopy.

4.2. Upbuilding pedogenesis and Holocene environments in central North Island, and spectroscopic methods for analysing SOM

4.2.1. Upbuilding pedogenesis associated with tephra deposition

A distinctive feature of many tephra-derived soils on stable sites is their multi-layered (multisequal) or composite nature (McDaniel et al., 2012). Such soils are formed by upbuilding pedogenesis during which soil evolution occurs via topdown processes whilst tephras are concomitantly added to the land/soil surface (Lowe and Palmer, 2005; Lowe and Tonkin, 2010; Inoue et al., 2011). The frequency and thickness of tephra accumulation (and other factors) determine how much impact topdown soil-forming processes have on the ensuing profile character, and if developmental or retardant upbuilding, or both, takes place (nomenclature after Johnson and Watson-Stegner, 1987; Almond and Tonkin, 1999). *Developmental upbuilding* occurs when the rate of addition of tephra to the land is incremental and sufficiently slow so that topdown pedogenesis effectively keeps pace as the land gradually rises. Nevertheless, the length of time each tephra is exposed to soil-forming processes at the land surface is limited, and hence the ensuing soil horizons typically are only weakly expressed (Birrell and Pullar, 1973; Stevens and Vucetich, 1984; Lowe and Tonkin, 2010). *Retardant upbuilding* occurs when a relatively thick layer of tephra is "instantaneously" added to the surface, or the rate of accumulation of thin tephras is sufficiently fast so that the original soil is rapidly buried (overwhelmed), and thus becomes a buried horizon isolated from the new materials at the land surface in which pedogenesis begins anew (Schaetzl and Sorenson, 1987; McDaniel et al., 2012). The central North Island consequently has huge stores of buried allophane-rich paleosols developed on sequences of well-dated tephra beds (Gibbs, 1971; Pullar et al., 1973; Newnham et al., 1999). The tephra layers provide isochrons (time-parallel marker beds) to connect and synchronize sequences and to assign relative or numerical ages using tephrochronology (Lowe, 2011).

When the soils on different tephra layers were at the land surface, the topsoils and upper subsoil horizons were able to adsorb the metabolized remains from organisms (e.g. fungi, bacteria, and plants) living at that time before their burial by new tephra deposits. The resultant allophane-rich paleosols in multi-layered tephra-soil sequences thus provide an ideal medium in which to study via

paleopedology the preserved organic materials that may reflect environments of the past, such as those of the Holocene, the past 11,700 calendar (cal.) years (Walker et al., 2009), and to examine the functional groups potentially preserved in such paleosols, as we have undertaken in the present study. Our work also helps test the concepts of upbuilding pedogenesis where chronostratigraphic control (via tephrochronology) is well established.

4.2.2. Holocene environments, central North Island

To date, Holocene environmental reconstructions of North Island, especially the history of vegetation, have focussed mainly on pollen studies together with analyses of plant macrofossils, phytoliths, speleothems, and various other climate-environment proxies (e.g. McGlone, 1983; Newnham et al., 1989; Horrocks and Ogden, 2000; Williams et al., 2004; Thorn, 2006). In broad terms, the early Holocene in central North Island was dominated by extensive podcarp-broadleaf forest and warm and wet conditions followed by gradual cooling and drying. The middle to late Holocene (since ca 7000 cal. yr BP) was dominated by podocarp-broadleaf and (in northern areas) kauri *(Agathis australis)* forest, and cooler and drier conditions and increased climate variability, until partial deforestation following the initial Polynesian settlement of New Zealand from ca AD 1280 (Hogg et al., 2003; Wilmshurst et al., 2008; McWethy et al., 2014). Therefore, there would have been an essentially constant input of organic matter, predominantly from forest vegetation and associated organisms, into soils through most of the Holocene.

4.2.3. Analysis of soil organic matter

Conventional studies of the nature of SOM have relied mainly on chemical (single or sequential) extractions (Baldock and Broos, 2012). However, it has become clear that the extractable components could only partially represent the nature of SOM (Schmidt et al., 2011; Heymann et al., 2014; Kleber et al., 2015). Modern spectroscopic techniques including ^{13}C nuclear magnetic resonance (NMR), Fourier transform infrared (FTIR), pyrolysis gas chromatography-mass spectrometry (pyrolysis-GC/MS), and C NEXAFS spectroscopies, are commonly used for characterising SOM (Solomon et al., 2005;

Verde et al., 2005, 2008; Buurman et al., 2007; Wan et al., 2007; Forouzangohar et al., 2013) in order to more closely analyse *in situ* organic matter by avoiding artefacts of the extraction techniques. Paramagnetic metals in soils interfere with ^{13}C NMR spectroscopy for SOM and thus HF has been used to dissolve the majority of soil minerals before such analysis (Parfitt et al., 1999; Smernik and Oades, 2002; Asano and Wagai, 2014). However, HF treatment results in the loss of a SOM that is held as organo-mineral complexes (Smernik and Oades, 2002). The investigation of the structural composition of SOM using FTIR spectroscopy is difficult also because of the overlap of absorption bands of organic matter and inorganic soil components (Lehmann and Solomon, 2010). Instead, C NEXAFS spectroscopy involves promotion of core electrons (in the K shell) to higher orbitals and allows monitoring of the emitted electrons and photons. Other soil components (e.g. clays and water) do not interfere with the analysis of carbon by C NEXAFS spectroscopy (Lehmann, 2005; Solomon et al., 2005; Lehmann and Solomon, 2010). Moreover, synchrotron radiation provides brighter light and tuneable X-ray energies, substantially improving the detection limits and spectra for soils or sediments or their components (Terzano et al., 2010), and the C NEXAFS spectra can be fitted with Gaussian curves to establish relative proportions of C functional groups (e.g. Fig. 4.1; Solomon et al., 2005; Sedlmair et al., 2012). Fig. 4.1 illustrates how different functional groups (multiple peaks) of soil humic substances have contributed a characteristic spectrum. Each provides a basis for comparison with spectra from our analyses (see also Table 4.2, below).

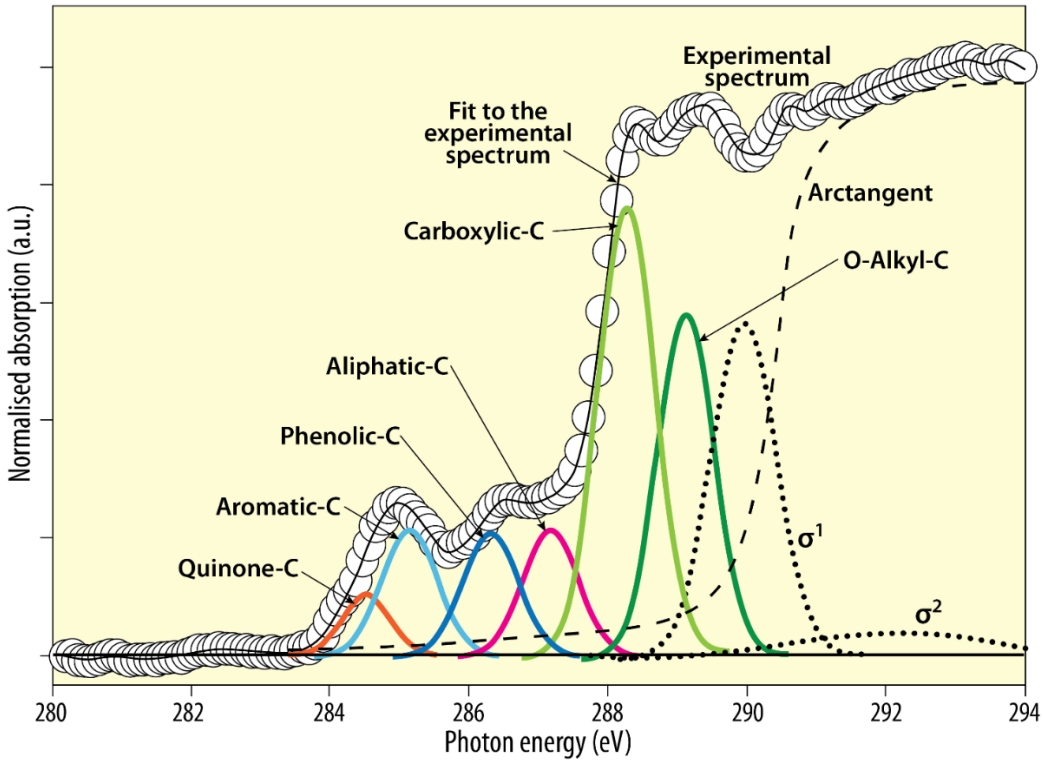

Fig. 4.1 C *K*-edge NEXAFS spectra for humic substances extracted from clay fractions of a soil from Wushwush, Ethiopia, and the spectra deconvolution showing the transitions (multiple peaks) of various carbon functional groups (redrawn from Solomon et al., 2005, p. 110, with permission from the Alliance of Crop, Soil, and Environmental Science Societies, publishers of *Soil Science Society of America Journal*) [CCC license number 3736690498274]. (For a colour version of this figure, see the web version of this article.)

4.3. Materials and methods

4.3.1. Allophane-rich soils/paleosols on Holocene tephras from volcanic sources in Taupo Volcanic Zone, central North Island

We selected allophane-rich soil material and paleosols developed on Holocene tephra-fall deposits of both rhyolitic (i.e. with high silica content, ≥~70 wt% SiO_2) and andesitic (with intermediate silica content, ~50–70 wt% SiO_2) composition at four sites in central North Island (Fig. 4.2), two relatively close to the main volcanic sources (proximal sites) and two farther away (distal sites).

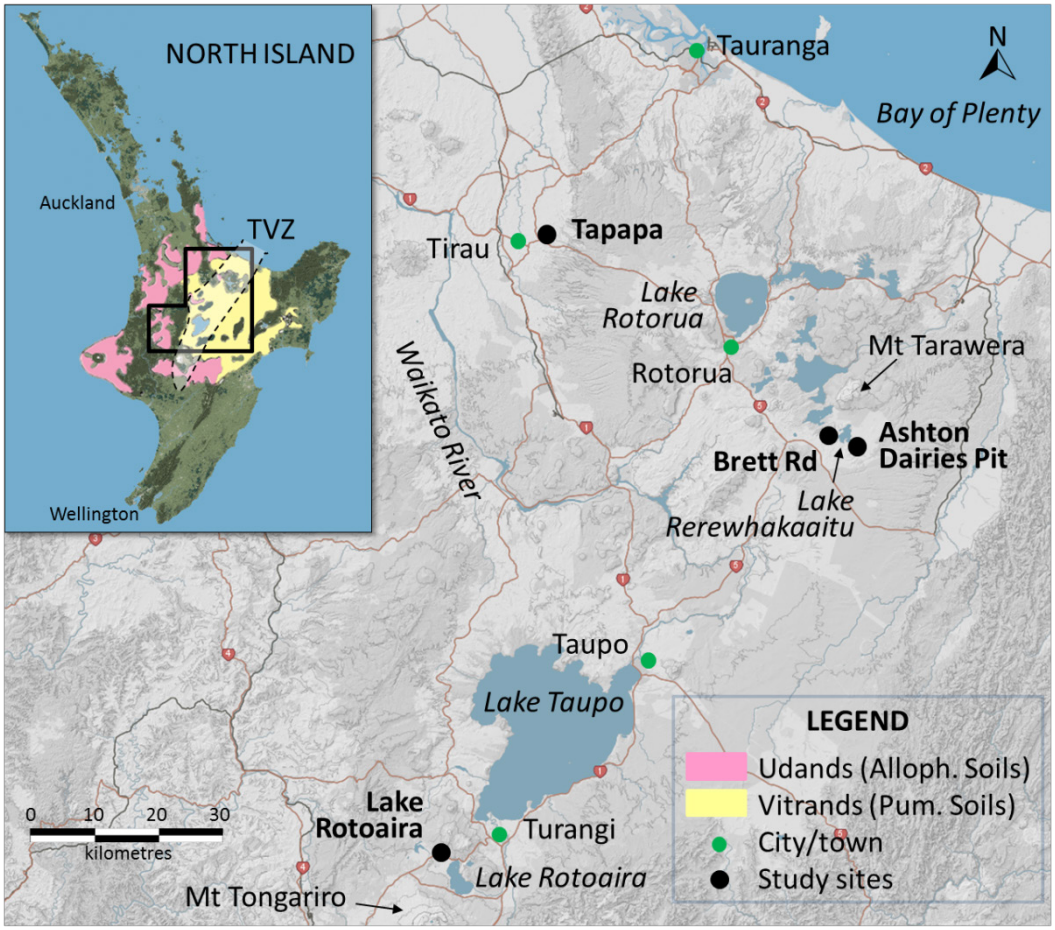

Fig. 4.2 Map of central North Island, New Zealand, showing the locations of the four study sites in the Taupo-Rotorua region. The inset map shows the distribution of Andisols, mainly Udands (equivalent to Allophanic Soils in Hewitt, 2010) and Vitrands (Pumice Soils in Hewitt, 2010), and the location of Taupo Volcanic Zone (TVZ) encompassing the volcanoes that erupted parent tephras for the soils and paleosols under study (Lowe and Palmer, 2005; see Chapter 2). State highways are numbered (red shields). (For a colour version of this figure, see the web version of this article.)

The multi-layered soils at the two distal sites were formed mainly by developmental upbuilding: *Tapapa* (formed on a composite of mainly rhyolitic tephras), near Tirau; and *Lake Rotoaira* (formed on a composite of mainly andesitic tephras), near Turangi and close to Mount Tongariro (Fig. 4.2). The multi-layered soils at the two proximal sites were formed on rhyolitic tephras via retardant upbuilding: *Brett Road and Ashton Dairies Pit*, both near Mount Tarawera to the southeast of Rotorua (Fig. 4.2). Roadside tephra-soil profiles (sections) at Tapapa and Lake Rotoaira were incised ca 50 cm laterally to remove any modern roadside plant material before sampling; the roadside profile at Brett Rd was incised ca 1 m laterally; and the profile at Ashton Dairies was in a pit

newly excavated using a digger. The stratigraphy and soil horizonation for each soil profile were established using tephrostratigraphy (described below) and conventional soil morphological examination (Fig. 4.3; Soil Survey Staff, 2014a).

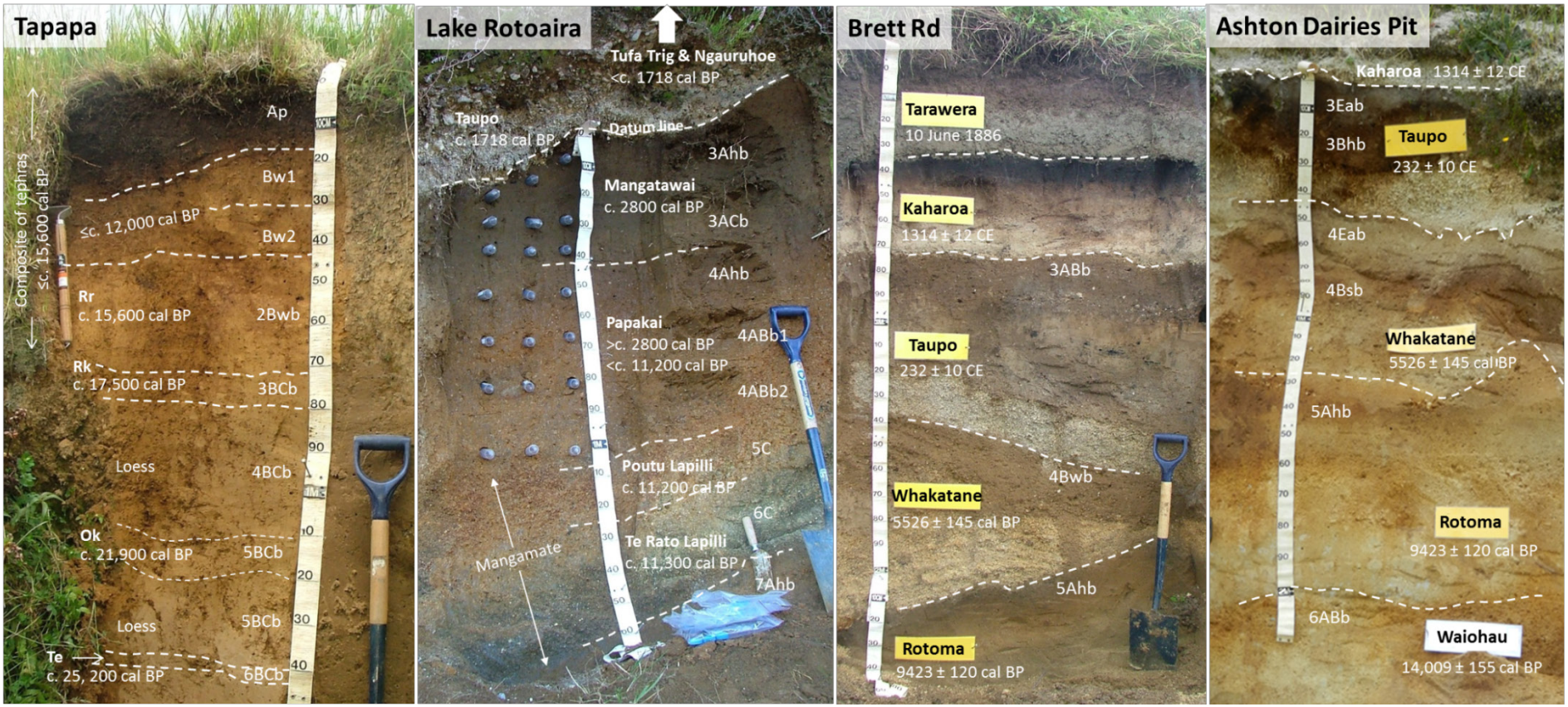

Fig. 4.3 Photos of soil profiles showing soil horizons and their notation, and the stratigraphy and names and ages of parent tephras (derived using tephrochronology) at Tapapa, Lake Rotoaira, Brett Rd, and Ashton Dairies Pit (Fig. 4.2). The suffix 'b' is used to denote an identifiable soil horizon with pedogenic features developed before its burial. More details are given in the text and Table 4.1. Abbreviations: Rr, Rotorua tephra; Rk, Rerewhakaaitu tephra; Ok, Okareka tephra; Te, Te Rere tephra; BP, before present. Scale divisions on tape = 10 cm; photos: D.J. Lowe.

4.3.1.1. Tapapa

This soil on a privately-owned farm-track cutting just off Tapapa Road near Tirau (Fig. 4.2) has developed mainly by developmental upbuilding on a composite of distal thin rhyolitic tephras and tephric loess deposits laid down incrementally (location: 37° 59' 41.01" S, 175° 52' 59.95" E, elevation 238 m above sea level [asl]). The soil stratigraphy shown in Fig. 4.3 and Table 4.1 is based on Lowe (1986, 1988) and Lowe et al. (2010, 2012); ages are from Lowe et al. (2013). The soil is a Medial, thermic Typic Hapludand (Soil Survey Staff, 2014a). Mean annual rainfall is ca 1524 mm (Leathwick et al., 2003). Two soil samples were collected from upper subsoils, namely the Bw1 (20−30 cm depth) and Bw2 (30−40 cm depth) subhorizons. The soil materials for these samples derive from a composite of weakly weathered tephras ≤ca 12,000 cal. yr BP in age that overlie Rotorua tephra (Fig. 4.3) and thus have relict properties (hence, strictly, they represent a 'relict' rather than a 'buried' paleosol using the nomenclature of Valentine and Dalrymple, 1976; Bronger and Catt, 1989). Current vegetation is pasture.

4.3.1.2. Lake Rotoaira

The soil occurs on State Highway 47 (Te Ponanga Saddle Road) near Lake Rotoaira (Fig. 4.2) (location: 39° 01' 07.08" S, 175° 41' 38.65" E, 580 m asl). The buried subsoil horizons of interest to us, depicted in Fig. 4.3, have formed mainly by developmental upbuilding pedogenesis on a composite of thin andesitic tephra beds that were deposited incrementally since the fall of Mangamate tephra. The horizons we sampled are overlain by ca 0.8 m of rhyolitic Taupo ignimbrite (its base provides the zero datum line for depth measurements) and (at the land surface) younger andesitic tephras (ca 0.3 m in thickness). These post-Taupo deposits thus indicate that both retardant as well as developmental upbuilding pedogenesis have occurred at this site. The soil stratigraphy and ages of tephras for the site follow Donoghue et al. (1995), Moebis et al. (2011), and Hogg et al. (2012). The soil profile in its entirety – from the land surface – is classified as an Ashy/pumiceous, glassy, mesic Typic Udivitrand (Soil Survey Staff, 2014a), with the buried soil horizons (as defined by Soil Survey Staff, 2014b) beneath the Taupo ignimbrite we examined having a high allophane content (Table 4.1) and thus strong andic soil properties (McDaniel et al., 2012). Mean annual rainfall is

ca 1944 mm (Leathwick et al., 2003). We sampled the 3Ahb and 3ACb subhorizons (on Mangatawai tephra ca 1718 to ca 2800 cal. yr BP) and the 4Ahb, 4ABb1, and 4ABb2 subhorizons (on Papakai tephra ca 2800 to 11,200 cal. yr BP) in the lower part of the ca 4-m-high section using Taupo ignimbrite (ca 1718 cal. yr BP) as the upper datum and Poutu Lapilli (ca 11,200 cal. yr BP) as the lower one (Fig. 4.3). The periods the soil horizons developed at the land surface were ca 1082 cal. years (3Ahb, 3ACb) and up to ca 8400 cal. years (4Ahb, 4ABb1, 4ABb2) (Table 4.1). Current vegetation is native podocarp-broadleaf forest.

4.3.1.3. Brett Rd and Ashton Dairies Pit

The soil on Brett Rd near Mount Tarawera (location: 38° 17' 53.53" S, 176° 28 52.83" E, elevation 452 m asl) (Fig. 4.2) has developed by retardant upbuilding on five relatively thick rhyolitic tephra beds as marked in Fig. 4.3. The (buried) soil stratigraphy is based on Lowe et al. (2010) and McDaniel et al. (2012); ages are from Lowe et al. (2013). Mean annual rainfall is ca 1464 mm (Leathwick et al., 2003). We sampled the 3ABb subhorizon (on Taupo tephra ca 1718 cal. yr BP), the 4Bwb subhorizon (on Whakatane tephra ca 5500 cal. yr BP), and the 5Ahb subhorizon (on Rotoma tephra ca 9400 cal. yr BP).

The soil at the Ashton Dairies Pit (on a privately-owned farm off Ash Pit Road, location: 38° 18' 09.82" S, 176° 32' 32.01" E, 444 m asl), with a mean annual rainfall ca 1428 mm (Leathwick et al., 2003), is very similar stratigraphically to the nearby Brett Rd profile. At Ashton Dairies, however, the Taupo tephra is overlain by a thicker deposit (ca 1.45 m) of Kaharoa tephra (its base provides the zero datum line for depth measurements in Fig. 4.3) and (at the surface) ca 0.45 m of Tarawera tephra. Further, several of the buried soil horizons on the Taupo and Whakatane tephras at Ashton Dairies Pit (Fig. 4.3) show morphological features associated with podzolisation (Lowe et al., 2012), an acidic soil-leaching process manifested as pale, enleached E horizons (denoted albic horizons) and as very dark brown to dark reddish-brown, humus- or sesquioxide-enriched Bh or Bs horizons (denoted spodic horizons) (Soil Survey Staff, 2014a). Consequently, uppermost B horizons on these tephras, rather than E horizons, were sampled: the 3Bhb subhorizon (on Taupo tephra ca 1718 cal. yr BP), the 4Bsb subhorizon (on Whakatane tephra ca 5500 cal. yr BP), and the 5Ahb subhorizon (on Rotoma tephra ca 9400 cal. yr BP) (Table 4.1). At both

sites, the amount of time the soil on Taupo tephra developed at the land surface was ca 1082 years (1718 minus 636 years [or AD 1314 minus 232]), Whakatane ca 3808 cal. years (5526 minus 1718 years), and Rotoma ca 3897 cal. years (9423 minus 5526 years) (Table 4.1). Both soils at Brett Rd and Ashton Dairies Pit are Fine-loamy/medial, mixed/glassy Typic Udivitrands (Soil Survey Staff, 2014a). Current vegetation at both sites is pasture.

Table 4.1 Depths, ages, and pH values of the soil and buried-soil horizons at the four sites, and their clay, allophane, and total organic carbon contents[a].

Site (horizon notation[b])	Depth	Age (range) of paleosol[c]	pH (H_2O)[d]	Clay	Allophane (fine-earth basis)	Total carbon (clay basis)
	cm	Cal. yr BP		wt %	wt %	wt %
Tapapa						
Bw1	20−30	≤c. 12,000	5.8	10.6	8.3	10.6
Bw2	30−40	≤c. 12,000	5.9	6.8	10.4	7.7[e]
Lake Rotoaira						
3Ahb	0−20[f]	2800-1718	6.3	9.3	11.3	7.0
3ACb	20−40[f]	2800-1718	6.2	8.1	18.3	6.9
4Ahb	40−60[f]	11,200-2800	6.2	16.6	32.7	6.7
4ABb1	60−80[f]	11,200-2800	6.2	19.0	18.6	6.5
4ABb2	80−100[f]	11,200-2800	6.1	17.2	14.5	7.0
Brett Rd						
3ABb	70−80	1718-636	6.6	3.4	3.0	11.9
4Bwb	140−150	5526-1718	6.8	3.9	5.3	10.2
5Ahb	210−220	9423-5526	6.6	5.0	2.5	6.3
Ashton Dairies Pit						
3Bhb	20−30[g]	1718-636	6.5	4.7	2.1	na
4Bsb	60−80[g]	5526-1718	6.5	2.9	1.2	10.5
5Ahb	130−140[g]	9423-5526	5.5	1.1	4.8	13.3[e]

[a]Means of two samples.
[b]Based on Soil Survey Staff (2014a, 2014b).
[c]Approximate length of time the tephra material was at or near the land surface undergoing weathering and pedogenesis by topdown soil processes before its burial by subsequent tephra(s) (ages derived from Lowe et al., 2013, using tephrochronology – see text).
[d]10 g of soil (<2 mm) in 25 mL of deionized (DI) water.
[e]From equivalent buried soil horizons on the same tephras at nearby sites as reported by Parfitt et al. (1981) and Lowe et al. (2010) (after Green, 1987).
[f]Below the datum line of the base of Taupo ignimbrite (Fig. 4.3).
[g]Below the datum line of the base of Kaharoa tephra (Fig. 4.3).
na: data not obtained.

4.3.2. Allophane field test and sample preparation

Samples at all sites were tested using the NaF-based allophane test in the field (Hewitt, 2010) to confirm that they were allophane-rich. In total, 13 samples of buried allophanic soil horizons (paleosols) were collected. They were sieved to obtain <2 mm-size (fine-earth) fractions and stored at 4°C in the dark.

4.3.3. Soil properties and extraction of organo-clay fractions

Soil pH values (solid/solution = 1/2.5) were measured in water following the method described in Blakemore et al. (1987) (Table 4.1). Allophane contents of soil samples were estimated by oxalate extractable Fe, Al, and Si as well as pyrophosphate extractable Al (Parfitt and Wilson, 1985; Blakemore et al., 1987). Before extraction of clay fractions, visible root remnants were removed. To obtain clay fractions (particle sizes <2 μm), each sample was dispersed mechanically by prolonged shaking for 16 h with DI water and 2-mm glass beads (Churchman and Tate, 1986), followed by sedimentation of particles >2 μm according to Stokes' Law and then clay was extracted via a pipette. The clay fractions were frozen quickly with liquid nitrogen and freeze-dried to preserve the nature of organic matter associated with the clay, and the freeze-dried (organo-) clays were ground and analysed for total organic carbon (TOC). Analysis of TOC was undertaken using a Leco TruSpec carbon/nitrogen analyzer. The pH values, clay (<2 μm), and allophane contents of samples collected from the paleosols are shown in Table 4.1, together with the total carbon contents (of clay fractions) and stratigraphic and age information.

4.3.4. Characterisation of SOM sequestered by clays using carbon K-edge NEXAFS spectroscopy

C NEXAFS spectra for (organo-) clays and for pure indium foils (as sample carriers) were collected at beamline 24A1 at the National Synchrotron Radiation Research Center (NSRRC) at Hsinchu, Taiwan. Fluxes of photons 5×10^{11} to 1×10^{10} per second were admitted to the chamber, and the beamline produced soft X-rays (energy range < 5 keV) so that the energy was tuneable over 10 to 1500 eV and allowed a focussed beam size of 0.7 by 0.3 mm. The end station comprised three chambers, including a pre-chamber, a transfer chamber,

and a main analytical chamber. The last is under very high vacuum (<1 $\times 10^{-8}$ torr). For C NEXAFS spectroscopy with high resolution, the beamline grating was set to 400 mm^{-1}, and the positions of two beamline silts were set to -200 and -20 on the dial to minimize the light source and focus the beam.

An extracted organo-clay fraction from each soil sample was pressed into indium foil (0.5 mm thick, manufactured by Puratronic®, 99.9975%) to be conductive so that the excited electrons could be transmitted from the sample surface to the detector. The surface of indium foil was fully covered with sample to avoid any extraneous carbon contribution from the indium foil itself, and the indium foil with attached sample was flattened with clean glass rods. The flattened indium foil containing the sample was attached to a clean sample holder, and the holder was placed into the pre-chamber for degassing for 2 h and then transferred to the main analytical chamber.

The C NEXAFS spectroscopy was performed with the X-ray energy set to increase from 275 to 340 eV with a step of 0.035 eV (2000 dwell points in total). Three spectra were collected for each (organo-) clay sample based on total electron yield and partial electron detection modes with an electron energy analyzer (SPECS PHOBOIS 150). Dwell time for every point was 1000 milliseconds to improve the counting statistics and reduce error. To normalise the signals from samples, the total electron yield from each sample was divided by the yield of a clean surface (I_0) measured concurrently with the sample of interest. I_0 is obtained by X-rays travelling through a golden grid which had been set up in the beam path in the ionization chamber ahead of the analytical chamber. The golden mesh is coated in-situ by ionized gold atoms ejected from a gold stick during a sputtering process before the experiment is carried out. The signal derived when the beam passes through the just-coated golden mesh is designated to be carbon-free, namely I_0, and the interference of carbon accumulating in the beam path with spectra normalisation could therefore be eliminated.

The replicate C NEXAFS spectra for each sample were merged, processed, baseline-corrected, and normalised using the Athena program, an interface to IFEFFIT (version 1.2.11). In our study we only show the spectra of total electron yield as they provided better resolution with high electron yield, and the characteristic peaks were distinct. The peaks representing various functional groups (or carbon speciation) were identified according to the X-ray energies

(Table 4.2), specifically quinonic (283.7−284.3 eV), aromatic (284.9−285.5 eV), phenolic (286.5−287.2 eV), aliphatic (287.1−287.8 eV), carboxylic (287.7−288.6 eV), and carbonyl (289.3−290.5 eV) groups (see also Fig. 4.1).

Table 4.2 Identification of functional groups via C *K*-edge NEXAFS spectroscopy[†]

Energy (eV)	C forms
283.7−284.3	quinonic C, protonated aromatic C
284.9−285.5	aromatic C
286.5−287.2	phenolic C
287.1−287.8	aliphatic C, aromatic carbonyl C
287.7−288.6	carboxylic C
289.3−290.5	carbonyl C, carbonate C

[†]After Lehmann (2005), Solomon et al. (2005), Wan et al. (2007) (cf. Fig. 4.1).

4.3.5. Characterisation of internal porous structure of allophane aggregates using transmission X-ray microscopy

To help envisage and explain the preservation of the SOM identified in the allophanic buried soils/paleosols, we used a synchrotron-based transmission X-ray microscope (TXM) which allows two dimensional (2D) imaging and 3D tomography at tuneable energies from 6 to 11 keV. The experiment was carried out at BL01B1 at NSRRC, and a natural allophane aggregate extracted from the Bw1 horizon of the soil at Tapapa (Fig. 4.3) was examined at 1.84 keV for silicon *K*-edge. 2D micrographs of the aggregate were generated using TXM with 60 s exposure times to obtain micrographs of high resolution; the 3D tomography datasets were reconstructed based on sequential image frames taken with azimuth angle rotating from −75° to +75° to obtain 151 2D micrographs of the aggregate, with 60 s exposure times for each 2D micrograph. The final 3D virtual structure (image) of the aggregate was generated from the tomographic dataset using the software Amira 5.01, Visage Imaging.

4.4. Results

4.4.1. Clay mineralogy

The allophane contents for each sample (Table 4.1) were generally consistent with findings of earlier work on the allophane-dominated clay mineralogical assemblages of paleosols on Holocene and late Pleistocene rhyolitic tephras in the Rotorua-Taupo region (Birrell and Pullar, 1973; Kirkman, 1976; Hodder et al., 1990; Lowe and Percival, 1993).

4.4.2. Testing the carbon in (organo-)clay samples and indium foil using C NEXAFS spectroscopy

The C NEXAFS spectra for carbon in (organo-) clays extracted from the Bw1 horizon at Tapapa (total carbon was ~10% on dry clay basis, see Table 4.1) and on indium foil (sample carrier) are shown in Fig. 4.4. The multiple peaks over the carbon X-ray absorption region (284−290 eV) indicate the presence of various functional groups in organic matter adsorbed on the clay sample and on the indium foil. The spectra revealed that the SOM held by the clay extracted from the Bw1 horizon at Tapapa was predominantly carboxylic carbon (absorption energy at ~287 eV), with subordinate contributions from quinonic and aromatic functional groups (over the 284−285 eV region). In contrast, carbon from the indium foil was characterised by only carboxylic and quinonic carbon, which could be from the impurities when the indium foil was refined and manufactured or from extraneous carbon adsorbed on indium foil, or both. Because these spectra from the clay and indium foil exhibit different baselines and intramolecular resonances (over the 290−296 eV region) for carbonaceous compounds, then the C NEXAFS spectrum for the clay sample attached to the indium foil could not have originated from the indium foil itself; the C NEXAFS spectroscopy at BL24A1 at the NSRRC was therefore able to characterise carbon from various sources.

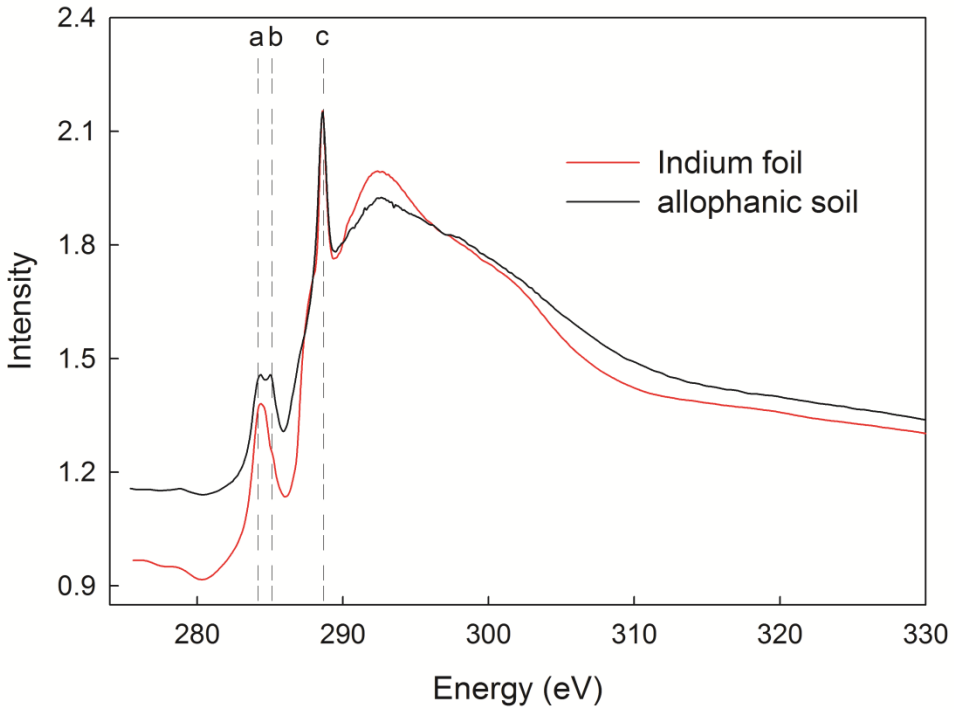

Fig. 4.4 C NEXAFS spectra for an organo-clay extracted from a natural allophanic soil (from the Bw1 subhorizon at the Tapapa site, Figs. 4.2 and 4.3) attached to indium foil and on soil-free indium foil (99.9975% purity). Spectral features identified by the vertical lines correspond to carbon in (a) quinonic, (b) aromatic, and (c) carboxylic functional groups (Fig. 4.1, Table 4.2).

4.4.3. Nature of SOM in clay fractions of paleosols at four sites

4.4.3.1. Developmental upbuilding sites: Tapata and Lake Rotoaira

At the Tapapa site, clay fractions of both the Bw1 (20−30 cm) and Bw2 (30−40 cm) horizons had sequestered similar C functional groups compositionally and proportionally (Fig. 4.5A). The C NEXAFS spectra were mainly characterised by quinonic (284 eV), aromatic (285 eV), and carboxylic (288.5 eV) carbon.

At Lake Rotoaira, the clay and allophane contents of the five buried horizons varied markedly despite consistent total carbon contents across these clays of ~7% (Table 4.1). The C NEXAFS spectra for adsorbed organic matter on the clay fractions from the different horizons showed similar compositions and proportions of functional groups, including a dominant peak in the carboxylic region (288.6 eV) and with contributions from quinonic (284.3 eV), aromatic (285 eV), and aliphatic (287.1 eV) carbon (Fig. 4.5B). This result shows that the

structures of preserved SOM in clay fractions of paleosols on Holocene tephras of different ages (Fig. 4.3, Table 4.1) have remained the same, and that clay and allophane contents, and time, have not significantly affected the constituent functional groups of the SOM adsorbed by clays in these paleosols.

4.4.3.2. Retardant upbuilding sites: Brett Rd and Ashton Dairies Pit

The soils at Brett Rd and Ashton Dairies Pit sites are developed on the same set of Holocene tephras, including Taupo, Rotoma, and Whakatane tephras. The clay-attached organics in paleosols on each of these tephras at the two sites, despite some soil morphological differences noted earlier (Fig. 4.3, Table 4.1), still have similar spectral characteristics (Fig. 4.5C), including substantial amounts of carboxylic groups (>40%) at 288.6 eV and relatively small contributions of quinonic (284.3 eV), aromatic (285 eV), and aliphatic (287.1 eV) groups despite differences in paleosol ages and depths (Fig. 4.3, Table 4.1). As at Tapapa and Lake Rotoaira, these similarities indicate that the structures of preserved SOM in the clay fractions of Holocene paleosols of different ages (Fig. 4.3, Table 4.1) have remained the same despite differences in clay and allophane content and age.

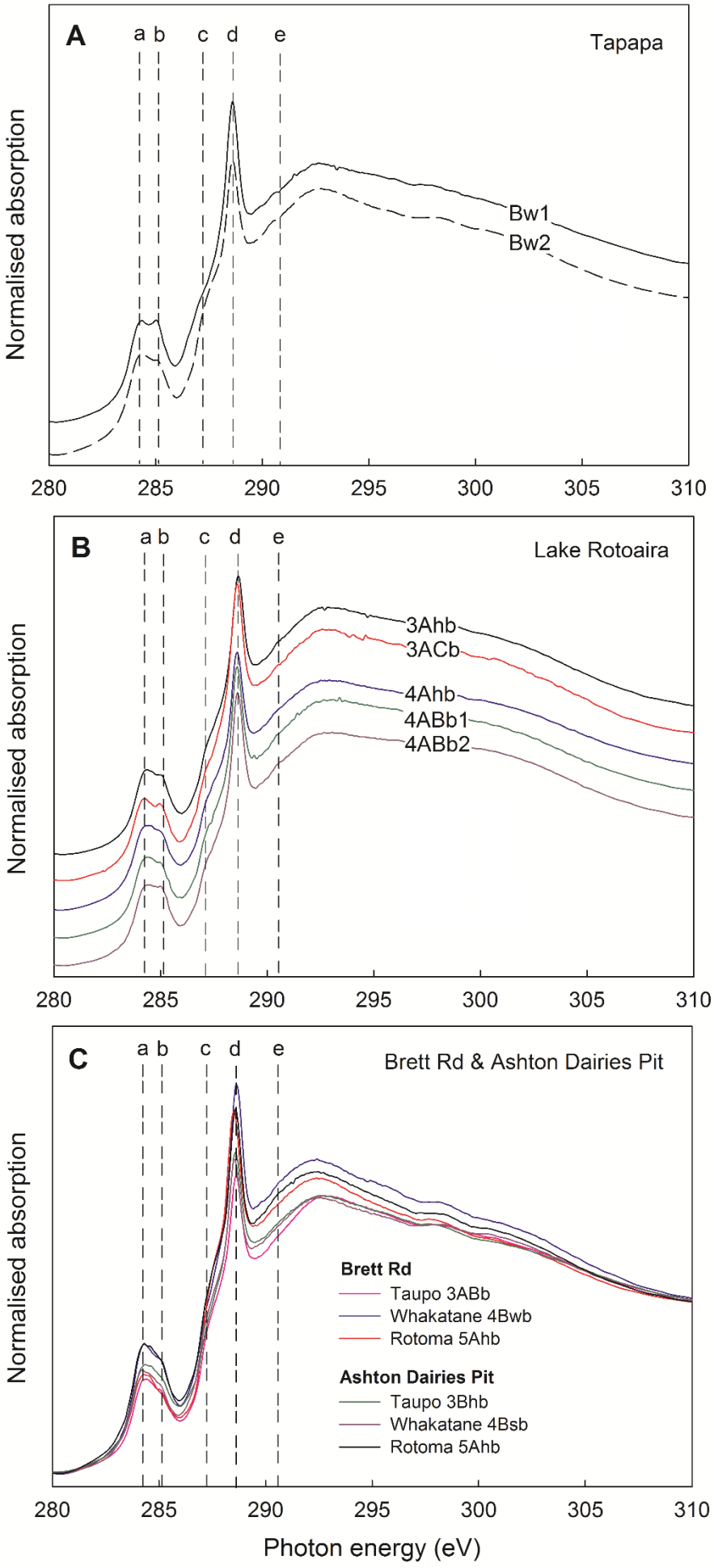
A
a b c d e
Tapapa
Bw1
Bw2
Normalised absorption
280 285 290 295 300 305 310
B
a b c d e
Lake Rotoaira
3Ahb
3ACb
4Ahb
4ABb1
4ABb2
Normalised absorption
280 285 290 295 300 305 310
C
a b c d e
Brett Rd & Ashton Dairies Pit
Brett Rd
Taupo 3ABb
Whakatane 4Bwb
Rotoma 5Ahb
Ashton Dairies Pit
Taupo 3Bhb
Whakatane 4Bsb
Rotoma 5Ahb
Normalised absorption
280 285 290 295 300 305 310
Photon energy (eV)

Fig. 4.5 C NEXAFS spectra for organo-clays in allophanic paleosols. (A) Clays extracted from the Bw1 horizon (20−30 cm depth) and from the Bw2 horizon (30−40 cm depth) above Rotorua (Rr) tephra at Tapapa. (B) Clays extracted from the five soil subhorizons denoted 3Ahb and 3ACb (representing ca 1082 yr of soil formation), and 4Ahb, 4ABb1, and 4ABb2 (representing up to ca 8400 yr of soil formation) at Lake Rotoaira. (C) Clays extracted from upper allophanic soil horizons on Taupo tephra (representing ca 1082 yr of soil formation), Whakatane tephra (representing ca 3808 yr of soil formation), and Rotoma tephra (representing ca 3897 yr of soil formation) at Brett Rd and Ashton Dairies Pit. Spectral features identified by the vertical lines correspond to carbon in (a) quinonic, (b) aromatic, (c) aliphatic, (d) carboxylic, and (e) carbonyl/carbonate functional groups (Fig. 4.1, Table 4.2). Note there were cartographic shifts of spectra in both A and B graphs to show each spectrum clearly, but no shift in graph C in order to observe the difference of intensity of X-ray absorbance between samples collected from the two near-adjacent sites.

4.4.4. Porous internal structure of an allophane aggregate from the soil at Tapapa

The 2D and 3D images of the examined aggregate are presented in Fig. 4.6. The 3D virtual reconstruction of the allophane aggregates showed that the microaggregate was highly porous and comprised many sub-microaggregates.

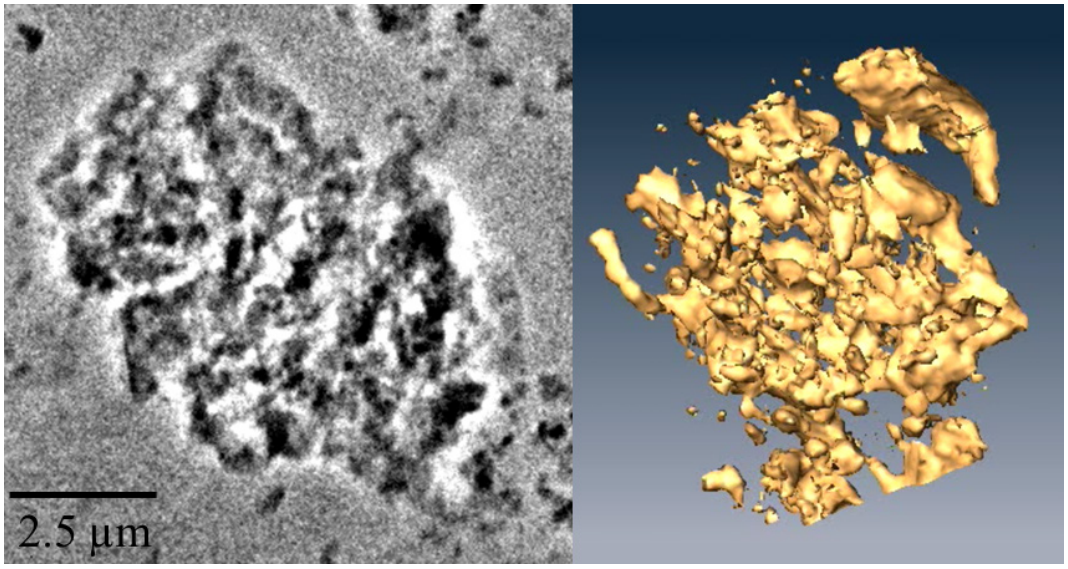

Fig. 4.6 (Left) A 2D micrograph of a natural allophane-dominated microaggregate (extracted from the Bw1 horizon of the soil at Tapapa) obtained using TXM. White areas are pores. (Right) A 3D virtual reconstruction of the microaggregate obtained from 3D tomography datasets, showing its high porosity and high degree of pore network tortuosity (see Huang et al., 2016). The scale is the same for the two images, but the images (stills) show the microaggregate from different

azimuth angles. (Video views of the 2D image and the coloured 3D reconstruction were submitted with the paper.)

4.5. Discussion

4.5.1. Explaining the similarity of carbon functional groups of SOM in paleosol clay fractions with time and depth of burial

In the present study, the shapes of spectra for SOM in clay fractions of the paleosols showed no significant increase or decrease in intensity of specific C functional groups between samples, revealing the high similarity of stabilized C in the clay fractions of the allophane-rich paleosols. Similarly, with the use of solid-state NMR spectroscopy, the SOM in 50, 100, 300, 700, and 2000 year-old soils collected from a paddy soil chronosequence in China showed similar compositions of carbon functional groups (Zhou et al., 2014), and this similarity of carbon functional groups was attributed to ongoing plant material input and wet-dry cropping alternations. In contrast, C NEXAFS experiments undertaken by Gillespie et al. (2014) showed a slight accumulation of ketones and a decrease of carbohydrate, phenolic, and carboxylic groups in buried organic horizons (no age data provided) in permafrost in Canada.

In our study, at both developmental and retardant upbuilding sites, neither clay content, allophane content, age, nor depth of burial seemed to influence the C functional group compositions of SOM of the clay fractions in Holocene paleosols on tephras. These similarities suggest that either (1) the SOM adsorbed as organo-clay in allophanic soils remained essentially constant with time throughout the Holocene under a persistent conifer-broadleaf forest vegetation and more-or-less stable climate apart from some changes in forest composition and climatic parameters (Section 4.2.2), or (2) the carbon compositions of paleosols were effectively homogenized throughout the sequences mainly by downward migration (leaching) of dissolved SOM through time, or both (1) and (2). A third possible explanation is that the soils themselves were markedly blended physically via pedoturbation processes such as tree overturn (Hogg and McCraw, 1983; Lowe, 1986). However, this (third) possible process is not likely to be of major significance because the visible tephra layering has remained largely conformable and intact at each of the sites (Fig. 4.3).

Although scenarios (1) and (2) are both plausible, we favour scenario (1) to explain the similarity of SOM composition, and its origin, through pedogenesis of these soils at or near the soil's surface with good drainage in an upbuilding setting (described in Section 4.2.1). In such settings, the inputs of modern organic matter and an active weathering environment, with pH values in the range 5 to 7, result in the dissolution of tephra-derived glass and primary minerals by hydrolysis where the dominant proton donor is carbonic acid, H_2CO_3 (formed from dissolved CO_2 via soil organism respiration), and the subsequent precipitation and synthesis (neoformation) of secondary minerals (Ugolini and Dahlgren, 1991, 2002; McDaniel et al., 2012). Where soil environments are rich in organic matter and pH values are <5, organic acids are usually the predominant proton donator (Shoji and Fujiwara, 1984; Ugolini and Sletten, 1991; Takahashi and Dahlgren, 2016), as probably occurred at times at Ashton Dairies Pit (with organic acids driving dissolution in upper, and carbonic acid, in lower soil horizons) (e.g. Ugolini et al., 1988, 1991). In either case, topdown processes including weathering predominate so that pedogenesis begins in newly-deposited tephra at or near the soil/land surface, allophane forms through neoformation (Hiradate and Wada, 2005; Churchman and Lowe, 2012), and it sequesters contemporary SOM from the modern organic cycle (e.g. Inoue et al., 2011). Ongoing tephra deposition then causes the land surface to rise, either incrementally or paroxysmally by deposition of a thick layer (depending on proximity of the site to volcanic source and volcanological and meteorological factors) so that the once-surface horizon becomes buried more deeply and hence increasingly isolated from the modern organic cycle so that the effects of pedogenesis become gradually or abruptly negligible (Schaetzl and Sorenson, 1987). The SOM adsorbed chemically and physically when the soil horizon was at the land surface is preserved despite burial, thus is relict from the soil-forming environment of that time. Allophane does not continue to form at depth in these multisequal Holocene soils once they are buried because the paleosol characteristics of late Quaternary tephras in the Rotorua-Taupo region, including those examined in our study, are widely preserved and typically weakly developed (Birrell and Pullar, 1973; Lowe, 1986; Hodder et al., 1990). They have only moderate to low amounts of clay because such clay contents correlate strongly with time for weathering of the tephra at the land surface, rather than age of eruption/deposition of the tephra, as expressed by the relationship:

$$y = 2.6752 + 3.2869x \ (R^2 = 0.818) \quad (1)$$

where y is clay content (<2 μm) and x is the time for weathering (years at land surface before burial) (Lowe and Percival, 1993, p. 65). Moreover, older buried paleosols developed on pre-Holocene tephras are dominated by halloysite, not allophane, this clay being formed under lower rainfall and reduced Si leaching during the Last Glacial period (Parfitt et al., 1984; Stevens and Vucetich, 1985; Churchman and Lowe, 2012).

These conclusions regarding the origins of the very stable SOM in the clay fractions of buried soils, and the gradual or sudden isolation of the buried soils from the modern organic cycle and surface weathering regime, are supported by findings from an earlier study (Limmer and Wilson, 1980) involving the fate of amino acids on a series of four buried paleosols on tephras of different ages in central North Island, New Zealand. Limmer and Wilson (1980) concluded that the amino acids extracted from the paleosols were "fossil", i.e. relict from when the soils were at the land surface, because of the simplicity of the composition of the amino acids in the older paleosols. The youngest paleosol (ca 80 years old) contained 14 identifiable amino acids, but there was a systematic disappearance of eight out of 14 of them over a period of ca 45,000 years. The lack of any recent contamination by invasion of plant roots, or downward leaching of amino acids, which would have involved all of the 14 identifiable amino acids, corroborates our view that the buried horizons of our study were isolated so that the preserved SOM in them must have originated from when they were at the land surface in the past, i.e. they faithfully represent environments of the past.

Near Naples in Italy, Calderoni and Schnitzer (1984) analysed humic substances extracted from six paleosols formed on a sequence of pyroclastic deposits dating from ca 30,000 to 7000 cal. yr BP. They found that there were essentially no differences in the chemical composition and structure of these substances in the paleosols despite the differences in age, and concluded that the paleosols appear to be "closed systems from the geochemical point of view" (p. 2049).

Studies on phytolith and clay mineral assemblages preserved in buried paleosols on late Quaternary rhyolitic tephras near Rotorua or Taupo and elsewhere in central North Island demonstrated that both these assemblages reflected the pre-burial climate at the time the soils were forming at the land

surface, not the climate subsequent to burial some thousands of years later (Parfitt et al., 1984; Sase et al., 1988; Kondo et al., 1994; Lowe and Percival, 1993; Churchman and Lowe, 2012). Similarly, Sedov et al. (2004) concluded that phytolith assemblages in buried late Quaternary paleosols on tephra deposits in Mexico reflected plant cover of the early stages of soil development before burial and are relict (i.e. the phytoliths represent "a soil memory").

4.5.2. Explaining the preservation of SOM in clay fractions of allophanic buried soils/paleosols

Previous studies on the intercalation of humic substances with interstratified mica-beidellite and mica-smectite have shown that such clays provide physical protection of the interlayer humic substances to resist decomposition (Theng et al., 1986, 2005), and the organic matter stored within such organo-minerals may be coincident with the formation of soil and absorption of humic substances by clay (Theng et al., 2005). Another study also showed that deoxyribonucleic acid (DNA) intercalated with expanding montmorillonite undergoes fewer attacks by enzymes (Hou et al., 2014).

SOM adsorbed by allophane and its nanoaggregates comprising nanopores (<100 nm in size) is likely to stay intact once the organo-aggregates or flocculates have formed and stabilized (Churchman and Tate, 1987; Asano and Wagai, 2014; Matus et al., 2014; Huang et al., 2016). The pore sizes of aggregates significantly influence carbon stabilization and storage in soils – the decomposition rate of labelled ^{13}C in soils with large volumes of pores with neck diameters <4 μm is much slower than that in soils with large volumes of pores with neck diameters ~15−60 μm (Strong et al., 2004).

It has also been suggested from several studies that the organic matter storage in Andisols is governed substantially by allophane: increasing allophane content and the extent of fractal nanopore distribution (described as "nanolabyrinthic") decreases organic matter bioavailability (Chevallier et al., 2010), and bomb ^{14}C studies have shown that carbon turnover in Andisols is slower than that in non-Andisols (Parfitt, 2009; Baisden et al., 2010). Studies by Parfitt et al. (2002) and Olson et al. (2014) demonstrated that soil carbon in both allophanic and non-allophanic soils decreased with depth because most inputs of fresh carbon occur at the soil surface, and the retention of old carbon in allophane-

dominated Andisols was 77−79% (Parfitt et al., 2002), greater than that (69−72%) in non-allophanic soils.

Although downward leaching of mobile younger SOM from the surface through the profiles occurs, we contend that such SOM would not be well protected chemically unless it was able to be encapsulated into the tortuous (nanolabyrinthic) pore network, which, however, would be largely occupied by older SOM encased physically during past periods of soil formation that included weathering and neoformation at the land surface. Thus, much of the SOM adsorbed and preserved chemically and physically by allophane in clay fractions of the buried paleosols on Holocene tephras in New Zealand is, we suggest, as described above in the model of upbuilding pedogenesis for our study, derived mainly from past environments when the organo-allophane aggregates were formed at the (paleo) land surface prior to their burial. This conclusion is supported by the earlier findings on amino acid degradation by Limmer and Wilson (1980), and other evidence relating to buried paleosol stability.

The SOM sequestered in clays of allophanic soils is thus likely to remain similar in structure after being buried for thousands years because the organic matter has been well preserved in such soil materials, no matter what the composition of functional groups of the organic matter were, and so therefore would likely increase in age with depth. Although there has been limited ^{14}C dating specifically of humic substances in Holocene paleosols in North Island (charcoal or woody material being preferred, e.g. Froggatt and Lowe, 1990), paleosol-derived humic samples dated using ^{14}C reported by Goh and Pullar (1977) (from studies on Holocene tephras in the Rerewhakaaitu area), and by Froggatt and Lowe (1990), are largely consistent chronologically with tephrostratigraphic ages and show a pattern of increasing age with depth through the Holocene, and therefore supporting our earlier contention that the SOM is relict and stable. Similarly, Inoue et al. (2011) showed that ^{14}C ages on humins that had formed in upbuilding soils on Holocene tephras in Japan increased with depth.

Using C NEXAFS spectroscopy, Heymann et al. (2014) showed that the proportions of aromatic C and O-alkyl C and in alkaline extracts from soils at different depths could be variable. Kleber et al. (2011) also showed that old, preserved SOM adsorbed by clays does not comprise a particular type of carbon, and a 680-year-old Inceptisol sequestered a large proportion of alkyl carbon

(aliphatic functional groups) that can be metabolized easily. In our study, the proportions of carbonaceous functional groups of SOM in the clay fractions of buried paleosols at Lake Rotoaira, Brett Rd, and Ashton Dairies Pit (Figs. 4.5B and 4.5C) are similar to those in the upper subsoil samples from Tapapa (Fig. 4.5A), and the SOM at different depths are both characterised by dominant carboxylic groups which are indicative of exposure and oxidation of carbon in soil (Lehmann, 2005). The predominance of carboxylic carbon in the allophanic soils/paleosols examined in our New Zealand study may result from (1) a strong association of carboxylic groups of humic substances with Al-OH defects on the surface of allophane by ligand exchange (Yuan et al., 2000; Theng et al., 2005; Matus et al., 2014), or (2) oxidation of organics in uppermost soil horizons (at the land surface under an active organic regime) (Lehmann, 2005) in the past and before the land surface was buried by new tephra deposits, or both.

A parallel conclusion was drawn by Calderoni and Schnitzer (1984) who suggested that the preservation of the humic materials (together with essentially constant levels of aromatic, carboxyl, phenolic, and carbonyl functional groups) in the sequence of six paleosols formed on late Quaternary pyroclastic deposits in Italy was a consequence of (1) very low biological activity after burial, and thus minimal or no significant inputs of fresh organic matter, and (2) probably because of "strong retention" of the humic substances by "amorphous" (i.e. nanocrystalline allophanic) minerals.

4.5.3. Quinone and its importance as a potential paleopedological biomarker

In comparison with published C NEXAFS spectra for organic matter in other soils or soil aggregates (Solomon et al., 2005; Wan et al., 2007; Kleber et al., 2011), our New Zealand allophanic paleosols studied here all contained a distinct amount of quinonic carbon (over 284 eV region). Natural quinones are common constituents of bacterial plasma membranes (Lester and Crane, 1959; Collins and Jones, 1981) and of pigments of chloroplasts (Karp, 2013), which are involved in cellular respiration and photosynthesis. Among natural quinones, isoprenoid quinones have been used as taxonomic markers (Collins and Jones, 1981) and are one of the most important groups of quinones because of their functions. Isoprenoid quinones are composed of a hydrophilic head group and an apolar isoprenoid side chain, giving the molecules a lipid-soluble character (Nowicka

and Kruk, 2010). These quinones are hydrophobic and particularly susceptible to breaking down in well-drained alkaline conditions and are photo-oxidized rapidly in the presence of oxygen and strong light (Green et al., 1960; Dunphy and Brodie, 1971). Naturally, the quinonic ring (an unsaturated ring containing two –C=O groups) undergoes reversible reduction, leading to more stable quinol ring (an unsaturated ring containing two –OH groups) (Nowicka and Kruk, 2010). Therefore, quinonic carbon is labile and highly susceptible to degradation and transformation in soils, which explains the absence or trace presence only of quinonic carbon in most soils examined in previous studies (e.g. Solomon et al., 2005; Wan et al., 2007). Similarly, easily-decomposable humic constituents in tephra-paleosol sequences in Japan are not preservable for long periods without protection mechanisms (e.g. Tsutsuki and Kuwatsuka, 1989). Hence, the presence of quinonic carbon in some soils has been attributed to its occlusion and thus protection within organo-aggregates – for example, Solomon et al. (2012) mapped the carbon functional groups of an ultrathin section of a soil microaggregate and showed the quinonic carbon occurred only in the inner and intermediate regions of the aggregate.

The encapsulation of organic matter within labyrinthic pore networks amidst allophane aggregates, readily envisaged from the 3D microtomographic image in Fig. 4.6, allows such organic matter to be occluded and thus remain intact despite acidic leaching and oxidising conditions at our sites (cf. McCarthy et al., 2008; Chevallier et al., 2010; Huang et al., 2016). Hence, the presence of quinonic carbon in the clay fractions of our allophane-rich paleosols suggests that it has been preserved both (1) by enclosure within tortuous pore network when allophane aggregates (either nanoaggregates or microaggregates) were formed, and (2) by burial by subsequent tephra-fall deposits. Both the enclosure and rapid burial would help cut out light (which is unlikely to penetrate beyond ca 10 mm: Tester and Morris, 1987) and thus reduce photodegradation of quinonic carbon by solar irradiance (Rutledge et al., 2010). The mean rates of tephra accretion at Brett Rd and Ashton Dairies Pit are ca 25 mm per century, and those at Tapapa and Lake Rotoaira are ca 5 to 12 mm per century, respectively (after Lowe, 2000), and so (on average) surface horizon components typically would be buried beyond light penetration within decades to a century.

We therefore suggest that the SOM in clay fractions of allophane-rich paleosols is very likely to derive largely from an environment of the past (in the Holocene) rather than as leachates of "young" organic matter, and the presence in particular of quinone effectively offers a paleopedological biomarker representing SOM formed contemporaneously with pedogenesis whilst parent tephra materials (now buried) were at or near the land surface. This conclusion will require further testing, such as by analysis of paleoenvironmental DNA in different soil/paleosol horizons (see Rawlence et al., 2014; see chapter 6), and by analysis of clay-associated SOM using pyrolysis-GC/MS (e.g. Buurman et al., 2007; Verde et al., 2008).

4.6. Conclusion

(1) The C NEXAFS spectra for SOM in the clay fractions of allophane-rich buried soils/paleosols on Holocene tephras in New Zealand ranging in age from ca 12,000 to 1718 cal. yr BP at sites characterised by both developmental and retardant upbuilding pedogenesis, and under persistent podocarp-broadleaf forest cover (currently extant at the Lake Rotoaira site) until Polynesian arrival in the late 13th century, showed considerable and consistent amounts of carboxylic carbon and subordinate contributions of quinonic, aromatic, and aliphatic functional groups. The compositions and proportions of carbonaceous functional groups of the SOM adsorbed by clays in allophanic paleosols did not change with time, depth of burial, clay, or allophane contents, composition (andesitic versus rhyolitic), current vegetation, and mode of soil genesis (developmental versus retardant upbuilding).

(2) The results mainly indicate that either (1) the passage of time would not result in the alteration of SOM in the clay fractions of buried allophane-rich paleosols and hence there is strong degree of preservation of organic matter in such soils, and no or negligible inputs of fresh organic matter because of the eventual or rapid isolation (via burial) of the soil horizons at depth, or (2) the downward leaching of dissolved SOM homogenizes the compositions of carbon functional groups of SOM in each horizon, effectively a process of diagenesis rather than pedogenesis in that it would occur after a soil was buried. However, the presence of labile quinonic carbon in the allophanic paleosols, which included strongly acidic to podzolic/spodic conditions at one site, may be attributable both

to the strong protection of SOM and quinones within the tiny nanolabyrinthic pores within allophane aggregates, and to rapid burial and isolation by ongoing tephra deposition. Therefore, the SOM is likely to be "old" or relict SOM of past environments.

(3) We envisage that the SOM (as expressed by the carbon functional groups) originated at the land surface via upbuilding pedogenesis and weathering dominated by hydrolysis (where the proton donor was mainly carbonic acid, and/or organic acids at Ashton Dairies Pit at times) in free-draining tephra. As soil genesis began in a newly-deposited tephra at the soil surface, allophane was precipitated and formed aggregates, physically sequestering contemporary SOM from the modern (surficial) organic cycle. Ongoing tephra deposition then caused the land surface to rise (incrementally → development upbuilding; or rapidly by deposition of a thick layer → retardant upbuilding) so that once-surface horizons were buried more deeply and hence became increasingly divorced from the modern organic cycle over time, eventually forming isolated paleosols. The SOM preserved in the paleosols at each of the four study sites thus derives from processes operating from early to late Holocene according to the age of burial of the (former) surface soils by subsequent tephra deposition, and not from modern surface processes or diagenesis.

(4) The novel study of carbon SOM functional groups using C NEXAFS spectroscopy in paleosols on tephra deposits, datable using tephrochronology, provides a new approach for studying environments of the past via paleopedology, with quinoic carbon effectively a paleopedological biomarker. The study also helps inform processes of carbon sequestration by multisequal Andisols and their polygenetic origins by upbuilding pedogenesis, and supports the important concepts espoused by Schaetzl and Sorenson (1987) about the burial and isolation of paleosols.

(5) We plan further applications of C NEXAFS spectroscopy to SOM extracted from clay fractions from older, late Pleistocene buried paleosols on tephras dating to ca 30,000 cal. yr BP in northern New Zealand including those formed under glacial conditions (e.g. Newnham et al., 1999; Barrell et al., 2013).

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Chapter 5 DNA adsorption by nanocrystalline allophane spherules and nanoaggregates, and implications for carbon sequestration in Andisols

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DNA adsorption by nanocrystalline allophane spherules and nanoaggregates, and implications for carbon sequestration in Andisols

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ABSTRACT

This study provides fundamental knowledge about the interaction of allophane, deoxyribonucleic acid (DNA), and organic matter in soils, and how allophane sequesters DNA. The adsorption capacities of salmon-sperm DNA on pure synthetic allophane (characterised morphologically and chemically) and on humic-acid-rich synthetic allophane were determined, and the resultant DNA–allophane complexes were characterised using synchrotron-radiation-derived P X-ray absorption near-edge fine structure (XANES) spectroscopy and infrared (IR) spectroscopy. The synthetic allophane adsorbed up to 34 μg mg^{-1} of salmon-sperm DNA. However, the presence of humic acid significantly lowered the DNA uptake on the synthetic allophane to 3.5 μg mg^{-1} by occupying the active sites on allophane so that DNA was repulsed. Both allophane and humic acid adsorbed DNA chemically through its phosphate groups. IR spectra for the allophane–DNA complex showed a chemical change of the Si–O–Al stretching of allophane after DNA adsorption, possibly because of the alteration of the steric distance of the allophane outer wall, or because of the precipitation of aluminium phosphate on allophane after DNA adsorption on it, or both. The aluminol groups of synthetic allophane almost completely reacted with additions of small amounts of DNA (~2–6 μg mg^{-1}), but the chemical adsorption of DNA on allophane simultaneously led to the formation of very porous allophane aggregates up to ~500 μm in diameter. The formation of the allophane nano- and microaggregates enabled up to 28 μg mg^{-1} of DNA to be adsorbed (~80% of total) within spaces (pores) between allophane spherules and allophane nanoaggregates (as "physical adsorption"), giving a total of 34 μg mg^{-1} of DNA adsorbed by the allophane. The stability of the allophane–DNA nano- and microaggregates likely prevents encapsulated DNA from exposure to oxidants, and DNA within small pores between allophane spherules and nanoaggregates may not be accessible to enzymes or microbes, hence enabling DNA protection and preservation in such materials. By implication, substantial organic carbon is therefore likely to be sequestered and protected in allophanic soils (Andisols) in the same way as demonstrated here for DNA, that is, predominantly by encapsulation within a tortuous network of nanopores and submicropores amidst stable nanoaggregates and microaggregates, rather than by chemisorption alone.

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1. Introduction

Andisols cover only approximately 0.8% of the ice-free surface in the world (Soil Survey Staff, 1999; McDaniel et al., 2012) but sequester upwards of ~1.8% of the total global soil carbon stocks (Matus et al., 2014; Takahashi and Dahlgren, 2016). Many Andisols are dominated by allophane and usually contain relatively large amounts of organic matter, up to ~8–12% organic carbon (McDaniel et al., 2012). Allophane is a nanocrystalline aluminosilicate with a formula $(1–2)SiO_2 \cdot Al_2O_3 \cdot (2–3)H_2O$ (Abidin et al., 2007; McDaniel et al., 2012) and it comprises hollow spherules ~3.5 to 5 nm in diameter with high specific surface areas (SSAs)[2] — from about 250 $m^2\ g^{-1}$ to as much as 1125 $m^2\ g^{-1}$ (e.g. Maeda et al., 1977; Parfitt et al., 1980; Wada, 1980; Allbrook, 1985; Parfitt, 1990; Ohashi et al., 2002; Iyoda et al., 2012). The high organic carbon content generally is significantly correlated with allophane content and SSA (Chevallier et al., 2010; Parfitt and Yuan, 2012), and carbon turnover in Andisols is slower than in other soils (Torn et al., 1997; Parfitt, 2009). Hence it is commonly acknowledged that Andisols can

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[2] All abbreviations are defined together at the end of the text.

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not only adsorb substantial organic carbon but also protect it from degradation (Dahlgren et al., 2004; Goh, 2004; Calabi-Floody et al., 2014). Allophane spherules are reportedly crucial in enabling Andisols firstly to store organic carbon and secondly to strongly adsorb phosphate (Parfitt, 1989, 2009; Calabi-Floody et al., 2011; Yuan and Wada, 2012).

Adsorption of organic matter by these Al-rich nanocrystalline minerals governs the mobility of organic matter in the Andisols (Harsh, 2012), and the chemical bonding between the active aluminol groups on allophane spherules and organic matter is then considered to allow carbon to be adsorbed and stored (Yuan et al., 2000; Buurman et al., 2007; Parfitt, 2009; McDaniel et al., 2012; Takahashi and Dahlgren, 2016). Moreover, allophane spherules tend to form clusters of subrounded "nanoaggregates" about 100 nm in diameter (Calabi-Floody et al., 2011), which could also stabilize organic carbon within aggregates and allow long-term carbon sequestration in allophane-rich soils (Six et al., 2000a; Blanco-Canqui and Lal, 2004; Lehmann et al., 2007; Chevallier et al., 2010).

Nanocrystalline aluminosilicates show a phosphate adsorption capacity up to two orders of magnitude greater than that of long-range-order phyllosilicates and Fe- and Al-oxides/hydroxides (Hesterberg, 2010). Andisols thus have a high affinity for deoxyribonucleic acid (DNA) (Hashizume and Theng, 2007; Saeki et al., 2010a, 2010b) as well as phosphate (Allbrook, 1983, 1985; Parfitt, 1989). The adsorption of DNA on clay minerals is one of the most important mechanisms of DNA retention in soils (Ogram et al., 1988; Paget et al., 1992), and allophane, along with organic matter, presumably could facilitate the preservation of environmental DNA in soils hence could, if extractable, enable reconstruction of past environments via the DNA preserved (Huang et al., 2012; Rawlence et al., 2014). Furthermore, Matsuura et al. (2014) have hypothesised that allophane is able to protect DNA and ribonucleic acid (RNA) from ultraviolet light and, using computer modelling, simulated the interaction between DNA and allophane. Their simulations illustrated that the DNA strands underwent elongation and the phosphate backbone of DNA altered after bonding to allophane (Matsuura et al., 2013), possibly as a result of chemical adsorption of DNA through its phosphate groups to aluminol groups at the wall perforations of allophane (Huang et al., 2014). However, a more detailed understanding of the adsorption mechanism of DNA on allophane has not been developed, and the driving factor allowing allophane to adsorb more DNA than other clay minerals has remained vague, thus providing impetus for the studies reported here.

In natural allophanic soil systems, the humic substances associated with allophane strongly bind to DNA and immobilize it (Saeki et al., 2011). However, some studies have contrarily shown that clay from which organic matter has been removed adsorbs more DNA than organic-matter-rich clay (Cai et al., 2006, 2007). Hence the level of influence of humic substances and organo-minerals on DNA adsorption in allophanic soils has been controversial and requires further examination.

1.1. Hypothesis

In this study it is therefore hypothesised that DNA is adsorbed by allophane both chemically and physically, and "physical adsorption" of DNA within pores amidst allophane spherules and nanoaggregates (defined here as aggregated clusters of allophane spherules up to ~100 nm in size) or microaggregates (defined here as aggregated clusters of allophane nanoaggregates up to several hundred micrometres in size, after Elliot, 1986) brings about the exceptionally high DNA adsorption capacity of allophane and enables DNA to be preserved. To test this hypothesis, the interactions between well-characterised synthetic allophane, salmon-sperm DNA, and humic acids are examined using P X-ray absorption near-edge structure (XANES) spectroscopy. XANES spectroscopy has been widely used in soil science to analyse the species of molecules, the oxidation state of a targeted atom, and the binding geometry of this atom with surrounding atoms in a molecule (e.g. Hesterberg, 2010; Lehmann and Solomon, 2010; Terzano et al., 2010). As well, the structural alterations of DNA and allophane spherules after they bind to each other are determined using infrared (IR) spectroscopy, which has been extremely useful to characterise chemicals and to describe structural or molecular alteration of chemicals adsorbed on clays (e.g. Farmer, 1968; Parfitt, 1989; Shin et al., 2004; Tahoun, 2014). Finally, the degree of aggregation/complexation of DNA and allophane are examined using high-resolution laser sizing.

The findings with respect to DNA provide a detailed mechanism to help explain carbon sequestration and its unique longevity in Andisols.

2. Materials and methods

2.1. Synthetic allophane

2.1.1. Allophane synthesis

Pure allophane was synthesised according to Ohashi et al. (2002) with two minor modifications as follows. The Si source, Na_2SiO_4, for synthetic allophane was replaced with Na_2SiO_3, and the alkalinity of the Si solution was adjusted by adding NaOH, given that the alkalinity of Si solution should be triple the Al molarity. The allophane precursor was made with a mixture of Si and Al solutions (at the same concentration but with an atomic ratio of Si/Al of 0.75) together with the addition of NaOH, and the precursor was incubated for 48 h. In the current study, two synthetic allophane products with 50 mmol L^{-1} and 100 mmol L^{-1} of initial solutions were prepared, and the products were labelled as *50-allophane* and *100-allophane*, respectively. After hydrothermal incubation for 48 h, the synthetic products were washed with deionised (DI) water until the pH became neutral. For the DNA adsorption experiments, the dispersed allophane in DI water was adjusted accurately to pH 6.5 and stored without drying.

2.1.2. Allophane analysis

Transmission electron microscopy (TEM) was employed to observe the morphology of the synthetic allophane. For TEM images of the synthetic allophane, 10 μL of diluted allophane dispersion was spread on a lacey formvar/carbon copper grid (200 mesh) followed by drying at 40 °C for moisture removal. Two TEM facilities were used, a Philips CM30 TEM and a JEOL TEM-3010 scanning TEM for images with varied magnifications. The synthetic allophane was damaged and melted in a fairly short time under the X-ray beam because allophane is heat-sensitive, and so the synthetic allophane was photographed as quickly as possible to avoid over-exposure of the samples to X-rays.

As well as TEM-based morphological observation, the allophane content of the synthesised product was determined. To accurately estimate its Si/Al ratio, the synthetic allophane was frozen rapidly with liquid nitrogen followed by freeze-drying. The allophane content was estimated by oxalate extraction of Al and Si and pyrophosphate extraction of Al (following Parfitt and Wilson, 1985; Blakemore et al., 1987). The results showed that the Al/Si ratio was 1.2 and the allophane yield was 95% of the total product. The SSA of the synthetic allophane was analysed via the Brunauer–Emmett–Teller (BET) method with nitrogen gas adsorption at −195 °C (Brunauer et al., 1938), and pore volume and pore size were evaluated via the Barrett–Joyner–Halenda (BJH) method (Barrett et al., 1951).

2.1.3. Preparation of humic-acid-laden synthetic allophane

To understand the influence of humic substances on the adsorption of DNA on allophane, the dispersed 50-allophane in DI water was incubated overnight with humic acid (Pahokee peat humic acid standard, which was obtained from the International Humic Substances Society) to allow humic acid (HA) adsorption on synthetic allophane. Prior to incubation, the dried HA powder was dissolved in diluted NaOH and then adjusted to pH 6.5 using 0.1 M HCl, and the resulting solution was then centrifuged at 8000 rpm (11,325 *g*) to remove insoluble HA. After incubation, the allophane–HA complex was repeatedly washed with DI

water three to five times to remove unbound HA. The allophane–HA complex was re-suspended completely and adjusted to pH 6.5 for the DNA adsorption experimentation.

2.2. Adsorption isotherm of salmon-sperm DNA on synthetic allophane

The double-stranded salmon-sperm DNA (Sigma-Aldrich product number D1626) was dissolved in sterile DI water to provide a 2 mg mL^{-1} DNA solution, and the DNA additions for adsorption experimentation were in the range of 0 to 200 μL. The DNA solution was added to two sterile 1.5-mL tubes, one containing a given volume of dispersed allophane in DI water comprising 10 mg of synthetic allophane, and the other sterile DI water of the same volume as used to disperse the allophane. The two tubes were centrifuged at 13,000 rpm (10,000 *g*) after shaking 3 h on a rotator at room temperature, and the supernatants were then collected and analysed spectrophotometrically at 260 nm for DNA concentration. The adsorbed DNA was calculated from the difference between the amounts of DNA with and without 10 mg of synthetic allophane. These experiments were conducted in triplicate.

The adsorptive affinity and maximum adsorption capacity of DNA on synthetic allophane were analysed using the Langmuir equation given below:

$$q = \frac{A_{max} \cdot k \cdot C}{1 + k \cdot C}$$

where q represents the adsorbed DNA, A_{max} is a constant representing the maximum adsorption capacity (monolayer coverage), k is an adsorptive constant which is often considered an affinity parameter or binding strength, and C is the equilibrium-dissolved concentration of DNA.

After removal of supernatant, the tubes with remaining allophane and salmon-sperm DNA complexes were placed in a vacuum desiccator for two days for drying, and the dried allophane–DNA complexes were stored at 4 °C for further chemical analysis. DNA adsorption on HA-rich allophane was performed following the same procedure as above.

2.3. Phosphorus X-ray absorption near-edge structure (P XANES) spectroscopy

The salmon-sperm DNA–allophane complexes were examined and characterised via synchrotron radiation-based P XANES. To obtain optimum signal-to-noise ratios of spectra, the air-dried allophane–DNA complexes were ground finely for uniformity and they were then compressed into pellets to increase the intensity of X-ray absorbance. The pellets of dried salmon-sperm DNA and allophane–DNA complexes were mounted on stainless steel sample holders and held in place with Kapton tape which has no X-ray absorbance over the P X-ray absorption region. The P X-ray absorption spectra were collected at beamline 16A1 Tender X-ray Absorption Spectroscopy at the National Synchrotron Radiation Research Center (NSRRC), Hsinchu, Taiwan. The beam current under standard operating conditions is 300 mA, and a Si(III) double-crystal monochromator is used for incident X-ray energy selection at a resolution of 1.5–2.1 × 10^{-4} $\Delta E/E$. For the samples used in the current study, the P X-ray absorption was carried out under fluorescence mode, along with the employment of a Lytle detector purged with nitrogen gas. The beam was optimised and calibrated in advance against the adsorption edge of metallic Zr at 2223 eV.

P XANES spectra for samples were collected with photon energies in the range 2106–2230 eV, which are 40 eV forward and 84 eV behind the P *K*-edge of 2146 eV, and two to three scans were completed for each sample to obtain a representative spectrum. Over the scanning region of 2138–2180 eV, an energy step size of 0.2 eV was used along with a dwell time of 4 s per step for elaborate analysis, and a step size of 0.5 eV and dwell times of 2 s were used for the remaining energy region. The spectra obtained were normalized and merged through the Athena program, an interface to IFEFFIT (version 1.2.11) (Ravel and Newville, 2005), and then plotted.

2.4. Infrared (IR) spectroscopy

IR spectra for samples were obtained using Fourier transform infrared (FTIR) microspectroscopy at beamline BL14A1 at the NSRRC. This beamline employs a Nicolet Magna 860 FTIR spectrometer equipped with a Continuum IR microscope (Spectra Tech), and the FTIR facility is configured with synchrotron light as an external light source for the spectrometer.

Pure salmon-sperm DNA, pure synthetic allophane, and aggregates of synthetic allophane with differing concentrations of salmon-sperm DNA spikes (see Section 2.2) were placed on a holder for analysis. Experiments were performed under the ring operation of top-up mode. Samples were analysed by IR in a range of 4000–600 cm^{-1}, with the co-addition of 128 scans. A spectrum of background signal (e.g. gases and moisture in atmosphere) without samples was collected for background removal. Data collection and background removal were completed via the program OMNIC™, and spectra were then processed using OMNIC™ and OriginPro 8.

2.5. Size distribution of DNA–allophane complexes

To examine the aggregation of synthetic allophane after adsorbing DNA, salmon-sperm DNA of varied concentrations were added into 30 mL of dispersed synthetic allophane in DI water for DNA adsorption. After allowing 3 h for complexation, allophane–DNA complexes with various DNA additions were collected without precipitation or centrifugation. The size distributions of allophane–DNA aggregates were determined by a Malvern Mastersizer 2000 laser diffractometer. Samples were injected into a vigorous stirring unit to homogenize them before throughput into the main measurement system (Sochan et al., 2012). DI water was used as the dispersant, where the dispersant refractive index was 1.33. Particle refractive index was set consistently at 1.5 for all samples.

3. Results and discussion

3.1. Physical properties of synthetic allophane spherules

The electron micrographs showed the spherical morphology of the synthetic allophane (Fig. 1) and indicated that the spherules had an external diameter about 10–15 nm (no significant size differences between the 50- and 100-allophanes were observed) and a wall thickness of ~1 nm (Fig. 1c). The particle size of the synthetic allophane was somewhat larger (by about three times) than the reported size of ~3.5–5 nm for natural allophane spherules (Creton et al., 2008; Parfitt, 2009), probably attributable to the unconstrained growth of synthetic allophane in the laboratory setting (Churchman and Lowe, 2012). It was also observed under TEM that allophane spherules coalesced naturally to form allophane nanoaggregates ~50–100 nm in diameter, and the aggregates then formed networks of large porous allophane aggregates in the size range of hundreds of nanometres (Fig. 1d).

The discrete synthetic allophane spherules (equivalent to "particles" in the terminology of Bergaya and Lagaly, 2013, p.13) and their networks are in accordance with properties of natural allophanes observed in soils from New Zealand and Japan (Fig. 2) and elsewhere (Henmi and Wada, 1976; Maeda et al., 1977; Wells and Northey, 1984; Parfitt, 1990; Karube et al., 1996; Kaufhold et al., 2010; Delmelle et al., 2015). Karube et al. (1996) showed that unit particles of allophane formed domains ("primary floccules", which are referred to as nanoaggregates in the current paper) about 100 nm in diameter "like strings of beads" (Fig. 2c); and micrometre-sized clusters of allophane nanoaggregates (which are referred to here as microaggregates) in a dilute "suspension",

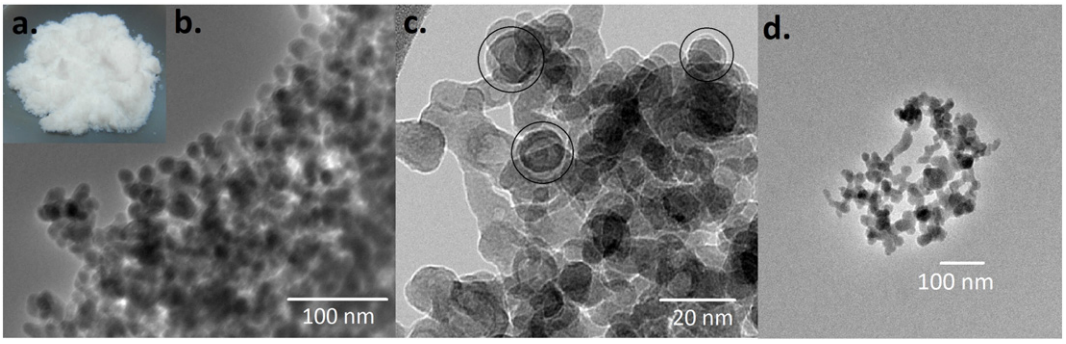

Fig. 1. Freeze-dried synthetic allophane (a) and the TEM images of the synthetic allophane showing the spherical and hollow morphology of allophane (b and c) as well as allophane nanoaggregates (d). The heat-sensitive allophane was damaged under the electron beam at high magnification, but the consistent spherical shape of allophane and the thickness of the allophane wall (in circles) are evident in photos b and c, respectively.

analogous to the characteristics of synthetic allophane shown in Fig. 1d. Earlier, Allbrook (1985) suggested that surficial moisture films allow allophane spherules to remain discrete (even when aggregated) rather than conjoining into large micelles that characterise crystalline (platy) clays, thereby explaining the high porosity (and low bulk density) of allophanic soils.

The spaces within the allophane nanoaggregates (inter-spherule spaces) were typically ≤~2 nm (according to measurements of the distances between spherules in TEM micrographs), whereas the spaces between the nanoaggregates (inter-nanoaggregate spaces) within microaggregates were of various sizes up to ~500 nm (see also Fig. 10C, below). The spaces (pores) could be further distributed into nanopores, which are defined here as <100 nm in diameter, and submicropores which are ~100 to 500 nm in diameter. The high volume of nanopores and submicropores potentially allow the allophane aggregates to be highly porous and adsorptive (see Section 3.5 below). Such numerous nanopores in Andisols were described by Chevallier et al. (2010) as having a fractal pore structure and therefore a pore network characterised by a high degree of tortuosity aptly called a "nanolabyrinth".

The estimated unit particle size of the synthetic allophane via BET was 16–23 nm (Table 1), slightly larger than the more accurate sizes estimated using TEM. The high pore volume of the two synthetic allophane samples corresponded with the observations made using TEM, but the measured SSAs and pore volumes and pore sizes of 50-allophane and 100-allophane were somewhat different (Table 1). The SSAs for 50- and 100-allophane were 257 and 374 $m^2\ g^{-1}$, and estimations of pore volume were 0.59 and 0.73 $cm^3\ g^{-1}$, and of pore size were ~10 and ~8 nm, respectively. Allophane synthesised by Ohashi et al. (2002) using the same method and similar concentrations of Si and Al solutions as employed in the current study, possessed similar pore volumes, up to 0.78 $cm^3\ g^{-1}$, but somewhat higher SSAs up to 550 $m^2\ g^{-1}$. However, Kaufhold et al. (2010) reported a SSA of 348 $m^2\ g^{-1}$ for synthetic allophane (made using the method of Ohashi et al., 2002) that closely matches that of the 100-allophane. The SSAs of some natural allophanes in New Zealand range from ~580 to 1125 $m^2\ g^{-1}$ (Parfitt and Henmi, 1980; Allbrook, 1983; Parfitt, 1990). The differences in the SSAs of natural versus synthetic allophane are likely to have resulted from other colloids (e.g. organic matter, ferrihydrite, and halloysite) contributing a range of SSAs in the natural samples, and the SSAs for synthetic allophane are mainly influenced by the sizes of spherules (assumptions regarding monolayer coverage are also possible factors) (Allbrook, 1983; Parfitt, 1990). The higher adsorptivity of the 100-allophane than that of the 50-allophane presumably resulted from the higher concentrations of initial Si and Al solutions in making the 100-allophane, which led to relatively more numerous active aluminol groups on it. The nitrogen adsorption–desorption isotherms of two synthetic allophane products both showed hysteresis loops (Fig. 3), which are indicative of the presence of mesopores (2–50 nm in diameter) with capillary condensation within allophane samples (Neimark et al., 2000; Iyoda et al., 2012).

3.2. DNA adsorption capacity of synthetic allophane

The adsorption isotherms of salmon-sperm DNA on 50- and 100-allophane (Fig. 4) can be classified as *L*-shaped isotherms, which are characterised by a decreasing slope while concentration increases because the vacant adsorptive sites become covered and saturated (Sposito, 1989). The calculated adsorption maximum (A_{max}) of DNA by 100-allophane, with a comparatively high SSA and pore volume, was 34 μg mg^{-1} allophane (adsorptive coefficient *k* was 0.0044), and A_{max} for DNA on 50-allophane was 26 μg mg^{-1} allophane (*k* was also

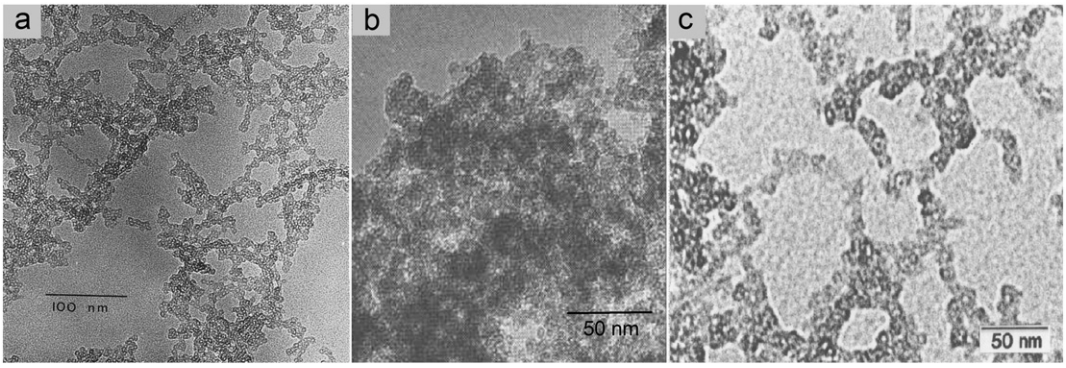

Fig. 2. Micrographs of natural allophane spherules occurring in (a) New Zealand, reproduced from Parfitt (1990), p. 345, with kind permission of CSIRO Publishing, Melbourne, Victoria, Australia, http://www.publish.csiro.au/nid/84/paper/SR9900343.htm (© CSIRO 1990); (b) Japan, reproduced from Henmi and Wada (1976), p.382, with kind permission of the Mineralogical Society of America; and (c) Japan, reproduced from Karube et al. (1996), p. 486, with kind permission of The Clay Minerals Society, publisher of *Clays and Clay Minerals*.

Table 1
Specific surface area and pore volume and pore size analyses of the two synthetic allophanes used in this study.

	50-allophane	100-allophane
Size of nanoparticles (nm)	23.31	16.02
BET specific surface area ($m^2\ g^{-1}$)	257	374
BJH pore volume ($cm^3\ g^{-1}$)	0.59	0.73
BJH pore size (nm)	9.92	7.67

0.0044). That the 50- and 100-allophane both had the same adsorptive coefficients indicated that their surface adsorptive energies are similar. The adsorption isotherms both showed a rapid and strong adsorption of DNA on to allophane when DNA additions were low, followed by weakening adsorption whilst DNA additions increased. However, the different DNA adsorption capacities for 50- and 100-allophane (26 and 34 μg mg^{-1}, respectively) mainly resulted from the different numbers of active sites of the two materials.

In contrast to the findings obtained here from DNA adsorption on moist synthetic allophane, Saeki et al. (2010a) reported the maximum DNA adsorption capacity for dried synthetic allophane at pH 6 to be only 5 μg mg^{-1}. Factors that influence the rate of DNA adsorption on allophane include acidity and ionic strength and also the moisture status of allophane. The drying process always generates non-reversible shrinkage of allophane aggregates and lowers the specific SSA (Rousseaux and Warkentin, 1976; Allbrook, 1992; Gray and Allbrook, 2002; Woignier et al., 2007; Kaufhold et al., 2010), potentially by ~40% according to Allbrook (1985). Consequently, it is concluded that drying would reduce DNA adsorption capacity of allophane and that it is ideal to keep synthetic allophane moist as a gel for subsequent applications.

3.2.1. Influence of humic acid on DNA adsorption capacity of 50-allophane

It was found that the presence of humic acid on the surface of synthetic 50-allophane significantly hampered the adsorption of DNA (Fig. 5), reducing the capacity from 26 to 3.5 μg DNA for 1 mg of synthetic allophane (note the 100-allophane had a maximum DNA uptake of 34 μg mg^{-1}). This result accords with those of Cai et al. (2006) and Saeki and Sakai (2009), who both showed that a decrease of organic matter raises DNA adsorption on clay minerals and colloid particles from soils. In the current study, the relatively low DNA adsorption on the humic acid-rich synthetic allophane indicated either that humic acid was competitive with DNA and/or that the humic acid had already occupied the active aluminol groups on surface of allophane and hence fewer adsorptive sites on allophane remained. This discovery — that humic acid effectively reduces the capacity of DNA adsorption by allophane — suggests that allophanic soils and paleosols, characterised typically by a high content of organic matter as noted earlier, may not be so favourable for DNA adsorption from a long-term perspective as previously considered.

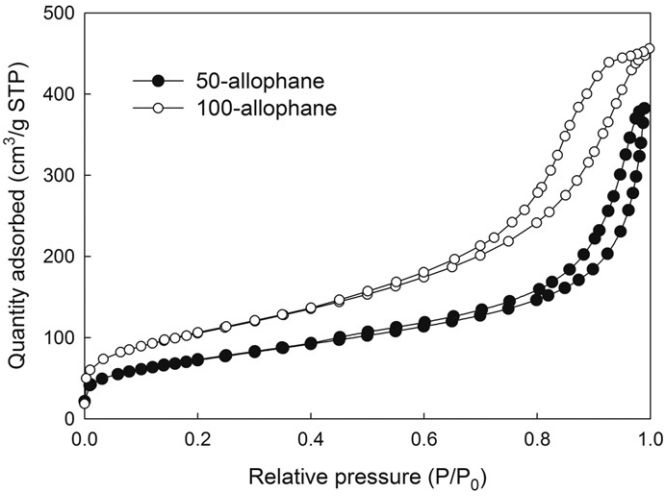

Fig. 3. N_2 gas adsorption-desorption isotherm on the two synthetic allophane products.

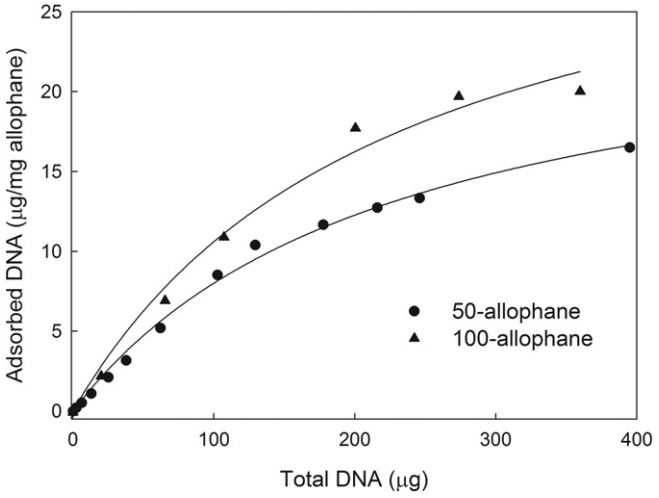

Fig. 4. Adsorption isotherms of salmon-sperm DNA on 10 mg of 50-allophane or 100-allophane. Reaction time was 3 h. The DNA adsorption isotherms were fitted to a Langmuir model, and the coefficients of determination (r^2) of Langmuir fitting for DNA adsorption on 50-allophane and 100-allophane were 0.987 and 0.995, respectively.

3.3. P XANES spectra for allophane- or humic-acid-associated DNA

The P XANES spectrum for pure salmon-sperm DNA (bold spectrum in Fig. 6) shows a sharp and strong white-line (WL) peak at 2153 eV, and a post-edge shoulder between 2160 and 2180 eV without showing pre-edge features. The P XANES spectrum for the allophane-associated DNA shows a pre-edge hump (2135–2141.1 eV) on the low-energy side of the absorption edge, along with a slight shift of the WL peak from 2153.0 eV to 2153.3 eV (Fig. 6). The WL peak for the allophane–DNA complex could be attributed to the propagation of P 1s electrons into P(3p)–O(2p)–Al(3p) antibonding molecular orbitals (Khare et al., 2005) where the nuclei are repelled by positive charges of Al ions. In comparing the two XANES spectra, the positive shift of the WL peak

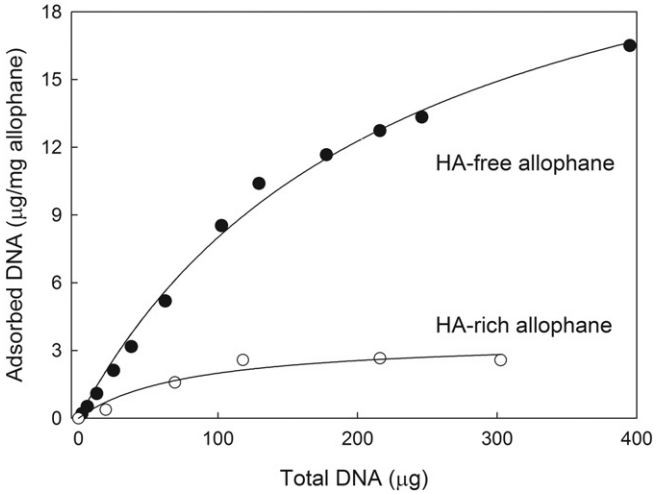

Fig. 5. The measured adsorption isotherms of salmon-sperm DNA on humic acid (HA)-free and HA-rich synthetic 50-allophane (both at pH 6.5) along with fitting to a Langmuir model (reprinted from Huang et al., 2014, p. 170, with kind permission of Springer). Each data point represents the average adsorption of samples in triplicate. The coefficients of determination (r^2) of Langmuir fitting for DNA adsorption on HA-free allophane and for HA-rich allophane were 0.995 and 0.946, respectively.

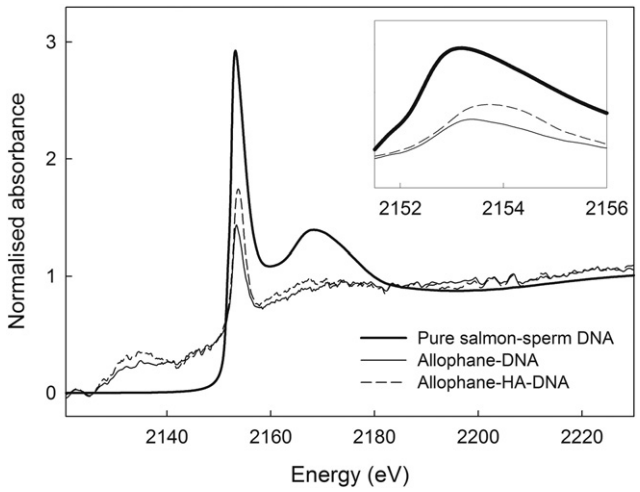

Fig. 6. Normalized P XANES spectra for pure salmon-sperm DNA, allophane–DNA complex, and allophane–HA-DNA complex. Two or three scans for one sample were obtained and then merged together for an average spectrum. The appended box shows detailed variations of spectra over the region of 2152–2156 eV.

for the allophane-associated DNA may be explained by the charge relocation from Al to P as a consequence of the strong electronegativity of DNA base pairs arising after the DNA was adsorbed chemically on to the allophane.

The behaviour of salmon-sperm DNA adsorption on humic-acid-rich allophane was illustrated by the P XANES spectrum as well (dashed spectrum in Fig. 6), whereby the WL peak for humic acid-associated DNA was shifted forward to high energy relative to allophane-associated DNA, along with amplified intensity of the WL peak and post-edge backscattering hump (see inset, Fig. 6). The DNA bound to the humic acid covering the allophane characteristically showed not only the chemical adsorption of DNA onto humic acid but also the oxidation of DNA molecules because of the decoupling between phosphate ligands of DNA and the adsorptive sites of humic acid, thereby confirming the high affinity of DNA and humic acid (Saeki et al., 2011). Accordingly, it was expected that the adsorption of DNA on organo–allophane complexes would help to retain environmental DNA in such soil materials. However, the negatively-charged humic acid (and its ligands) atop allophane suppressed the charge relocation from phosphorus within the DNA molecules to the adsorptive ligands of humic acid, a process illustrated by the higher WL intensity for DNA bound to the humic-acid-rich allophane compared with the WL intensity for DNA on the humic-acid-free allophane. Hence it is concluded that humic acid instead reduces DNA adsorption in allophanic soils by not only attaching to active sites on the allophane spherules (thus precluding DNA from binding) but also by repelling the negatively-charged phosphate groups in the DNA molecules by electrostatic repulsion.

Even though humic acids inhibit the adsorption of DNA by allophane, the organo–allophane complex in soils retains an ability to stabilize some environmental DNA by chemical adsorption and possibly store the DNA indirectly. Using P XANES, Huang et al. (2014) analysed a natural allophanic soil sample, to which salmon-sperm DNA had been added, in order to ascertain specifically how DNA was adsorbed on allophane. However, the result was not conclusive because it was unclear whether the added DNA was chemically bound to the allophane or to organics, or both. In the present study, however, the spectrum from humic acid-associated DNA can be distinguished from that for allophane-associated DNA. Accordingly, the distinct WL peak at 2153.8 eV for DNA associated with humic acid indicates that the salmon-sperm DNA added into the natural allophanic soil (as reported by Huang et al., 2014) was in fact chemically bound to humic acid or organics in soils rather than to the allophane spherules directly because the active sites on allophane had been naturally occupied by organics.

3.4. Infrared spectra for pure DNA, allophane, and DNA–allophane complexes

The identifiable infrared spectral features from low wavenumbers for pure salmon-sperm DNA (Fig. 7) correspond to P═O stretching (at 1098 cm^{-1}), O–P–O bending (at 1240 cm^{-1}), CH_2 and CH_3 bending (at 1300–1500 cm^{-1}), C═C stretching within amine bases (at 1450–1600 cm^{-1}), C═O stretching within amine bases (at approximately 1700 cm^{-1}), and finally OH and NH stretching (at 3000–3400 cm^{-1}) (Brown and Poon, 2005). With the use of infrared spectroscopy, the DNA molecule was characterised mainly by absorbance of amine bases and phosphate groups, whereas the absorbances of methylene bending, methyl bending, and aliphatic stretching were not distinct. Freeze-dried synthetic allophane was characterised by strong infrared absorption between 900 and 1000 cm^{-1}, ascribable to the predominance of Si–O–Al bonds of allophane spherules and by the medium absorption around 3000 to 3700 cm^{-1} relating to the hydroxyl groups on the surface of allophane, together with the contribution of organic impurities near 1600 cm^{-1} (Parfitt and Henmi, 1980, 1982). After DNA adsorption on synthetic allophane, the addition of 0.2% salmon-sperm DNA induced a steric alteration in Si–O–Al stretching of allophane: the strong absorption band at 1200 cm^{-1} of allophane was split into two broad sub-bands at 990 and 1080 cm^{-1} (bold line in Fig. 7), and the PO_2^- signal of the DNA molecule at 1240 cm^{-1} was not observed. The disappearance of P–O stretching within the DNA molecule after it is bound to allophane supports the conclusion that allophane binds to DNA through its phosphate groups (especially through the deprotonated P–O sites). On the other hand, the alteration of Si–O–Al stretching of allophane after DNA adsorption confirmed that DNA was attached to the reactive aluminol (AlOH) defects on the surface of allophane (Nanzyo, 1984; Parfitt, 1989), and the signal of Si–O–Al stretching shifted or disappeared because of the spatial inflexion of the allophane wall or because of the precipitation of aluminium phosphates (Parfitt, 1989) (or both).

With an increase in concentration of additional DNA (0.2, 0.7, and 1.1%) onto synthetic allophane, the intensity of the infrared absorption band at 1080 cm^{-1} increased and shifted gradually but the absorbance at 990 cm^{-1} remained the same (Fig. 8). Consequently, the absorbance at 990 cm^{-1} could be assigned to the chemical change of Si–O–Al bonding on the surface of allophane spherules after DNA adsorption because the peak at 990 cm^{-1} did not alter with the rising concentration of additional DNA. This result suggests that almost all the reactive defects of allophane spherules had reacted and altered chemically after the addition of only a small amount of DNA (e.g. 0.2% of DNA spike). More DNA, however, could still be taken up slowly even though the reactive sites had been saturated and would not change chemically further, and so other DNA adsorption mechanisms must have taken place to enable the uptake of more DNA by allophane.

3.5. Formation of allophane nano- and microaggregates and physical adsorption of DNA within pores

During DNA adsorption on synthetic allophane, it was observed that chemical adsorption of DNA on allophane brought about further aggregation/complexation of allophane nanoaggregates simultaneously (Fig. 9) so that the size of allophane nanoaggregates increased to micron-sized aggregates (microaggregates). This phenomenon has been attributed to the chemical adsorption of allophane nanoaggregates on DNA strands, as described by Matsuura et al. (2013), followed by conjoining of these aggregates by the polymeric DNA, with porous allophane microaggregates formed as a result. The microaggregates comprised assemblages of allophane nanoaggregates with numerous spaces (pores) of both nano- and submicron scale. Consequently, DNA fragments could be readily enclosed during the formation of allophane microaggregates, or adsorbed within the nanopores between allophane spherules (i.e., within inter-spherule spaces) or within nano- or submicropores between allophane nanoaggregates (i.e., within inter-

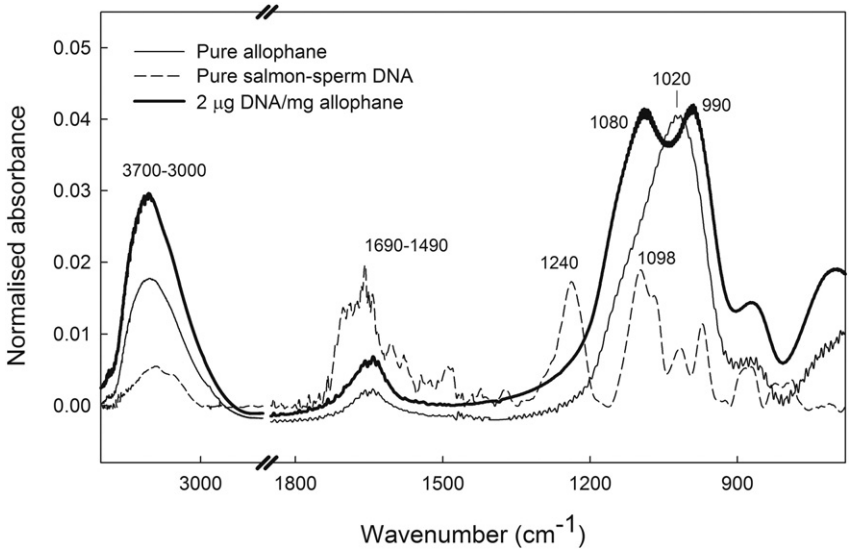

Fig. 7. Normalized infrared spectra for pure salmon-sperm DNA, synthetic allophane, and allophane–DNA complex. Identified spectral features at various wavenumbers correspond to OH group (3700–3000), C═C in the base planes of DNA (1690–1490), PO_2^- of DNA (1240), P═O (1098), and stretching Si–O–Al of allophane (1020).

nanoaggregate spaces), effectively as a form of physical adsorption as noted earlier.

The size of allophane–DNA clusters increased with the addition of DNA (Fig. 9). Allophane microaggregates could be up to 500 μm in diameter and the dominant size of such aggregates with high DNA loading (>4.4 μg DNA per mg allophane) onto synthetic allophane was 100–300 μm. Increasing the DNA adsorbed from 4.4 μg mg^{-1} to 8.5 μg mg^{-1} had no significant impact on the overall size distribution of aggregates, but the result showed a reduction in the volume of nanoaggregates <100 μm in diameter and a predominance of microaggregates with a size range 100–300 μm in diameter.

The DNA adsorption isotherm on synthetic allophane (Fig. 3) reflected the fact that further adsorption became weak when adsorbed DNA was more than 6 μg mg^{-1} allophane (0.6%), which could be attributable to the saturation of the DNA chemisorption on the surface of allophane spherules and then physical adsorption of DNA in nano- or submicropores within and between allophane nanoaggregates. The DNA uptake by synthetic allophane eventually attained up to 34 μg mg^{-1} allophane (3.4%) whilst the allophane aggregates had continuously grown up to 500 μm in diameter and inter-nanoaggregate spaces had become saturated with DNA. Hence the proportion of chemically-adsorbed DNA to physically-adsorbed DNA is 6 to 28 (34 minus 6), indicating that ~80% of DNA is adsorbed physically in nano- and submicropores.

Oades and Waters (1991) showed that soil microaggregates are bound together into macroaggregates by organic matter in most soils, and later Six et al. (2000b) further demonstrated that macroaggregates sequester 1.65 times more carbon than microaggregates. Using an experimental approach earlier, Churchman and Tate (1987) showed that the stability of macroaggregates in allophanic soils on tephra is highly related to carbon content in such soils. Aggregates at nano- and micron scales have also been found to be crucial in helping to govern carbon sequestration in Andisols (Huygens et al., 2005; Asano and Wagai, 2014) in which the large allophane aggregates possess higher volumes of inter-nanoaggregate interstice and hence more room for the physical storage of carbon than in small aggregates. In Fig. 9 it is shown that allophane nanoaggregates could be assembled by DNA molecules acting as strong binding agents, and such aggregation has enabled much DNA to

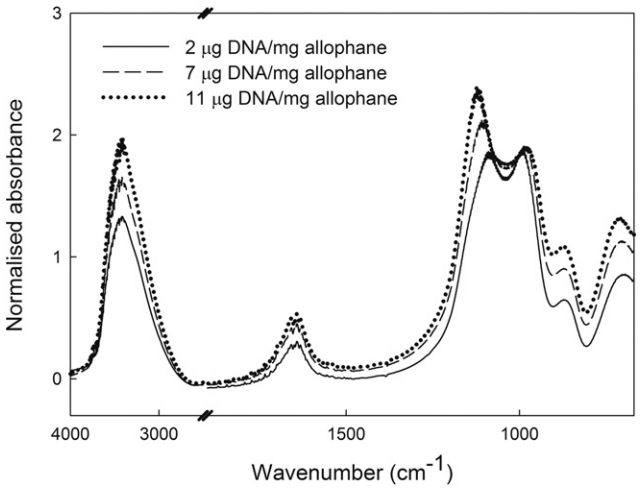

Fig. 8. Normalized infrared spectra for three allophane–DNA complexes with 2 (0.2%), 7 (0.7%), and 11 (1.1%) μg mg^{-1} of DNA additions.

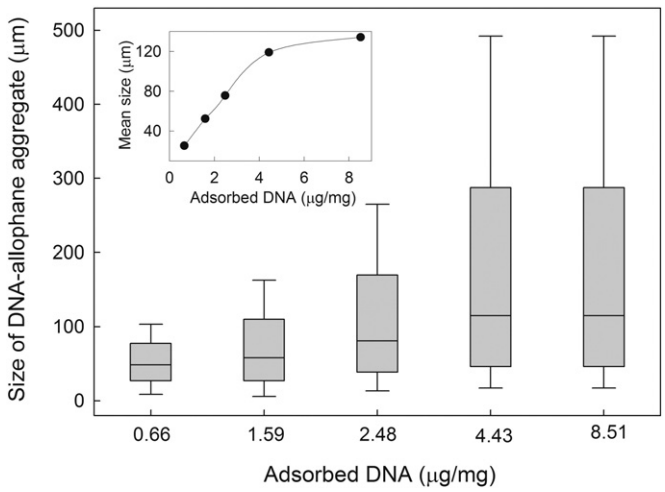

Fig. 9. Size distribution of allophane–DNA aggregates responding to gradually increasing additions of DNA. The 50-allophane was used for this set of experiments. The allophane–DNA aggregates (with differing amounts of DNA additives) were vigorously homogenized in a stirring unit, and hence are demonstrably stable, being unable to be broken down easily. The concentration of DNA added and the mean sizes of the allophane–DNA aggregates are reported alongside each histogram.

be adsorbed by the allophane microaggregates in the spaces (nano- and submicropores) between aggregates rather than just on the limited aluminol groups on allophane spherules — i.e., physical adsorption within and between allophane aggregates seems to account for much more DNA sequestration than chemical adsorption directly on the surfaces of allophane spherules.

It is therefore proposed that environmental DNA in Andisols or allophane-rich soils could be adsorbed by (1) allophane directly via chemisorption, (2) humic acid (or organic matter) covering on allophane and then attached to allophane indirectly, and (3) pores within and between allophane nanoaggregates (inter-spherule and inter-nanoaggregate spaces) via physical adsorption (Fig. 10), of which physical adsorption is considered to be the crucial mechanism allowing substantial environmental DNA to be sequestered in such soil materials because of the high porosity of allophane aggregates. The stability of the allophane–DNA complexes was noticeable because the allophane–DNA microaggregates could not be broken up by turbulent stirring, indicating that DNA (and hence organic matter as well in the natural soil system) would likely be well protected and stored within allophane aggregates. Natural humus-allophane aggregates in Andisols are similarly very stable (e.g. Goh, 1980, 2004; Nanzyo, 2002; Ugolini and Dahlgren, 2002; Matus et al., 2014). The pores at nano- or submicrometre scale between allophane spherules or within aggregates seem to be the main reservoir for DNA adsorption and probably a refuge for DNA because some of the pores are so small (~2–100 nm) that they are not accessible to enzymes or microbes.

Therefore, the formation of allophane aggregates and the physical adsorption of DNA within nanopores amidst allophane aggregates may be indicative of better preservation of environmental DNA in natural allophanic soil materials than in other (non-allophanic) mineral soils (Rawlence et al., 2014).

3.6. Implications for carbon sequestration in Andisols

The findings relating to DNA may also pertain to soil organic matter and organic carbon in that the porous and stable allophane aggregates potentially allow much organic carbon to be adsorbed physically, being encapsulated within the small spaces (pores), thereby leading to slow carbon turnover in Andisols (Parfitt et al., 2002; Parfitt, 2009; Baisden et al., 2010, 2013). Previously, the high organic carbon content

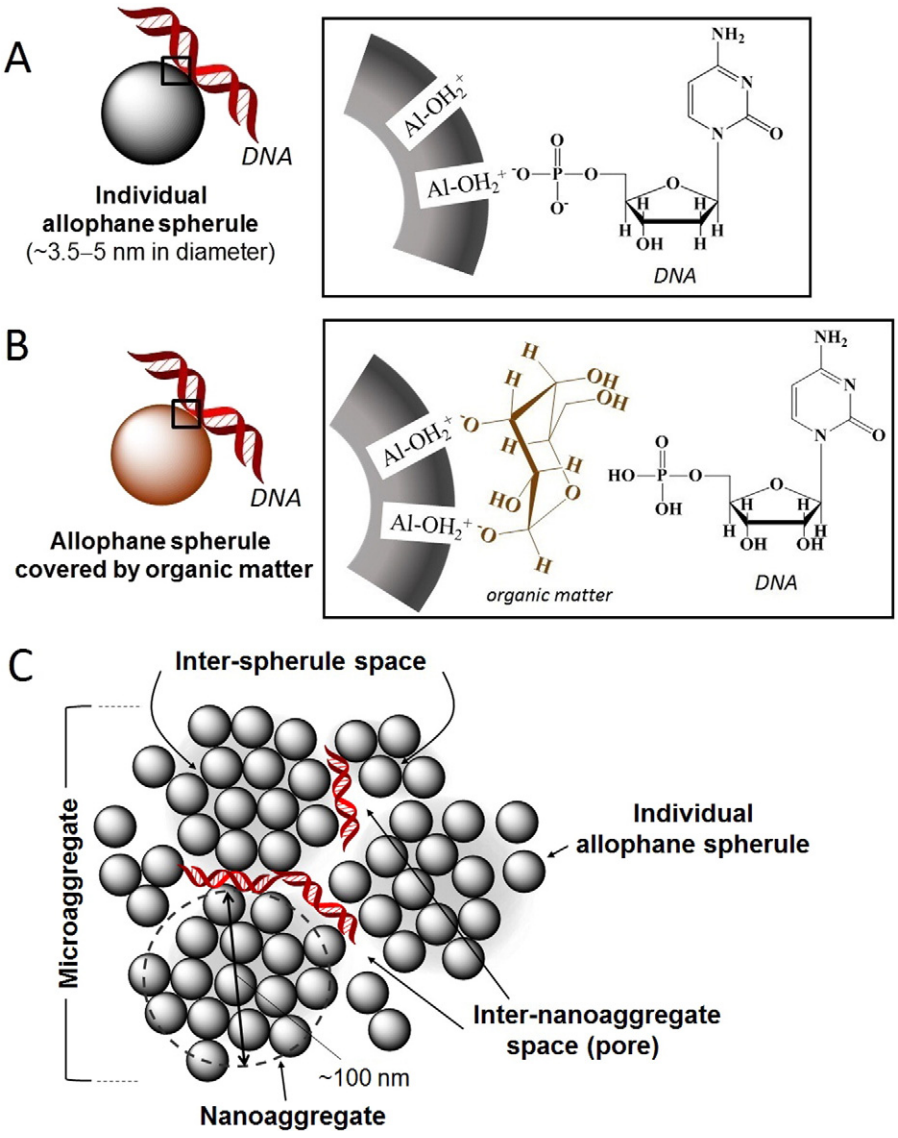

Fig. 10. Illustration of proposed mechanisms for chemical and physical adsorption of DNA by nanocrystalline allophane spherules and allophane nanoaggregates: (A) direct chemical adsorption of DNA on allophane through the phosphate group of DNA; (B) indirect chemical adsorption of DNA on a covering of organic matter on allophane; and (C) physical adsorption of DNA in the spaces (nano- to submicropores) of allophane nanoaggregates and microaggregates (modified after Huang et al., 2014, p. 165, with kind permission of Springer). The natural DNA fragment is ~20 Å (2 nm) in diameter and the DNA grooves (spaces between helical strands) are 12–22 Å (1.2–2.2 nm) wide.

of Andisols had been ascribed mainly to the very high SSA and the variable surface charge characteristics associated with allophane (e.g. Harsh, 2012; McDaniel et al., 2012), and to the strong propensity of allophane to form nanoaggregates up to 100 nm in diameter that enable carbon to become stabilized and protected (e.g. Goh, 2004; Chevallier et al., 2010; Calabi-Floody et al., 2011; Matus et al., 2014). In some Andisols, carbon, mainly as humus, becomes stabilized through complexation and precipitation with Al (e.g. Percival et al., 2000; Yuan et al., 2000; Nanzyo, 2002; Basile-Doelsch et al., 2005; Chevallier et al., 2010; McDaniel et al., 2012; Takahashi and Dahlgren, 2016). However, the findings obtained here with respect to DNA – up to 80% of which was physically adsorbed (and presumably protected) on synthetic allophane — provide a mechanism to explain carbon sequestration more specifically in Andisols: a high proportion of organic carbon is encapsulated within myriads of small to tiny pores, effectively within a nanolabyrinth, both (1) amidst nanoaggregates of allophane spherules (inter-spherule spaces), and (2) in pores between nanoaggregates (inter-nanoaggregate spaces) within microaggregates, of which the microaggregates possess higher volumes of interstitial space for carbon adsorption than nanoaggregates. For the synthetic allophane examined in this study, the dominant pore size of the inter-spherule spaces was ~3 nm (nanopores), and that of the inter-nanoaggregate spaces (nano- to submicropores) was ~30 to 100 nm. For natural allophane, the equivalent pore dimensions within and between nanoaggregates were ~2 nm and ~50–500 nm (based on micrographs of natural allophane), respectively. Such sizes ranges amidst very stable aggregates (both nano- and microaggregates) provide natural 'havens' for organic carbon to be encapsulated more or less permanently, free from attack because the Al-rich allophane spherules encircling the nanopores are non-bioavailable to most microorganisms (because of Al toxicity), enzymes themselves may be adsorbed, microbes are deprived of phosphorus (because of strong P retention) (Tate and Theng, 1980; Ugolini and Dahlgren, 2002; Matus et al., 2014), and because the nanopores and their openings are both too small and too tortuous for enzymes and microbes to access.

Using small angle X-ray scattering (SAXS), Chevallier et al. (2010) showed for Andisols in Martinique (French West Indies) that an increasingly tortuous nanopore network (defined as the extent of fractal range or cluster, ξ/a, where ξ is the size of allophane aggregates and a is 3.5 nm, the mean size of natural allophane spherules) resulted in decreasing carbon bioavailability. A larger ξ/a ratio indicates a more tortuous pore network. Similar research was undertaken by Woignier et al. (2008). Filimonova et al. (2011) used ^{129}Xe nuclear magnetic resonance (NMR) spectroscopic studies of xenon gas adsorption of a non-allophanic Andisol (i.e. an Andisol dominated by Al- and Fe-humus complexes: Takahashi and Dahlgren, 2016) to show that its porous structure comprised interconnected micro- and mesopores formed by agglomerated nano-sized Al-rich clusters, the micropores, critically, being very narrow (~0.44–0.88 nm). In the current study, the synthetic allophane spherules were ~10–15 nm in diameter (mean size: ~12.5 nm), and the allophane nanoaggregates were between ~50 and ~100 nm in diameter, and so it is estimated that the ξ/a ratio is between 4 (50/12.5) and 8 (100/12.5). Chevallier et al. (2010) reported that a ξ/a ratio of about 10 "implies the existence of a large tortuous labyrinth built of allophane particles" (p. 184). Thus, assuming the results for natural allophane apply to the synthetic allophane in the current study, then the ξ/a ratios of ~4 to 8 imply that pore networks in the synthetic allophane nanoaggregates (without any added DNA) have at least a moderately high degree of tortuosity.

Parallel conclusions have been reported previously in various studies that examined the nature of the relationship between minerals and organic carbon/organic matter in non-allophanic soils (e.g. Six et al., 2000a, 2000b; Lehmann et al., 2007; Baldock and Broos, 2012; Churchman and Lowe, 2012). For example, Mayer and Xing (2001); Kaiser and Guggenberger (2003), and Chenu and Plante (2006) concluded that most organic matter was stabilized in soils by close associations with clays in very small microaggregates, either through adsorption or by entrapment (occlusion). Wan et al. (2007), using scanning transmission X-ray microscopy (STXM), showed that organic matter existed as distinct particles within microaggregates more typically than as coatings on minerals. And McCarthy et al. (2008), who used SAXS to directly observe pores and their constituents, found that most organic matter was held within pores, and that it was encapsulated, rather than adsorbed, by minerals. Consequently, the results of the current study relating to DNA adsorption on synthetic allophane strongly imply that such mechanisms of encapsulation of organic carbon within pores in nanoaggregates apply to allophanic soils including Andisols.

In comparing the world's soil orders based on *Soil Taxonomy* (with the exclusion of Histosols) (Soil Survey Staff, 1999), the foremost ability of Andisols to sequester carbon, and the primacy of allophane nanominerals rather than long-range-order and other crystalline clay minerals in affecting such sequestration, are therefore attributable largely to the very high fractal-scale porosity (at nano- and submicron scales) and stability of their constituent nanoaggregates and microaggregates.

4. Conclusions

(1) The synthetic allophane spherules made in this study were uniformly 10–15 nm in diameter and with a SSA up to 374 $m^2\ g^{-1}$. Generally, 1 mg of synthetic allophane could adsorb up to 34 μg of salmon-sperm DNA in total.

(2) P XANES and IR spectra for salmon-sperm DNA–allophane complexes affirmed that the chemical adsorption of DNA by allophane is through its phosphate groups (direct chemical adsorption of DNA on allophane spherules). The DNA adsorption on allophane induced an alteration of the surface chemistry of allophane whereby the characteristic Si–O–Al stretching frequency of allophane shifted from 1020 cm^{-1} to 990 cm^{-1}. This alteration and shift could be attributable to either the change of interatomic distances of the allophane wall or the precipitation of aluminium phosphates on the surface of allophane, or both.

(3) Humic acid hampered the DNA adsorption capacity on synthetic allophane by occupying the active sites on allophane and suppressing the charge relocation between DNA and humic acid-rich allophane. However, some ligands of humic acid bound to DNA chemically through its phosphate groups and DNA became attached to allophane spherules indirectly (indirect chemical adsorption on allophane).

(4) The adsorptive sites on the surface of allophane spherules became saturated despite the addition of only small amounts of DNA (~2–6 μg mg^{-1} allophane), but much more DNA (up to 28 μg mg^{-1}, ~80% of the total DNA adsorbed) was able to be adsorbed by allophane physically (i.e., in nanopores and submicropores) when the porous allophane–DNA aggregates (including nano- and microaggregates) were formed. The aggregation of DNA and allophane spherules and great stability of such aggregates thus explain why allophanic soil materials are able to sequester much DNA, and hence slow the degradation of DNA in such materials.

(5) These findings relating to DNA very likely appertain to soil organic matter in that the stable, highly porous allophane nano- and microaggregates allow much organic matter and organic carbon (potentially up to ~80%) to be adsorbed physically. The carbon is effectively encapsulated and protected within a nanolabyrinthic network of nanopores (<100 nm) and submicropores (100–500 nm), enhanced because of the network's high degree of tortuosity, thereby leading to the especially slow turnover of carbon in Andisols and other allophanic soils.

Abbreviations

BET	Brunauer–Emmett–Teller
BJH	Barrett–Joyner–Halenda
C	carbon
DI	deionised
DNA	deoxyribonucleic acid
FTIR	Fourier transform infrared
HA	humic acid
IR	infrared
NMR	nuclear magnetic resonance
NSRRC	National Synchrotron Radiation Research Center
RNA	ribonucleic acid
SAXS	small angle X-ray scattering
SSA	specific surface area(s)
STXM	scanning transmission X-ray microscopy
WL	white line
XANES	X-ray absorption near-edge structure

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Chapter 6 A new method to extract and purify DNA from allophanic soils and paleosols, and potential for paleoenvironmental reconstruction and other applications

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Abstract

Andisols, developed from late-Quaternary tephra (volcanic ash) deposits and dominated by the nanocrystalline aluminosilicate mineral, allophane, contain large stores of organic matter and are potential reservoirs for DNA[1]. However, DNA recovery from Andisols and other allophane-rich soils has been difficult and inefficient because of strong chemical bonding between DNA and both allophane and organic matter. We have therefore developed a new two-step DNA isolation method for allophanic soils and buried paleosols which circumvents these problems. The method centres on (1) chelating DNA and blocking adsorptive sites on allophanic materials using EDTA and phosphate, respectively, and (2) the novel application of acidified ammonium oxalate (Tamm's reagent) to dissolve allophane to release bound DNA. Ammonium oxalate has not previously been applied to soil DNA extraction. DNA yields up to 44.5 $\mu g\ g^{-1}$ soil (oven-dry basis) were obtained from three field-moist natural allophanic soil samples from northern New Zealand using this two-step method. We also evaluated different DNA purification methods both before and after DNA extraction. Gel electrophoresis of the extracted DNA followed by gel purification of the DNA from agarose gel, despite DNA loss, was the only purification method that sufficiently removed humic substances for successful DNA amplification using the polymerase chain reaction (PCR) of multiple gene regions. Sequencing of PCR products obtained from a buried allophanic paleosol at 2.2-m depth on a Holocene tephra yielded endemic and exotic plants that differed from the European grasses growing currently on the soil's surface. This difference suggests that the DNA extraction method is able to access (paleo)environmental DNA derived from previous vegetation cover. Our DNA extraction and purification method hence may be applied to Andisols and allophane-rich paleosols, potentially offering a means to isolate paleoenvironmental DNA and thus facilitate reconstruction of past environments in volcanic landscapes, datable using tephrochronology, and also aid biodiversity understanding of andic soils and paleosols.

Keywords:

Andisols; allophane; DNA extraction; DNA purification; paleopedology; tephra

[1]*Abbreviations*:

asl, above sea level; bp, base pair; BP, before present (present = AD 1950); cal, calendar or calibrated; CTAB, cetyltrimethylammonium bromide; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; *g*, gravitational acceleration (9.81 m s^{-2}); humics, humic substances including humic acids; HTS, high-throughput sequencing; IPTG, isopropyl β-D-1 thiogalactopyranoside; kb, kilo base pair; LB, Luria-Bertani broth; pa, per annum; ODS, oven-dry soil; PalEnDNA, paleoenvironmental DNA; PEG, polyethylene glycol; PCR, polymerase chain reaction(s); PVP, polyvinylpyrrolidone; Rai's lysis buffer, buffer of Rai et al. (2010); rpm, revolutions per minute; SDS, sodium dodecyl sulphate; TAE, tris-acetic-EDTA; TE, 10 mMTris-HCl, 1 mM EDTA; TEM, transmission electron microscope; UV, ultraviolet; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

6.1. Introduction

Allophane-containing soils, which include most Andisols, strongly sequester organic matter (Dahlgren et al., 2004; McDaniel et al., 2012; Matus et al., 2014) and strongly adsorb deoxyribonucleic acid (DNA) (Cai et al., 2006; Saeki et al., 2008, 2011; Huang et al., 2014), and hence enable carbon storage and possible preservation of DNA. Consequently, Andisols and allophanic soils on Holocene or late Pleistocene tephras potentially represent a valuable source of soil environmental DNA which could be used to reconstruct past environments or climates of the late Quaternary period through paleopedology. In addition, the extraction of environmental DNA from Andisols, which can comprise fungal, microbial, plant (including recombinant plant DNA), insect, and animal DNA (e.g. Fierer and Jackson, 2006; Levy-Booth et al., 2007; Staudacher et al., 2011; Perdersen et al., 2015), would offer enhanced insight into the biodiversity of these soils as well as possible application to forensic soil analysis (e.g. Fitzpatrick, 2013; Young et al., 2014). However, the potential use of such a resource has been hampered because of the difficulty in extracting adsorbed DNA from allophanic materials (see section 6.2). An effective method for DNA recovery from Andisols and buried allophanic paleosols (soils of past environments or landscapes) is therefore required to potentially enable paleoenvironmental DNA (Rawlence et al., 2014) to be investigated, and to aid research involving soil biodiversity. The development of such a method, and its potential application including paleoenvironmental reconstruction, form the main focus of this paper.

The key roles of allophane and organic matter in DNA adsorption and extractability are summarised before we outline the basis of our new approach to extract and purify DNA from allophanic soils or paleosols (section 6.2). Our extraction method was developed using initial experiments involving synthetic allophane and salmon-sperm DNA and is based on chelating DNA and blocking adsorptive sites on allophanic materials using ethylenediaminetetraacetic acid (EDTA) and phosphate, respectively, and then releasing (rather than displacing) DNA by dissolving the allophane using acidified ammonium oxalate (section 6.3). The results of the DNA extraction and purification (from both synthetic allophane–salmon-sperm-DNA complexes and field-moist natural allophanic soils), including the successful polymerase chain reaction (PCR) amplification and sequencing of plant DNA extracted from a buried Holocene paleosol, are

reported in section 6.4. The efficacy of the method and some implications are then discussed (section 6.5) before the conclusions (section 6.6).

6.2. Roles of allophane and organic matter in DNA adsorption and extractability

Allophane provides Andisols, and other allophanic soils and buried paleosols, with many of their unique chemical and physical properties (Qafoku et al., 2004; McDaniel et al., 2012; Yuan and Wada, 2012; cf. Takahashi and Dahlgren, 2016). Such properties are defined as "andic soil properties" in *Soil Taxonomy* and these help delimit the Andisol order (Ahrens and Arnold, 2012; McDaniel et al., 2012; Soil Survey Staff, 2014). Allophane is a nanocrystalline aluminosilicate comprising tiny spherules ~3.5 to 5.0 nm in diameter and with a chemical composition in the range $(1–2)SiO_2 \cdot Al_2O_3 \cdot (2–3)H_2O$ (Abidin et al., 2007; Harsh, 2012). It has been shown previously that extracting DNA from allophane-rich volcanic soils is more difficult than from non-allophanic (non-volcanic) soils using the same extraction method (Rai et al., 2010). The strong bonding between the allophane (and other nanocrystalline aluminium-rich and iron-rich clays) and DNA (Hashizume and Theng, 2007; Huang et al., 2014) can be attributed to the small particle size of such clay minerals, an extremely high specific surface area (up to ~1125 $m^2\ g^{-1}$) (Allbrook, 1985; Parfitt, 2009; Kaufhold et al., 2010; Yuan and Theng, 2012), and chemical attractions with variable surface-charge characteristics that arise via $(OH)Al(OH_2)$ groups at wall perforations of its outer gibbsitic octahedral sheet $[Al(OH)_3]$ (Cai et al., 2006; Buurman et al., 2007; Saeki et al., 2010; Calabi-Floody et al., 2011; Harsh, 2012; Matsuura et al., 2014). Huang et al. (2014, 2016) showed that the DNA phosphate group was bound to the outer gibbsitic octahedral sheet on the surface of allophane spherules or to organic matter, or both.

In addition, allophane spherules tend to form nanoaggregates up to about 100 nm in diameter (Calabi-Floody et al., 2011; Huang et al., 2016). Associated nanopores (tiny gaps between nanoaggregates and between spherules within nanoaggregates), which are too small and tortuous in structure to be accessible to microbes and enzymes (Chevallier et al., 2010; Filimonova et al., 2011; Huang et al., 2016), demonstrably provide "refuges" to any disseminated DNA in Andisols as long as the DNA has bonded to allophane spherules and remains enclosed in

the nanopores amidst the nanoaggregates (Huang et al., 2016). Such a protection mechanism enables DNA to be stored and preserved in soils dominated by allophane.

Furthermore, the abundant organic matter in Andisols is also highly adsorptive and has a strong affinity for DNA (Saeki and Sakai, 2009). Adsorptive humic substances including humic acids ("humics" hereafter) in soils can be co-extracted with DNA and prevent DNA amplification by inhibiting the enzyme activity in PCR (Lloyd-Jones and Hunter, 2001; Saeki et al., 2011; Young et al., 2014). Most commercial soil DNA extraction kits involve bead-beating, which disrupts both microbial cells and soil aggregates, and various extraction reagents (Young et al., 2014), but do not produce sufficient yields for samples with highly adsorptive minerals, such as allophane or ferrihydrite, or efficiently purify DNA from samples with high amounts of humics (Lloyd-Jones and Hunter, 2001; Cai et al., 2006; Saeki and Sakai, 2009; Saeki et al., 2011). Also, methods that utilise commercial columns, flocculation, and gel electrophoresis for inhibitor removal lead to considerable loss of DNA (Miller et al., 1999) and so they are not ideal for purifying environmental DNA. Therefore, an effective DNA extraction and purification scheme for allophane must be able to efficiently extract DNA and remove PCR inhibitors whilst optimising DNA yield.

A limited number of studies have evaluated DNA extraction from "volcanic materials" including allophanic clays (Herrera and Cockell, 2007), but the common reagents involving ion/cation exchange or chelation could neither release bound DNA from allophane nor isolate DNA from humics (Herrera and Cockell, 2007; Miao et al., 2014). Consequently, DNA extraction methods applicable to allophanic soils have required ongoing development (e.g. Ikeda et al., 2004; Takada-Hoshino and Matsumoto, 2004; Dong et al., 2006). Currently, one method able to extract environmental DNA from allophanic soil materials uses skim milk, which functions as an adsorption competitor (Takada-Hoshino and Matsumoto, 2004, 2005), but thus far only clay- and organic-rich topsoils (soil 'A' horizons; typically 0 to 15 cm depth) comprising DNA from microbes, insects, and plants, have been tested but with limited efficacy with respect to DNA extractability.

6.2.1. A new approach to extract and purify DNA from adsorptive allophanic soils

As noted earlier, we posit that much more genetic information could be obtained by applying a more efficient method of DNA extraction to most Andisols or to allophanic paleosols. We have therefore developed a two-step DNA isolation method for allophanic materials that breaks down the strong bonding between allophane and chemically-bound DNA and liberates relatively high yields of amplifiable DNA. Our method is based on the premise that DNA is likely to be more easily extractable if the allophane spherules and nanoaggregates are physically disrupted and, notably, chemically dissolved to release the DNA.

Our approach uses an alkaline lysis buffer extraction (based on Rai et al., 2010, described below) followed by acidified ammonium oxalate, also known as Tamm's reagent (Tamm, 1922). The alkaline lysis buffer has been shown to extract DNA quantities of up to 20−40 μg g^{-1} soil from Japanese allophanic soils (Rai et al., 2010), whereas acidified ammonium oxalate has been widely used to extract Al and Si, and other metals such as Fe, from nanocrystalline minerals including allophane and ferrihydrite by means of their dissolution (e.g. Parfitt and Childs, 1988; Wada, 1989; Parfitt, 2009; Churchman and Lowe, 2012). To our knowledge, this key ingredient, Tamm's reagent, has not previously been used to release adsorbed DNA by dissolution of the allophane.

Furthermore, we tested the procedure using field-moist soil rather than soil that had been dried in the laboratory. Fine pores are largely lost, the texture of the soil becomes coarser, and dispersion difficult when allophanic soils are oven-dried, and these effects are irreversible (Allbrook, 1983; Churchman and Payne, 1983; Gray and Allbrook, 2002; Harsh et al., 2002). In addition, drying of soils strengthens bonds between minerals and organic matter, which tend to increase in covalent character (Kleber et al., 2015). Hence it is likely that prior drying of allophanic soils alone could prevent the complete removal of adsorbed DNA. Results involving the DNA extractions (yields) from the soil samples are expressed relative to the mass of oven-dry soil (ODS), however.

We also explored several DNA purification techniques prior to, and after, DNA extraction. The techniques trialled included (1) manufactured activated charcoal prior to DNA extraction to absorb soluble humics, (2) two commercial DNA clean-up kits after DNA extraction, and (3) gel electrophoresis followed by use of GenScript QuickClean II Gel Extraction Kit.

6.3. Materials and methods

6.3.1. Samples for DNA extraction

6.3.1.1. Synthetic allophane-salmon-sperm DNA complex

For proof of principle, synthetic allophane and salmon-sperm DNA were used to test whether our DNA extraction method was able to extract DNA strongly bound to synthetic allophane. Pure aluminosilicate allophane was synthesized following the method of Ohashi et al. (2002), and chemical analysis of the fine white product (Fig. 6.1a) with both oxalate and pyrophosphate extractions showed an allophane yield of 95% with an Al/Si ratio of 1.2. Ultra-high-resolution transmission electro microscope (TEM) images of the synthetic allophane (after Huang et al., 2016) showed that the allophane was consistently spherical in shape with an average diameter of 10−15 nm (Fig. 6.1b and 6.1c), approximately two- to three-fold larger than that reported for natural allophane spherules (typically ~3.5 to 5 nm) (e.g. McDaniel et al., 2012). This larger spherule size is presumably a consequence of the precipitation of synthetic allophane in the laboratory setting without growth constraints imposed in the milieu of natural soil microenvironments (Churchman and Lowe, 2012). Because allophane spherules are heat-sensitive (Woignier et al., 2007), the synthetic product was frozen quickly with liquid nitrogen, followed by freeze-drying for subsequent analysis.

To carry out DNA adsorption, 90 μg of salmon-sperm DNA was added into a suspension containing 25 mg of synthetic allophane and mixed on a rotator for 3 h. Then the DNA-clay suspension was centrifuged at 13,000 rpm (10,000 *g*) and the supernatant was analysed spectrophotometrically to ascertain that all additive DNA had bonded to the synthetic allophane with no detectable DNA remaining in the supernatant. The complex of synthetic allophane and salmon-sperm DNA was stored at 4 °C in the dark for subsequent DNA extraction.

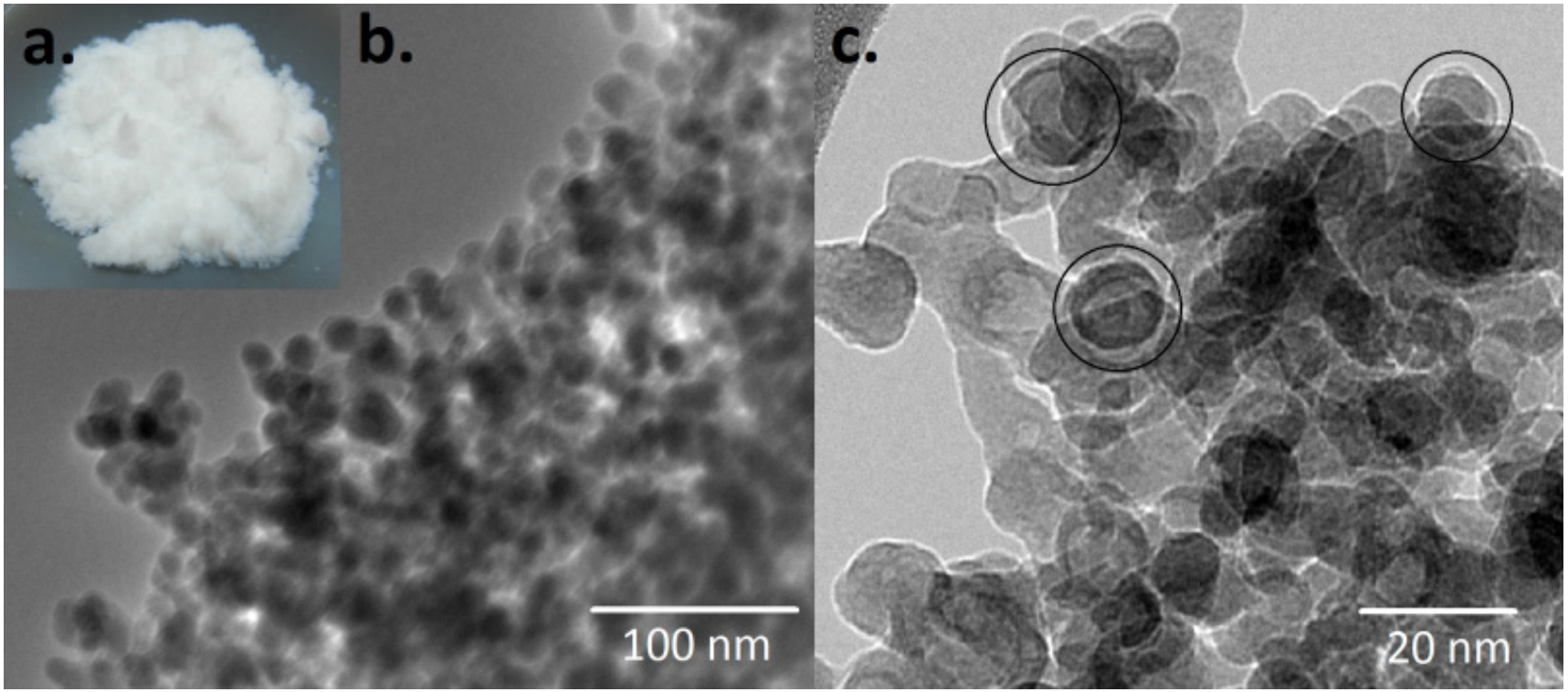

Fig. 6.1 Synthetic allophane (**a**) and its spherical morphology seen using transmission electron microscopy (TEM) (**b**) and (**c**), reproduced from Huang et al. (2016, p. 43) with permission from Elsevier [*licence no. 3771690390133*].

6.3.1.2. Natural allophanic soil samples

We selected a site near Rotorua in central North Island, New Zealand, where a well-drained Andisol had previously been characterised (Cole, 1970; Lowe et al., 2010, 2012). This multilayered soil profile of the Rotomahana soil series (Fig. 6.2) was excised laterally in two stages for close to 1 m to remove surface (roadside) contamination. Allophane-rich materials were sampled from buried soil horizons developed on three Holocene rhyolitic (siliceous) tephras, namely Taupo, Whakatane, and Rotoma, details of which (including ages) are given in Table 6.1. The depths of samples below the modern land surface were ~0.8 m (3ABb horizon developed on Taupo tephra), ~1.4 m (4Bwb horizon developed on Whakatane tephra), and ~2.2 m (5Ahb soil horizon developed on Rotoma tephra) (Fig. 6.2).

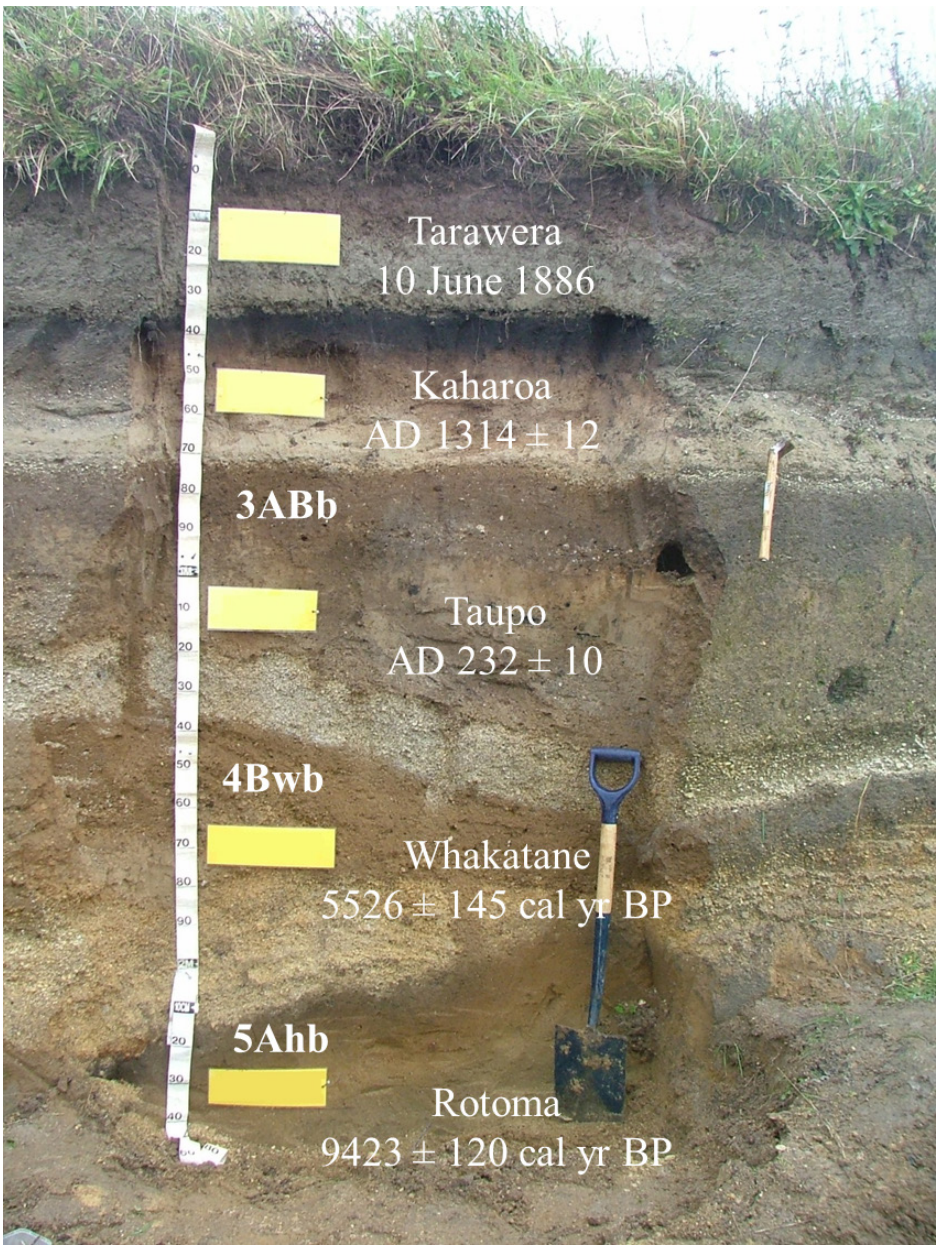

Fig. 6.2 Multilayered Andisol at Brett Rd near Mt Tarawera comprising five separate tephra deposits (names and ages/dates as marked; see also Table 6.1) and associated soil horizons. We sampled the buried allophanic soil horizons on Taupo (denoted 3ABb), Whakatane (4Bwb), and Rotoma (5Ahb) tephras (horizonation from McDaniel et al., 2012, after Soil Survey Staff, 2014). The suffix 'b' denotes an identifiable soil horizon with pedogenic features developed before its burial (Soil Survey Staff, 2014). Scale divisions on tape = 10 cm. Photo: D.J. Lowe.

Modern root growth was observed through all horizons and tephra layers of the profile; the current land surface underlies roadside pasture. Therefore, samples were sieved through a 2-mm mesh to remove gravel and visible plant roots, and the <2-mm fractions were thoroughly homogenised and stored at field-

moist conditions in plastic bags at 4 °C prior to analysis. Moisture contents (to enable analyses to be expressed on an oven-dry soil [ODS] basis) and pH were measured using the methods described by Blakemore et al. (1987). Total carbon contents of the freeze-dried clay fractions were examined using a Leco TruSpec carbon/nitrogen analyzer. Allophane and ferrihydrite contents were estimated according to the oxalate/pyrophosphate-dissolution based methods of Parfitt and Wilson (1985) and Parfitt and Childs (1988), as documented by Blakemore et al. (1987).

Table 6.1 Properties of allophanic material from three buried soil horizons of an Andisol[a], northern New Zealand.

Buried soil (horizonation)	pH (H_2O)	Clay content[a] %	Allophane content[b] %	Ferrihydrite content[b] %	Total carbon %
Taupo (3ABb)[c]	6.6	3.4	39	6.1	11.9
Whakatane (4Bwb)[d]	6.8	3.9	38	9.5	10.2
Rotoma (5Ahb)[e]	6.6	5.0	47	4.9	6.3

*Well-drained multisequal soil (of Rotomahana soil series) located on Brett Road, Rerewhakaaitu (near Mt Tarawer)a, North Island (Fig. 6.2), at 38° 17.905' S, 176° 28.823' E, elevation 454 m asl, rainfall ~1500 mm pa (Lowe et al., 2012; McDaniel et al., 2012, p. 33-38). It is a Fine-loamy/medial, mixed/glassy, active, mesic Typic Udivitrand (Soil Survey Staff, 2014). Note that the uppermost material, labelled Tarawera tephra in Fig. 6.2, comprises mainly pre-weathered materials of the so-called Rotomahana Mud Member of the Tarawera Tephra Formation (Froggatt and Lowe, 1990) erupted on 10 June 1886. With a clay content of ~20% (Parfitt et al., 1981), the clays include interstratified montmorillonite/mica, montmorillonite, mica, kaolinite, and mordenite, as well as allophane (Kirkman, 1976).

[a]<2 μm size fraction determined using the pipette method after prolonged shaking of samples with glass beads (method after Churchman and Tate, 1986).

[b]As percentage of clay fraction.

[c]Taupo tephra deposited AD 232 (± 10 years); soil formed on it represents ~1082 years at land surface before its burial by Kaharoa tephra in AD 1314 (± 12 years).The 3ABb horizon sampled is ~0.8 m below the modern land surface (Fig. 6.2).

[d]Whakatane tephra deposited 5526 ± 145 calendar (cal) years before present (BP) (present = AD 1950); soil formed on it represents ~3808 years at land surface before its burial by Taupo tephra in AD 232 (± 10 years). The 4Bwb horizon sampled is ~1.4 m below the modern land surface (Fig. 6.2).

[e]Rotoma tephra deposited 9423 ± 125 cal years BP; soil formed on it represents ~3897 years at land surface before its burial by Whakatane tephra 5526 ± 145 cal years BP. The 5Ahb horizon sampled is ~2.2 m below the modern land surface (Fig. 6.2).

All ages or dates in footnotes c-d-e are 95% probability ranges from Lowe et al. (2013).

6.3.2. DNA extraction

Three independent one-step extraction methods were initially applied to the previously prepared synthetic allophane–salmon-sperm-DNA complexes, and to the natural allophanic soils on tephras (Taupo, Whakatane and Rotoma): (1) alkaline lysis buffer, (2) alkaline lysis buffer with bead-beating, and (3) ammonium oxalate extraction. Subsequently, we applied a two-step DNA extraction method using the alkaline lysis buffer followed by ammonium oxalate extraction to both the synthetic allophane and natural allophanic soils. For each extraction, 25 mg of synthetic allophane–salmon-sperm-DNA complex and 0.5 g of natural field-moist allophanic soil material were processed. All DNA extractions were carried out in triplicate.

6.3.2.1. Alkaline lysis buffer

The alkaline DNA extraction buffer of Rai et al. (2010) ("Rai's lysis buffer" hereafter) consisted of 1% sodium dodecyl sulphate (SDS), 100 mM Tris-HCl, 200 mM EDTA, and 500 mM Na_2HPO_4 (final pH at 8.6). Sample material was mixed with 500 µL of Rai's lysis buffer and incubated at 95 °C with shaking for 10 min. For the synthetic allophane, the supernatant was collected by centrifugation at 13,000 rpm (10,000 *g*) for 10 min. 3 M sodium acetate (1/10 the volume to the mixture) and phenol-chloroform (equal volume to the mixture) were specifically added only to the natural allophanic soil samples, followed by vigorous mixing, 10 min shaking, and centrifugation at 13,000 rpm (10,000 *g*) for 10 min, to generate two immiscible liquid layers and to separate the aqueous phase containing DNA (upper layer) from an organic phase (bottom layer), and the upper aqueous supernatant was collected.

6.3.2.2. Alkaline lysis buffer (Rai's lysis buffer) with bead-beating

After adding 500 µL of Rai's lysis buffer to synthetic allophane and natural allophanic material, three 2-mm glass beads (provided by Biospec, USA) (Li et al., 2011) were added, followed by beating three times at maximum speed for 30 s with an interval of 30 s between each period of beating, and then samples were incubated at 95 °C with shaking for 10 min. The extracts from synthetic

allophane and natural allophanic soils were collected following the methods described in section 6.3.2.1.

6.3.2.3. Ammonium oxalate extraction

To chemically dissolve synthetic allophane and natural allophanic soil material to release bound DNA, oxalate extraction was carried out. Usually, for analysing the Al/Si ratio of allophanic samples, the oxalate extraction is undertaken with 0.2 M oxalate solution (pH 3) for 4 hours in dark conditions (Blakemore et al., 1987; Dahlgren and Saigusa, 1994). Here, we increased both the concentration of oxalate solution and the reaction time to enhance the efficiency of oxalate extraction for DNA. 1 M acidified ammonium oxalate salt was prepared by homogenising 18 g of ammonium oxalate and 10.8 g of oxalic acid powder in sterile deionised water and making the volume up to 200 mL (pH 3). 500 µL of this oxalate solution was added to each sample (0.5 g of field-moist soil), followed by overnight shaking for allophane dissolution. The extracts from synthetic allophane and natural allophanic soils were collected following the methods described in section 6.3.2.1.

6.3.2.4. Two-step DNA isolation: alkaline lysis buffer (Rai's lysis buffer) followed by ammonium oxalate extraction

We developed our two-step DNA extraction method consisting of (step 1) DNA extraction using Rai's lysis buffer, followed by (step 2) overnight DNA extraction using acidified ammonium oxalate extraction from the residual material (i.e. the synthetic or the natural allophane 'pellet' remaining after step 1 of the extraction) (Fig. 6.3). After step 1 of the extraction, the 3 M sodium acetate (1/10 the volume to the mixture) and phenol-chloroform (equal volume to the mixture) were specifically added only to the natural allophanic soil samples, and the aqueous supernatant (upper layer) was collected and remaining phenol-chloroform (bottom layer) was removed by a fine transfer pipette; the remaining soil underwent step 2 of the extraction (ammonium oxalate extraction). In contrast, synthetic allophane was directly treated with acidified ammonium oxalate after extraction by step 1. The supernatant collected after extraction by step 1 is denoted as the '1st extract' in Fig. 6.3, and the supernatant obtained from step 2 is

recorded as the ‘2nd extract’. The extracts from the first and second extractions were kept separate.

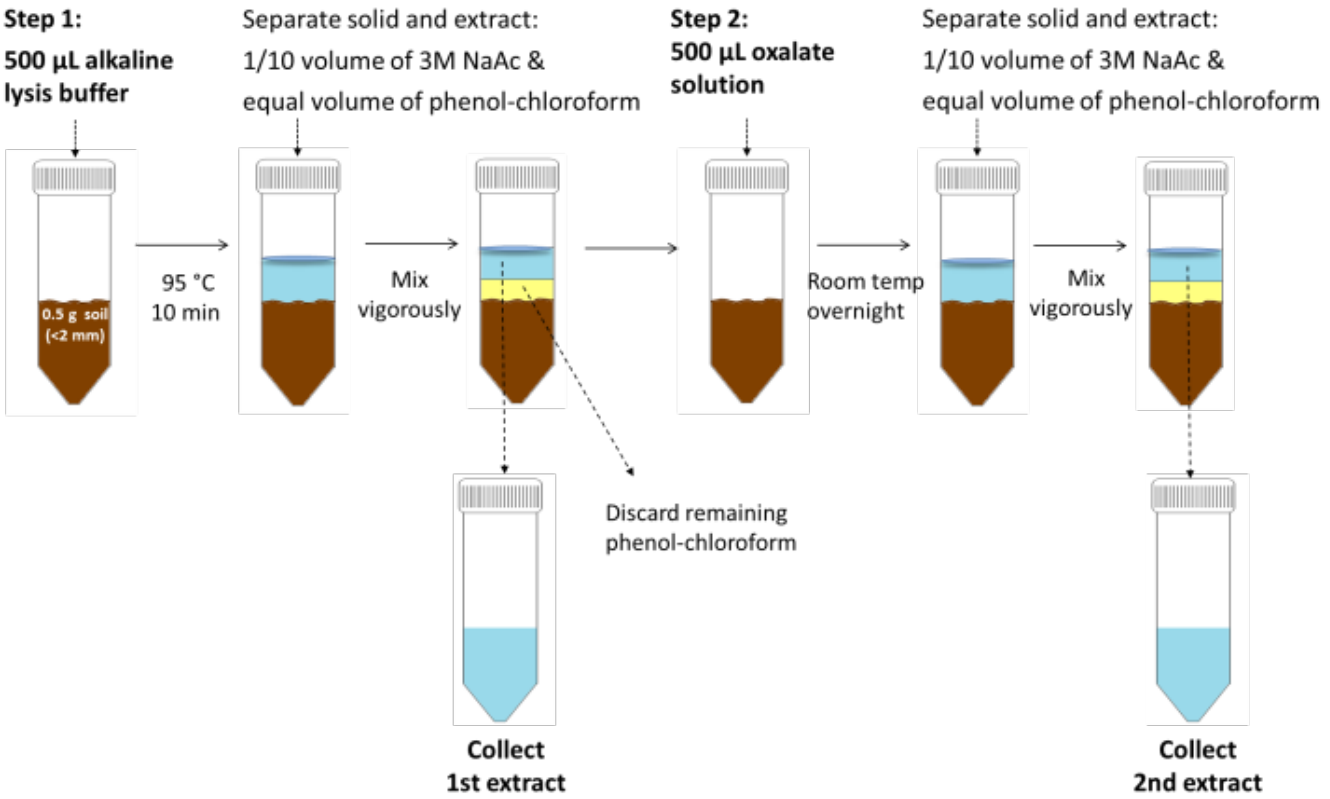

Fig. 6.3 Simplified schematic depicting the two-step DNA extraction method involving Rai's lysis buffer (step 1) followed by acidified ammonium oxalate treatment (step 2) for DNA extraction from natural allophanic soil samples.

6.3.3. DNA quantification and sample purification

The fragment lengths of extracted DNA were assessed via gel electrophoresis and ethidium bromide staining, using 1 kb Plus DNA Ladder (Invitrogen) as a reference. For the extracts from synthetic allophane, DNA yield was evaluated via gel electrophoresis together with a 1 kb Plus DNA Ladder (Invitrogen). DNA extracts derived from the natural allophanic soils on tephras (Taupo, Whakatane, and Rotoma) were evaluated spectrophotometrically, and the reading (in ng μL^{-1}) was multiplied by the final volume of extract (μL) and then divided by the oven-dry mass of soil material used for the DNA extraction. Note that the co-extracted humics substantially interfered with the spectrophotometric measurement at 260 nm, and therefore the quantification of DNA yields may be overestimated.

To remove co-extracted humics and impurities from the natural allophanic soils, each extract (described in sections 6.3.2.1 to 6.3.2.4) was gently mixed with 5 M NaCl (1/3 volume to the collected supernatant) and 10% cetyltrimethylammonium bromide (CTAB) (1/3 volume to the collected supernatant), followed by 65 °C incubation for 10 min on a thermomixer. The mixture was then thoroughly mixed with 750 μL of chloroform for 10 min at room temperature, and then centrifuged at 13,000 rpm (10,000 × g) for 10 min. The upper layer was collected and gently mixed with an equal volume of 20% polyethylene glycol 8000 (PEG 8000), incubated at room temperature for 20 min followed by centrifugation for 15 min at 13,000 rpm (10,000 × g). The supernatants were removed, and the precipitates were washed with 1 mL of 100% ethanol and 1 mL of 70% ethanol followed by centrifugation at 13,000 rpm (10,000 × g) for 2 min. After the ethanol had been decanted, the precipitates were air dried and then resuspended with 30 μL TE (10 mM Tris-HCl and 1 mM EDTA). DNA concentrations in 30 μL of TE were evaluated at 260 nm spectrophotometrically using a NanoDrop2000 spectrophotometer (Thermo Scientific, USA), and DNA yields from the natural allophanic soils were then calculated by the volume of TE and sample concentration. PCR amplification of the DNA in TE was carried out with selected primers (see section 6.3.4). However, the A_{260}/A_{280} NanoDrop readings and the poor PCR amplification rate indicated that further purification to remove inhibitors was required.

6.3.3.1. Further DNA purification of DNA extracts from buried soil horizon on Rotoma tephra for polymerase chain reaction (PCR)

DNA extracts derived from the buried soil on Rotoma tephra using the two-step DNA extraction method were further purified for PCR and subsequent DNA sequencing. The soil horizon (5Ahb) developed on the Rotoma tephra was selected for testing DNA purification for two main reasons: (1) it represents nearly c. 4000 years of development and weathering whilst at the land surface (before being buried by the Whakatane and other tephras since c. 5500 cal years ago) and so has a relatively high clay and allophane content (Table 6.1); and (2) it is now at a depth of c. 2.2 m and so is beyond the range of most topdown pedogenic processes (Fig. 6.2) (e.g. Schaetzl and Thompson, 2015; Huang et al., in review).

To further purify the DNA extracts from the buried soil horizon on Rotoma tephra, two commercial kits (Zymo Research Genomic DNA Clean & Concentrator Kit and GenScript QuickClean PCR Purification Kit) were used following the manufacturers' protocols. A third approach involved mixing the DNA extract with 5 μL of nucleic acid stain GelRed for gel electrophoresis (1% agarose gel). The gel was illuminated with a blue-light illuminator (Safe Imager, Invitrogen) and the DNA band or smear was extracted for gel purification using the GenScript QuickClean II Gel Extraction Kit. In addition, the use of humic-acid removal solution and activated charcoal prior to DNA extraction was also included (described below in section 6.3.3.1.1). The efficiency of each of these five purification techniques (Table 6.2) was assessed with regard to final DNA yield, purity, and amplification success using four primer pairs (section 6.3.4).

Table 6.2 DNA yield, purity, and PCR amplification success observed from DNA extracts from the buried soil (5Ahb horizon) on Rotoma tephra subjected to five different purification methods following the two-step DNA isolation method.

Purification method	DNA yield[a] (µg g^{-1} ODS basis)	DNA purity		PCR amplifications with selected primers[b]				
		A_{260}/A_{280}	A_{260}/A_{230}	16s rDNA	Lep	rbcL	trnL c−d	trnL g−h
1. Two-step DNA extraction followed by chloroform treatment	8.7	1.6	0.6	−	−	−	−	−
2. Two-step DNA extraction followed by Zymo Research Genomic DNA Clean & Concentrator Kit	3.2	1.2	0.2	+	−	−	−	−
3. Two-step DNA extraction followed by GenScript QuickClean PCR Purification Kit	8.6	1.3	0.2	+	−	−	−	−
4. Humics-removal prior to two-step DNA extraction followed by chloroform[c]	10.0	1.6	0.9	−	−	−	−	−
5. Two-step DNA extraction followed by gel electrophoresis and GenScript QuickClean II Gel Extraction Kit	1.9	1.6	1.7	+	−	+	+	+

[a]See text, section 6.3.3. ODS, oven-dry soil.

[b]Presence (+) or absence (−) of amplicons on agarose gel after amplification with touchdown PCR.

[c]DNA precipitated from the humics removal step remained inhibiting to DNA amplification, presumably because of the presence of unknown inhibitors.

6.3.3.1.1. Humic-acid removal using activated charcoal prior to two-step DNA extraction

The combination of 10 mmol L^{-1} $MgCl_2$ and 1% activated charcoal has been found to effectively remove most humics and PCR inhibitors from soil samples (Sharma et al., 2014). $MgCl_2$ precipitates organic matter by chemical flocculation and the porous charcoal adsorbs soluble humics and other impurities. Activated charcoal is usually manufactured after pyrolysis at 400−500 °C for 5 to 10 hours (Kloss et al., 2012), and sometimes up to 800 °C (Gaskin et al., 2008), and hence DNA from any original tissues are likely to be destroyed after treatment at such temperatures. To test this assumption, we attempted to extract DNA from the residual activated charcoal used in our study, but no quantifiable DNA was detected nor amplified.

A humics-removal solution (Li et al., 2011) was used in combination with a charcoal suspension and $MgCl_2$ solution prior to cell lysis/DNA isolation (i.e. as the initial step of the DNA extraction). The humics-removal solution comprised 0.1 M Tris, 0.1 M $Na_4P_2O_7$, 0.1 M Na_2EDTA, 1% polyvinylpyrrolidone (PVP) (w/v), 0.1 M NaCl, and 0.05% Triton X-100 (v/v), pH 10. 400 µL of the humics-removal solution, 50 µL of 10% charcoal suspension, and 50 µL of 100 mM $MgCl_2$ were added to 0.5 g of moist soil, followed by vortexing or shaking for 5 min. The humics-rich supernatant was removed after centrifugation at 13,000 rpm (10,000 × g) for 2 min. The remaining soil in the reaction tube was then treated with the two-step DNA extraction method, and extracted DNA was purified with chloroform and ethanol (see section 6.3.3) for DNA quantification and PCR amplification.

6.3.4. Polymerase chain reaction (PCR) and DNA sequencing of extract from the buried soil horizon on Rotoma tephra

Four universal primers (Table 6.3) were used to assess PCR amplification success resulting from the five different purification methods (Table 6.2). PCR amplification was carried out in a total volume of 50 µL, containing 43.75 µL of master mix (1.5 mM of Mg^{2+}, 200 µM of each dNTP, 1× Hot Fire Buffer), 1 µL of primers (20 pmol/µL), 0.25 µL of Taq DNA polymerase (Solis BioDyne), and 5 µL (~20 ng) of purified DNA template. To increase specificity and sensitivity, PCR amplifications were performed using a touchdown protocol (Korbie and

Mattick, 2008) as follows: (1) heating at 95 °C for 15 min to activate the polymerase; then (2) 10 cycles consisting of 95 °C for 20 s, 60 °C for 20 s , and 72 °C for 1 min, with the annealing temperature being decreased by 1 °C after each cycle; then (3) 30 cycles consisting of 95 °C for 20 s, 50 °C for 20 s, and 72 °C for 1 min; and (4) a final extension step at 68 °C for 10 min. PCR products were visualised by electrophoresis on a 1% (w/v) agarose gel in 1× TAE buffer. The rbcL and *trn*L PCR products derived from the two-step extraction followed by gel purification were prepared for DNA sequencing. For each primer pair, PCR products from the first and second extraction were combined before being ligated into pBluescript SK+ vectors according to the manufacturer's instructions, followed by cloning into *Escherichia coli* DH5 α and Sanger sequencing with T3 and T7 primers.

NCBI BLAST and an unpublished database of New Zealand endemic and exotic plants (held at Landcare Research, Lincoln, New Zealand, a central government-owned research centre) were used to analyse the sequences.

Table 6.3 Universal primers used for PCR amplification from the buried soil horizon (5Ahb) on Rotoma tephra. The primer pairs of rbcL and *trn*L include rbcLb F−rbcLa R, *trn*L c−h, and *trn*L g−h.

Name	Sequence	Target taxa*	Reference
16s F	GGTTAAGTCCCGCAACGAGCGC	Bacteria	Greisen et al. (1994)
16s R	AGGAGGTGATCCAACCGCA	Bacteria	Greisen et al. (1994)
Lep F1	ATTCAACCAATCATAAAGATATTGG	Insects	Hebert et al. (2013)
Lep R1	TAAACTTCTGGATGTCCAAAAAATCA	Insects	Hebert et al. (2013)
rbcLb F	AGACCTWTTTGAAGAAGGTTCWGT	Plants	Dong et al. (2014)
rbcLa R	GTAAAATCAAGTCCACCRCG	Plants	Micklos et al. (2013)
*trn*L c F	CGAAATCGGTAGACGCTACG	Plants	Taberlet et al. (2007)
*trn*L h R	CCATTGAGTCTCTGCACCTATC	Plants	Taberlet et al. (2007)
*trn*L g F	GGGCAATCCTGAGCCAA	Plants	Taberlet et al. (2007)

*Length of target fragment with 16s primers: ~450 bp; with Lep primers: ~658 bp; with rbcLb F−rbcLa R primers: ~350 bp; with *trn*L c−h primers: ~200 bp; with *trn*L g−h: ~40 bp.

6.3.5. Summary of new method

The two-step DNA extraction and gel purification method is summarised in Table 6.4 (see also Fig. 6.3). The development of this protocol forms a key part of our paper, and its efficacy and performance are discussed further in section 6.4.

Table 6.4 Summary of method for DNA extraction and its purification from Andisols and allophanic soils or buried paleosols.

Procedure	Description
	0.5 g field-moist soil (< 2-mm fraction)[a] in a 2-mL centrifuge tube
Humics removal	Add 400 µL of humics-removal solution, 50 µL of 10% charcoal suspension, and 50 µL of 100 mM $MgCl_2$, and then vortex or shake for 5 min. Centrifuge at 13,000 rpm (10,000 × *g*) for 2 min. Decant the supernatant.
1st step of DNA extraction	Add 500 µL DNA extraction buffer to the soil pellet, vortex, and then incubate the tube at 95 °C for 10 min with shaking. Add 1/10 volume of 3 M sodium acetate and equal volume of phenol-chloroform into the tube. Mix the sample vigorously and shake for 10 min and decant the supernatant after another centrifugation at 10,000 × *g*. Collect the top aqueous layer into a new 2-mL tube for subsequent DNA purification.
2nd step of DNA extraction	In the original tube, decant the remaining phenol-chloroform and then add 500 µL of acidified ammonium oxalate to the residual soil pellet (i.e. soil remaining from 1st extraction). Put sample on a rotator for overnight mixing. Add 1/10 volume of 3 M sodium acetate and equal volume of phenol-chloroform into the tube. Mix the sample vigorously and shake for 10 min. Decant the supernatant after centrifugation. Collect the top aqueous layer into a new 2-mL tube for subsequent DNA purification.
DNA gel purification	Mix the supernatant with an equal volume of 20% PEG 8000, and leave sample at room temp for at least 10 min. After centrifugation for 15 min at 10,000 × *g*, discard supernatant carefully and keep the precipitate. Rinse the precipitate with 1 mL of 100% ethanol, and re-suspend the precipitate with 30 µL of TE[b]. Load the DNA solution on a 1% agarose gel and electrophoresis for 30 min, and then cut out the DNA band on the gel using a sterile blade. Place the DNA band in the agarose into a 1.5-mL tube and add 3 volumes of DNA binding buffer from the GenScript QuickClean II Gel Extraction Kit, and solubilise the gel at 55 °C for 10 min, and purify the DNA using the kit instructions.

[a]Field-moist soil is used to prevent irreversible changes to the allophane-organic complexes and to help reduce possible bond strengthening between these on drying (see text). Yields are expressed relative to the mass of oven-dry soil, however, and hence moisture contents (oven-dry) need to be measured.

[b]TE buffer contains 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

6.4. Results

6.4.1. DNA extraction from synthetic salmon-sperm DNA-allophane complexes

Using the synthetic allophane–salmon-sperm-DNA complex, only acidified ammonium oxalate was able to successfully extract DNA from synthetic allophane (Fig. 6.4), indicating that acidified ammonium oxalate treatment indeed provides a means to dissolve allophane spherules and release DNA bound to the allophanic matrix. In contrast, Rai's lysis buffer, both alone and with bead-beating, was ineffectual, yielding no DNA (Fig. 6.4).

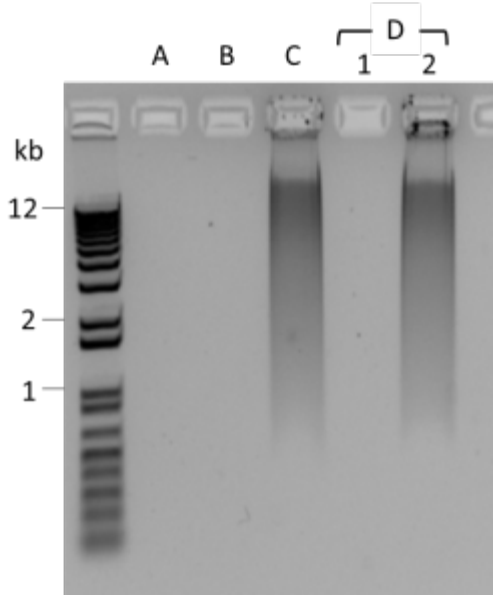

Fig. 6.4 Gel photo of salmon-sperm DNA extracted from synthetic allophane using four different methods. A: Rai's lysis buffer; B: Rai's lysis buffer with bead-beating; C: ammonium oxalate extraction; D-1: first step of two-step DNA extraction; D-2: second step of two-step DNA extraction (Fig. 6.3).

6.4.2. DNA extraction from the natural allophanic soils on Taupo, Whakatane, and Rotoma tephras

6.4.2.1. Effects of Rai's lysis buffer and bead-beating on DNA yield and fragment length

DNA extraction from three soil samples using Rai's lysis buffer allowed 26.8, 4.7, and 7.1 μg g^{-1} soil (ODS basis) of DNA to be extracted from soil horizons on Taupo, Whakatane, and Rotoma tephras, respectively, and bead-beating combined with Rai's lysis buffer seemed not to increase DNA yield from the three soil samples (Fig. 6.5A). Instead, the mechanical lysis step involving bead-beating appeared to fragment (shear) the DNA from >12 kb to 2−12 kb (Fig. 6.5A), particularly evident in the Taupo extracts.

6.4.2.2. Effects of oxalate extraction on DNA yield

The effect of ammonium oxalate extraction differed between synthetic allophane and natural allophanic soils. Using the synthetic allophane–salmon-sperm-DNA complex, acidified ammonium oxalate yielded a higher DNA yield than treatment with alkaline lysis buffer (Fig. 6.4). In contrast, when a one-step ammonium oxalate treatment was applied to natural allophanic soils, no DNA yield was observed (Fig. 6.5B). This finding indicates that the natural allophanic system differs somewhat from the synthetic model. However, when ammonium oxalate was used in the two-step DNA isolation method, DNA was obtained upon ammonium oxalate treatment at step 2 for all three soil horizons on the tephra layers (Fig. 6.5C). This result shows that the use of Rai's lysis buffer prior to ammonium oxalate treatment is essential for effectively releasing chemically bound DNA from natural allophanic soils. We deduce that this finding is likely to be a consequence of residual alkaline buffer from step 1 increasing the pH of the solution in step 2 (soil pH at this step was measured as 6). The additional DNA obtained from each soil upon step 2 with ammonium oxalate treatment is likely to be DNA that was chemically bound to the allophane, as demonstrated for the synthetic allophane–salmon-sperm-DNA complex previously, whereas the DNA obtained in the first step (using Rai's alkaline buffer) was unbound DNA.

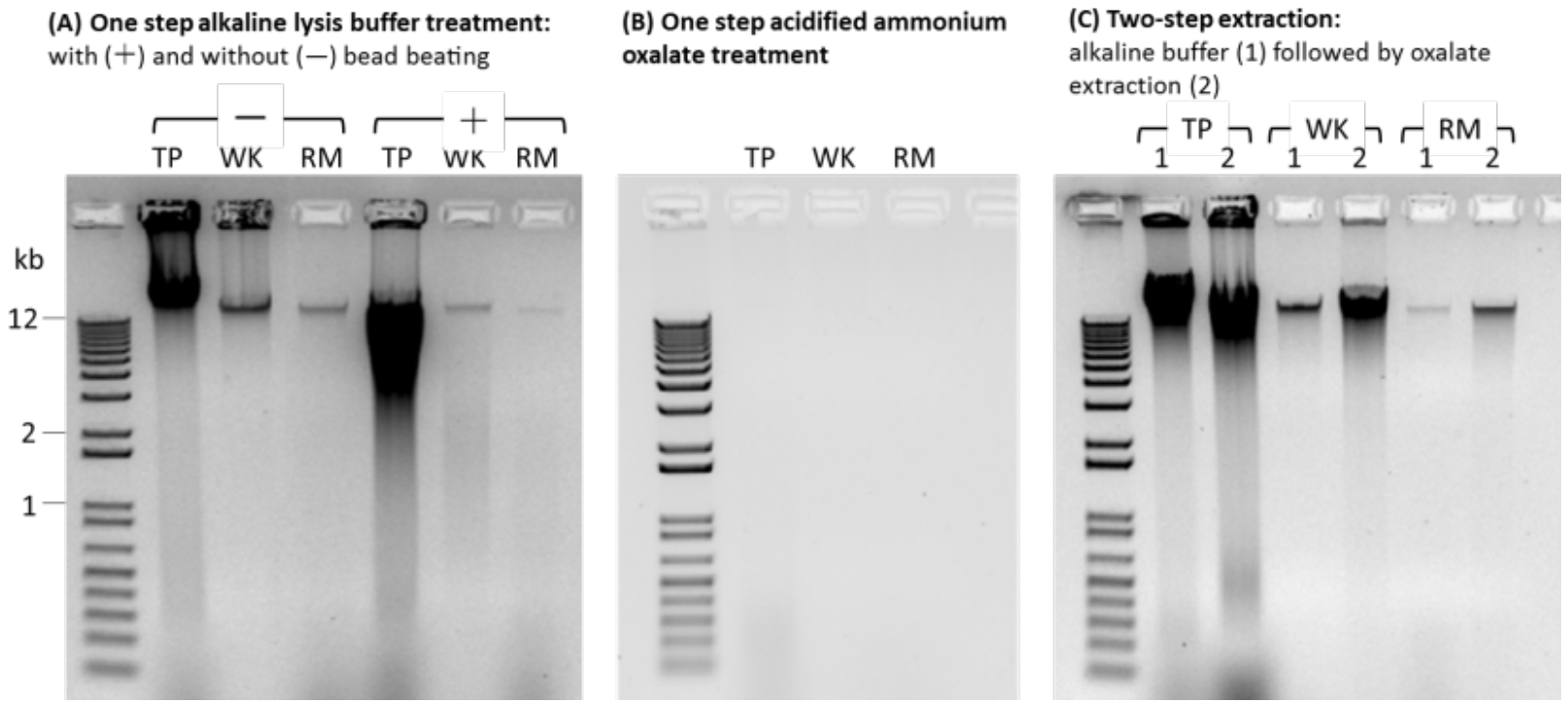

Fig. 6.5 Gel photos of extracted DNA from samples of natural allophanic soil horizons on Taupo (TP), Whakatane (WK), and Rotoma (RM) tephras using different methods, showing the relative concentrations and lengths of extracted DNA.

6.4.3. DNA purification, PCR amplification, and DNA sequencing of the buried allophanic soil on Rotoma tephra

Purification of the DNA extract from the soil horizon (5Ahb) on Rotoma tephra using NaCl, CTAB, and chloroform (see section 6.3.3) resulted in 8.7 μg g^{-1} soil (ODS basis) of DNA, but the purified DNA was not amplifiable with selected primers (Table 6.2). The poor A_{260}/A_{230} reading of the purified DNA using NaCl, CTAB, and chloroform showed a significant presence of impurities at 230 nm which inhibited PCR. Attempts to remove humics using the humics-removal solution and charcoal prior to the two-step DNA extraction resulted in no significant improvement in DNA purity nor amplification success (Table 6.2). Nevertheless, the use of the humics-removal process prior to two-step DNA extraction increased the overall DNA yield by ~1 μg g^{-1}.

Following the use of Zymo Research and GenScript DNA clean-up kits on extracted DNA from the soil horizon on Rotoma tephra, 3.2 and 8.6 μg g^{-1} soil (ODS basis) of purified DNA were obtained, respectively. However, in both cases PCR amplification was only successful for one of the five primer pairs, 16S rRNA (Table 6.2 and Fig. 6.6). This result is expected because bacterial DNA is ubiquitous and generally present in a much higher concentration than insect or plant DNA (e.g. Pietramellara et al., 2009), and so is more easily amplified than targets of lower abundance. In contrast, the gel purification approach led to successful PCR amplification of four primer pairs (except Lep primers) from the soil horizon on Rotoma tephra (1.6 of A_{260}/A_{280} and 1.7 of A_{260}/A_{230}), despite lower total DNA yields (1.9 μg g^{-1} soil, ODS basis).

Successful PCR amplification of the plant chloroplast *trn*L and rbcL genes allowed us to identify five plant families, including native New Zealand forest and scrub genera/species (Table 6.5) and an exotic species (*Eucalyptus*).

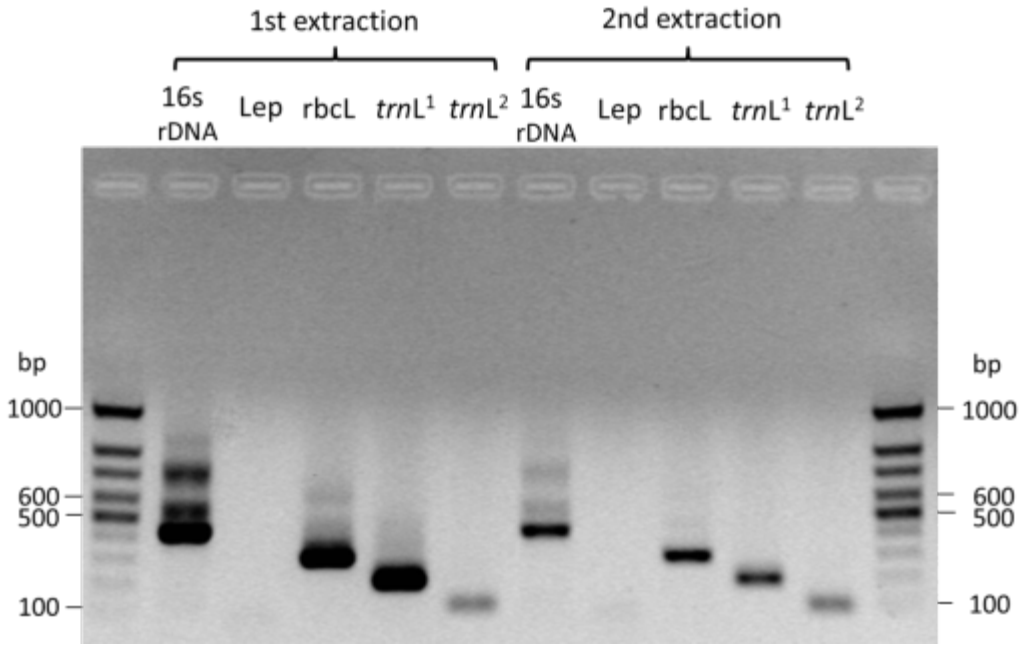

Fig. 6.6 Image of gel electrophoresis of PCR products with 16S rDNA (bacterial), Lep (insect), rbcL (plant), *trn*L c−h (plant)[1], and *trn*L g−h (plant)[2] primers. The DNA templates were extracted from the buried allophanic soil horizon (5Ahb) on Rotoma tephra (Fig. 2) using the two-step extraction. Each extract was purified using gel electrophoresis followed by GenScript QuickClean II Gel Extraction Kit and amplified separately with different primers. PCR was undertaken using DNA extracted from multiple subsamples from the same soil horizon on Rotoma tephra, and the PCR amplicons were very similar to the results shown here.

Table 6.5 New Zealand endemic plants detected in the buried soil horizon (5Ahb) on Rotoma tephra subjected to the two-step DNA extraction protocol, gel purification method, and PCR with selected primers.

Family	Plant taxa	Primer pair and % similarity to reference
Myrtaceae	*Leptospermum scoparium**	*trn*L g−h (98−99%) and *rbcL* (99%)
Araliaceae	Multiple genera	*trn*L c−h (99%) and *trn*L g−h (100%)
Griseliniaceae	*Griselinia lucida**	rbcL (98%)
Araliaceae	*Hydrocotyle*	rbcL (97%)
Podocarpaceae	*Podocarpus*	*trn*L c−h (95−98%)

* NCBI accessions of *trn*L and rbcL genes of *Leptospermum scoparium* are KF591267 and HM850121; NCBI accession of rbcL gene of *Griselinia lucida* is L11225.

6.5. Discussion

6.5.1. DNA extraction

For proof of principle, salmon-sperm DNA extraction from synthetic allophane using various methods showed that only ammonium oxalate extraction was effective at releasing salmon-sperm DNA, whereas Rai's lysis buffer − either with or without mechanical bead-beating − showed no significant DNA yield. Previous studies have shown that the use of bead-beating (in conjunction with chemical extraction) can increase DNA yield from some soils (e.g. Ikeda et al., 2004; Li et al., 2011). The bead-beating is a mechanical approach to break down soil aggregates, break down cells, and release intracellular DNA into solution. Additionally, the use of high concentrations of EDTA (a chelating agent) and phosphate (a blocking agent) in Rai's lysis buffer prevents such intracellular DNA from being adsorbed onto allophane, or humics, immediately following cell lysis. However, in our study, the use of a synthetic allophane–salmon-sperm-DNA complex highlights that EDTA and phosphate (i.e. Rai's lysis buffer) together with bead-beating are not sufficient for the isolation of extracellular DNA that has already become bound to allophane because of the highly adsorptive nature of allophane. Conversely, the increase in DNA yield from synthetic allophane using oxalate extraction to solubilize nanocrystalline clays, such as allophane and

ferrihydrite, suggests that ammonium oxalate dissolution releases extracellular DNA bound to the allophane by disrupting chemical bonds between DNA phosphate groups and Al- hydroxyl groups (Parfitt, 1980, 1990; Wada, 1989). This is an important finding because it demonstrates that the use of ammonium oxalate not only enables environmental DNA to be extracted from allophanic soils but also offers a potential approach for differentiating between (1) ancient extracellular DNA chemically bound to the matrices and (2) modern or near-modern intracellular DNA.

The application of ammonium oxalate extraction on natural allophane soils was only successful following the initial extraction with Rai's lysis buffer. The DNA yield obtained upon one-step ammonium oxalate extraction was much lower, or negligible, than that derived in the two-step ammonium oxalate extraction. This outcome indicates that Rai's lysis buffer is required prior to oxalate treatment to enable effective dissolution of natural allophane and hence the release of DNA from allophanic soils. The first extraction using Rai's alkaline lysis buffer appeared to buffer the pH of the soil solution so that the ammonium oxalate extraction (at pH 3) would not severely damage the extracted DNA because the extract obtained after the second step of DNA extraction remained at pH 6. In fact, the DNA yield obtained using Rai's buffer was significantly higher for the natural allophane soils compared with that from the synthetic allophane where DNA yield with Rai's buffer was negligible. This difference may be partially due to the increase in intracellular or unbound DNA present in natural allophanic soil materials compared with the synthetic allophane, which contained only chemically bound DNA. This result further suggests that Rai's lysis buffer targets intracellular, unbound DNA in soils whereas oxalate targets extracellular, chemically bound DNA. Analyses to test this hypothesis are currently in progress.

For reasons noted earlier in section 6.2.1, we used field-moist rather than laboratory-dried soil in applying our extraction method to natural allophanic soils. It is probable that any prior drying of an allophanic soil sample could, in itself, prevent the complete removal of adsorbed DNA from it.

6.5.2. DNA purification

Following ammonium oxalate treatment (either as a single treatment or as step 2 of the two-step extraction), the abundance of co-extracted humics inhibits

the PCR reaction and therefore the removal of such PCR inhibitors is imperative. In comparing various DNA purification methods, we found that gel electrophoresis followed by DNA recovery from the gel was the most efficient method for successful PCR amplification across four gene regions (one bacterial region and three plant regions), even though the final DNA yield was reduced. This result accords with the findings of Harnpicharnchai et al. (2007) and Miao et al. (2014). After gel electrophoresis, we observed that the brownish humics were separated from DNA by migrating faster than the extracted DNA to the positively-charged electrode.

Despite the high DNA loss, gel electrophoresis followed by gel purification enabled the DNA extracted from the buried soil horizon (5Ahb) on Rotoma tephra at the Brett Rd site to be successfully amplified, cloned, and sequenced via Sanger sequencing. From this, DNA of several New Zealand indigenous plants, and an exotic plant, were identified in the 5Ahb horizon (Table 6.5). However, the plants currently growing on the land surface at the Brett Rd site today are largely European grasses (composed mainly of *Poaceae*, *Fabaceae*, *Asteraceae, Ranunculaceae*, *Oxalidaceae*, and *Plantaginaceae*). Therefore, the plant DNA extracted from the buried soil horizon on Rotoma tephra may have originated from previous vegetation growing at the site.

Other DNA purification methods used in this study were less efficient at removing humics and, as a result, PCR amplification was largely unsuccessful. Chloroform, used in the purification of the extracts from the soil horizons on Taupo, Whakatane and Rotoma tephras, forces a separation of the organic and aqueous phases. DNA is soluble in the aqueous phase because the negatively-charged DNA backbone makes the molecules chemically polar. Conversely, proteins and the hydrophobic humics remain in the organic phase (chloroform) of two immiscible liquids. The chloroform treatment, however, was not able to eliminate hydrophilic humics in solution or humics bound to DNA, and hence PCR of the treated DNA remained inhibited and PCR amplification was thus unsuccessful. The humics-removal undertaken before the two-step DNA extraction increased the DNA yield from the soil sample on Rotoma tephra; however, PCR amplification of all five gene regions failed. This result suggests that the removal of co-extracted humics prior to DNA extraction may improve DNA recovery from allophanic soils, but further purification post-extraction is

still required. The use of two commercial DNA purification kits brought about substantial loss of DNA, and the poor A_{260}/A_{230} readings following purification indicate the presence of impurities which resulted in unsuccessful PCR amplification in three of the five gene regions. The poor purification observed using these commercial kits could be due to simultaneous binding of both humics and DNA to the silica gel/column and co-elution of both into the buffer (Miao et al., 2014).

6.5.3. Potential application of the DNA extraction method

DNA sequencing results from the buried soil horizon (5Ahb) on Holocene-aged Rotoma tephra showed that vegetation dating to a time before that of the present can be accessed using the DNA extraction protocols developed in this study (Table 6.4, Fig. 6.3). Therefore, our method should be useable to access paleoenvironmental DNA (PalEnDNA) that has been protected within such soils, enabling the novel study of past terrestrial environments or paleoclimates using such DNA extracted from successions of allophanic paleosols on tephra deposits of known age. Even though New Zealand's late Quaternary vegetation and past climates have been generally well reconstructed from pollen and plant macrofossils preserved in peats and lake sediments, and from various other climate-environment proxies such as phytoliths and speleothems (e.g. Kondo et al., 1994; Newnham et al., 1995, 1999, 2013; Williams et al., 2010; Barrell et al., 2013), (paleo)environmental DNA preserved in subfossils and sediments is able to provide considerable insightful information about past environmental change not always evident using the classical methods (Jørgensen et al., 2012; Pedersen et al., 2013; Rawlence et al., 2014; Birks and Birks, 2015).

Our new extraction and purification method thus potentially adds an important new PalEnDNA-based tool for studying climatic and environmental change through paleopedology using andic soils and buried paleosols in extensive volcanic terrains. The effectiveness of such studies is enhanced because tephra layers provide a powerful means – tephrochronology – both for dating the soils and paleosols and for connecting them chronostratigraphically to other paleoenvironmental sites (e.g. Sanborne et al., 2006; Lowe, 2011; Barrell et al., 2013).

6.6. Conclusions

(1) We have developed a new two-step DNA isolation method for allophanic soils (including Andisols) and buried paleosols that successfully bypasses previous difficulties in extracting DNA from such materials relating to the strong chemical bonding between DNA and allophane and organic matter. The method centres around (1) chelating DNA and blocking adsorptive sites on allophanic materials using EDTA and phosphate, respectively, and (2) the novel application of acidified ammonium oxalate (Tamm's reagent) to dissolve allophane to release bound DNA. DNA yields up to 44.5 $\mu g\ g^{-1}$ (ODS basis) were obtained from three field-moist natural allophanic soil samples (i.e. samples were not dried prior to extraction) in northern New Zealand using this two-step method.

(2) We evaluated different DNA purification methods both before and after DNA extraction. Gel electrophoresis followed by gel purification of the DNA, despite DNA loss, was the only purification method that sufficiently removed humics for successful PCR of multiple gene regions.

(3) Sequencing of PCR products obtained from a buried allophanic paleosol at 2.2-m depth on a Holocene tephra in northern New Zealand yielded 1.9 $\mu g\ g^{-1}$ soil (ODS basis) of amplifiable DNA comprising endemic and exotic plants that differed from the European grasses growing currently at the land surface.

(4) The DNA extraction and purification protocols we have developed offer a means to extract DNA and successfully amplify multiple gene regions from allophane-rich soils and paleosols to study past environments or climates in volcanic terrains where such soils are widespread and potentially datable using tephrochronology and other geochronological methods. Further, the method potentially provides a way of studying biodiversity in such soils/paleosols as well as having possible application in forensic soil analysis.

(5) The use of acidified ammonium oxalate, in conjunction with Rai's lysis buffer, could be the key for releasing adsorbed DNA chemically bound to allophane-rich soils enabling detailed pictures of past environments and biodiversity changes through time to be obtained. However, to further support the hypothesis of intracellular versus extracellular DNA isolation using Rai's alkaline lysis buffer and ammonium oxalate, additional investigations using high-

throughput sequencing (HTS) are required to determine the DNA quality and taxonomic profiles recovered in the different DNA isolation steps.

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Chapter 7 Synthesis and Conclusions

7.1. Introduction and review of thesis objectives

Volcanic ash-derived Andisols cover only about 0.84% of the ice-free surface of the world (Soil Survey Staff, 1999; McDaniel et al., 2012) but sequester upwards of ~1.8% of soil organic carbon (Eswaran et al., 1993; Dahlgren et al., 2004; Matus et al., 2014). In New Zealand, ~3.2 million hectares of Andisols are located in North Island, largely supporting agriculture or forestry (Lowe and Palmer, 2005). The North Island's Andisols are dominated by the nanomineral, allophane, which confers adsorptive properties (Allbrook, 1985) so that soil organic matter (SOM) is strongly adsorbed and the decomposition rate of soil organic carbon (SOC) is slowed (Parfitt and Henmi, 1980; Parfitt, 1990, 2009). Baisden et al. (2011) showed nearly a doubling of soil carbon residence time (SCRT) and a doubling of soil carbon stock in comparing Andisols (~17 years SCRT) with non-Andisols (~9 years SCRT).

Active rhyolitic and andesitic volcanism in central and western North Island has added numerous tephra deposits to the land surface so that soil formation has been dominated by upbuilding pedogenesis, forming multi-layered (multisequal) or composite Andisols (Lowe and Palmer, 2005; Neall, 2006; Lowe and Tonkin, 2010). The process of upbuilding pedogenesis leads to the occurrence of organic carbon in lower parts of pedons that has become isolated from most (topdown-dominated) surface processes as the land-surface rose, and therefore carbon is stored deeply in allophanic soils (McDaniel et al., 2012). The allophanic paleosols formed on well-dated Holocene tephras in northern New Zealand sequester much organic matter (shown as relatively high total carbon contents in Table 4.1) and may also act to sequester environmental DNA from the past environments. The main aim of this thesis was to establish a scientific basis for (1) the potential of allophanic soils and Andisols to preserve organic materials including DNA over a long period of time, and (2) the potential of palaeoenvironmental DNA (PalEnDNA) (if it exists) in paleosols on Holocene tephras to reveal past terrestrial environments in northern New Zealand (i.e., using a new method).

The aim has been addressed mainly using an experimental approach and the four objectives involving studies of SOM and PalEnDNA in Andisols/allophanic soils as summarised in Chapter 1 (section 1.2) were:

(1) To test the ability of synchrotron radiation-based techniques to characterise the preserved SOM in allophanic soils and to interpret the interactions between allophane and DNA in an allophanic soil.

(2) To characterise the SOM in paleosols on Holocene tephras of different ages and origins to investigate the alteration of SOM over time and to determine which carbonaceous functional groups are persistent in allophanic soils, and to explain why.

(3) To examine the adsorption capacity and adsorption mechanisms of salmon-sperm DNA with respect to synthetic allophane and to evaluate the implications for DNA storage and carbon stabilisation in allophanic soils and Andisols.

(4) To develop a technique to effectively extract DNA (including intercellular and extracellular DNA) from allophanic paleosols and to assess the taxonomic diversity of plants recorded in DNA extracted from a c. 4000-year-old paleosol formed on Rotoma tephra (deposited c. 9400 cal years BP) at a site near Mt Tarawera in central North Island.

7.2. Summary of conclusions

Detailed conclusions are embedded in each of Chapters 3 to 6. The main finding from these studies are summarised below.

7.2.1. The use of synchrotron radiation to study carbon storage and DNA adsorption in allophanic soil material (Chapter 3)

The extracted organo-clays from two allophanic horizons (20 and 30 cm in depth) at a site at Tapapa (where the Andisol is a representative of the Tirau series) were directly analysed using C NEXAFS spectroscopy without any chemical treatment. The spectra of the organo-clay showed that the SOM sequestered by the clays extracted from two horizons were similar and comprised quinonic, aromatic, aliphatic, and carboxylic carbon (in order of their X-ray absorption

energies). The presence of organic matter in natural allophanic soils reduced adsorption of DNA on clay, and humic-acid-free synthetic allophane adsorbed seven times more DNA than humic-acid-rich synthetic allophane. The P XANES spectroscopy also demonstrated that the clays adsorbed the additional salmon-sperm DNA through its phosphate groups; however, it was unclear if the latter were bound to the surface of allophane spherules or to organic matter, or both.

7.2.2. Characteristics and preservation of SOM in allophanic paleosols on Holocene tephras in northern New Zealand (Chapter 4)

Allophanic paleosols from multisequal soils on Holocene tephra sequences (aged from c. 12,000 to 1718 cal years BP) at four sites were sampled for analysis. Two of the soils were formed mainly by developmental upbuilding pedogenesis (soils at Tapapa, mainly rhyolitic; and soils at Lake Rotoaira, mainly andesitic) and two were formed mainly by retardant upbuilding pedogenesis (soils at Brett Road and Ashton Dairies Pit, both mainly rhyolitic). The C NEXAFS spectra for organo-clays extracted from the allophanic paleosols showed that very similar carbon functional groups of SOM were preserved at all sites and at all depths. The SOM was dominated by carboxylic functional groups, with subordinate amounts of quinonic, aromatic, and aliphatic groups. Differences in clay and allophane contents, stratigraphic position (depth), age, parent-tephra composition (andesitic versus rhyolitic), current vegetation, and mode of soil origin (retardant versus developmental upbuilding pedogenesis) seemed not to have affected the characteristics of SOM in the different paleosols. The similarity, and thus origins and preservation, of the SOM can be explained by a model of upbuilding pedogenesis: as soil genesis begins in a newly-deposited tephra at the soil surface, allophane forms and it sequesters SOM; ongoing tephra deposition then causes the land surface to rise and the sequestered SOM is gradually buried more deeply in the sequence, becoming divorced from the modern (surficial) organic cycle. The presence of the quinonic carbon in the allophanic paleosols, normally easily degraded including by exposure to light, indicates that it and the buried SOM (derived from when the tephra was at the land surface) can be at least partially isolated from oxygen, light, microbes, and enzymes in allophanic soils and hence preserved, in turn suggesting that genetic material such as DNA might also be protected. Previous work, and a 2D TXM microtomograph with a 3D virtual

tomographic reconstruction of a natural allophanic aggregate obtained in my study, suggested that a fractal pore network of allophane-dominant aggregates had encapsulated and shielded the quinonic carbon and relict SOM from degradation.

7.2.3. DNA adsorption capacity and adsorption mechanisms on allophane, and implications for carbon stabilisation (Chapter 5)

Synthetic allophane adsorbed up to 34 μg g^{-1} of salmon-sperm DNA; however, the presence of humic acid significantly lowered the DNA uptake on the synthetic allophane from 34 to 3.5 μg g^{-1} by occupying the active sorption sites of allophane. The mechanisms of DNA adsorption on nanocrystalline allophane spherules and allophane nanoaggregates were: (1) direct chemical adsorption of DNA on allophane through the phosphate groups of a DNA molecule, (2) indirect chemical adsorption of DNA on a covering of organic matter on allophane, and (3) physical adsorption of DNA in the interstices (nanopores) of allophane nanoaggregates. Approximately 80% of the 34 μg g^{-1} DNA was adsorbed/protected physically within the nanopores, and only 20% of the total was adsorbed chemically on the aluminol groups of allophane spherules. The DNA within the nanopores may not be accessible to enzymes, microbes, or oxidants because the allophane aggregates are stable and their pore networks have at least a moderately high degree of tortuosity. The findings relating to DNA adsorption mechanisms on allophane very likely apply to SOM stabilisation in allophanic soils. The highly porous allophane nano- and microaggregates potentially allow considerable SOM and SOC (up to ~80%) to be adsorbed physically, and the carbon is effectively encapsulated and protected within a nanolabyrinthic network of nanopores, thereby partially explaining the especially slow carbon turnover in allophanic soils and Andisols.

7.2.4. Extraction of environmental DNA from allophanic paleosols and assessment of taxonomic diversity of plant DNA in a buried allophanic paleosol (Chapter 6)

The potential assessment of taxonomic diversity of plant DNA in allophanic paleosols relied on (1) the development of an effective method for DNA extraction from adsorptive allophanic soils, and (2) a successful purification

technique to clean up the extracted DNA for PCR amplification and sequencing. Based in part on preliminary experiments using synthetic allophane and salmon-sperm DNA, a novel two-step method was developed for DNA extraction from allophane-rich soils. The method is based on (1) chelating DNA and blocking adsorptive sites of allophanic soils using a lysis buffer containing mainly EDTA and phosphate, and (2) dissolving allophane using acidified ammonium oxalate. DNA yields up to 44.5 μg g^{-1} soil (oven-dry basis) were obtained from three natural (field-moist) allophanic samples from Holocene paleosols on Taupo, Whakatane, and Rotoma tephras in northern New Zealand using the two-step DNA extraction method. In comparison to commercial DNA purification kits, gel electrophoresis followed by gel purification of the DNA was the most effective technique that enabled successful PCR of multiple gene regions of bacteria (16s rDNA) and plants (rbcL and *trn*L). The sequencing of the purified DNA extracted from a buried allophane paleosol at 2.2 m-depth on Rotoma tephra deposited c. 9400 cal years BP at a site near Mt Tarawera in northern New Zealand showed endemic (*Leptospermum scoparium*, *Griselinia lucida*, *Hydrocotyle*, and *Podocarpus*) and exotic (*Eucalyptus*) plants that differed from the European grasses growing currently on the land surface.

7.3. Linking results of soil organic matter and DNA in relation to allophane

The similar characteristics of SOM in allophanic paleosols of different ages over four sites in northern New Zealand, and especially the presence of distinctive quinonic carbon, both demonstrate that allophanic soils have an aptitude for SOM preservation. This finding was in line with other studies that showed that rates of decomposition of SOC were slower in Andisols than in non-Andisols (Parfitt et al., 2002; Baisden et al., 2010, 2011), and that there was a decrease in the bioavailability of SOM in fractal allophane aggregates (Chevallier et al., 2010). The quinonic carbon represents not only the labile carbon (Green et al., 1960; Dunphy and Brodie, 1971) but also the material that comes from living organisms (Nowicka and Kruk, 2010), hence an implication of its preservation is the possibility of preservation of genetic materials (e.g. DNA) in allophanic soils (which formed an underlying hypothesis of this project).

In my thesis, the research undertaken on DNA adsorption mechanisms on allophane spherules and nanoaggregates has helped to explain the stabilisation of carbon in allophanic soils and buried andic paleosols. The chemical adsorption of DNA (and flocculants or organic matter in soils) brings about the aggregation of allophane spherules and nanoaggregates, and the porous, large aggregates (micro- and macroaggregates) so formed provide enormous amounts of space (but of nanoscale dimensions and with a high degree of tortuosity) for the physical adsorption of DNA and SOM. Similar experimental findings obtained from non-allophanic soils were demonstrated by Six et al. (2000): the organic binding agents in soils result in the aggregation of microaggregates (<250 μm in diameter) and the formation of macroaggregates (>250 μm in diameter), and the macroaggregates sequester ~1.65 times more carbon than microaggregates. Thus, the formation of organo-mineral complexes, and their fractal shape, are the most important mechanisms of carbon stabilisation storage in Andisols (this study; see also Panichini et al., 2012). Moreover, because physical adsorption is the driving mechanism of SOM storage in allophanic soils (by implication from the findings of DNA adsorption on allophane presented in Chapter 5), there should be no specific types of 'persistent' carbon in such materials, which explains the similar characteristics of SOM in allophanic paleosols of different ages (i.e., old SOM does not possess specific types of functional groups) (see Chapter 4). With the growth of an allophane aggregate, the adsorbed SOM and DNA become enclosed, and the continuous accumulation of SOM and DNA in the outer region of an aggregate encapsulates the SOM and DNA inside the aggregate, forming a 'fort' against attacks from microbes and enzymes in soils, and presumably reducing photodegradation by blocking light. As a result, the adsorbed SOM and DNA are protected from degradation, and they remain isolated from modern contamination such as leachates which have little capacity to enter the stable aggregates and these leachates remain vulnerable to decomposition by microbes.

At the Brett Rd site near Mt Tarawera, the current surface vegetation is comprised of European grasses, the dominant pastoral vegetation in the area was established decades ago, and no trees have been at the site since the early 1980s at least (see Appendix B). However, the plant DNA fragments extracted from a 2.2 m-depth buried soil horizon at that site were identified as New Zealand native trees and exotic *Eucalyptus* (introduced during European settlement from around 1860 AD), but no European grasses were recorded (Chapter 6). These differences

between the current surface vegetation and that identified from the DNA extracted from the buried soil horizon (which was at the land surface between c. 9400 and c. 5500 cal years BP) on Rotoma tephra supports the concept that SOM and DNA originating from past environments can be preserved in allophanic soils, even though it is not clear yet how old the DNA extracted from the soil was, and for how long (a period of time) DNA can be preserved in such materials. The absence of exotic grass-derived DNA in the buried horizon indicates that either (1) the invasion of plant roots through the soil has had little or no impact on the biological diversity in lower horizons of allophanic soils, or (2) the recent DNA moving downwards was not protected in buried horizons because the protective sites in aggregates have been occupied by older SOM and thus there is no or limited protection for newly-added DNA and SOM, or both (1) and (2). In addition, the modern carbon input into allophanic soils under European grasses may occur mainly at the soil/land surface (Parfitt et al., 2002) and may be easily degraded in deeper horizons.

One of my experiments (Chapter 5) also suggested that humic acid occupied active sites on synthetic allophane and so reduced DNA adsorption capacity of synthetic allophane. This result demonstrated that the adsorption capacity of allophane was not unlimited and indicated the adsorption ability of allophane in soils reduces when it becomes saturated with organic matter. In turn, the findings are in line with the concept of carbon saturation deficit of soils (the potential of soils to store additional soil carbon) (Beare et al., 2014) in that each soil has a maximum soil carbon storage capacity. Therefore, the adsorption of environmental DNA is influenced by the presence of SOM, so that SOM in allophanic soils hampered DNA adsorption and protection, probably leading to difficulties in the search for possible PalEnDNA in allophanic soils. These difficulties contrast with the positive findings regarding the potential preservation of environmental DNA inferred from the presence of protected quinonic carbon (see section 7.2.2. and Chapter 4).

7.4. Value of studying allophanic paleosols and possible future work

My experiments have confirmed that SOM and DNA in buried allophanic soils can be isolated and protected by (1) ongoing tephra deposition that increasingly divorces the buried horizons from the modern organic and biological

cycle over time, and (2) encapsulation and occlusion (and thus physical protection) of SOM and DNA within the allophane aggregates in soils. Therefore, allophanic paleosols in New Zealand and elsewhere are valuable repositories of SOM and potentially environmental DNA, and the buried allophanic soils developed on sequences of precisely-dated tephras could provide a valuable paleobiological laboratory for ancient DNA analysis of past populations and environments. Comparisons with findings derived using other methods for paleoenvironmental reconstructions, including phytolith studies and palynology (e.g., Kondo et al., 1994; Carter, 2007; Wilmshurst et al., 2014), would be enhanced using tephrochronology.

More experiments could be undertaken to test the degree of preservation of genetic materials in allophane aggregates and in allophanic soils, and to examine the rate of vertical (and potentially lateral) migration of DNA in allophanic soils to identify or rule out possible contamination. Possible future studies could involve examining DNA (by extracting DNA and purifying it using the method developed in chapter 6 of this thesis) in a vertical succession of paleosols formed on distinct tephras up to ~30,000 years old to assess the changes in taxonomic diversity and composition of vegetation and fauna recorded since the last glacial maximum (LGM) ~25,000 years ago through the last glacial-interglacial transition (LGIT) to the Holocene and the present (e.g. see Newnham et al., 2007, 2013; Barrell et al., 2013). High throughput sequencing (HTS) will be required to properly determine the taxonomic profiles, however.

Identifying the first occurrence and extent of human impacts since the arrival ~730 years ago of Polynesians in New Zealand (c. 1280 AD), and of later European arrivals in the 1800s, is another potential application (especially involving the Kaharoa tephra, a key marker for early Polynesian settlement) (Hogg et al., 2003; Wilmshurst et al., 2008, 2011; Lowe and Pittari, 2014; Anderson, 2015). Such studies could also help inform the current debate about the magnitude and timing (start) of the so-called Anthropocene epoch (e.g., Bostock et al., 2015; Steffen et al., 2015; Waters et al., 2016).

A challenging project would be to extract DNA from a range of dated paleosols on tephras and to attempt date the DNA using an AMS-based radiocarbon approach (Spalding et al., 2005). The ^{14}C ages acquired could then be compared with those of the paleosols that are known from tephrochronology.

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Appendix A Sample preparation for analysis via synchrotron radiation; sampling for PalEnDNA

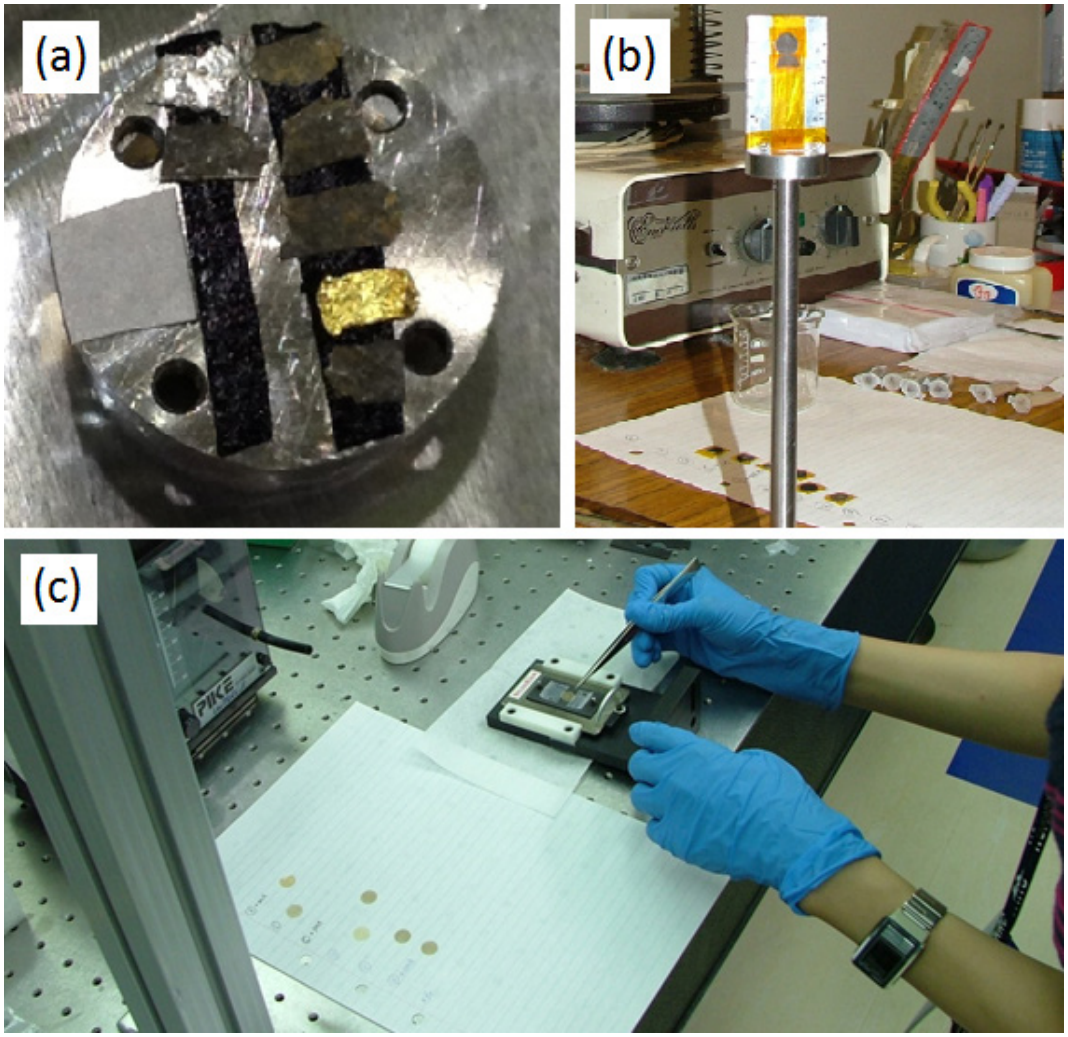

Fig. A1 Sample preparation for analysis using synchrotron radiation: (a) organo-clay samples extracted from New Zealand paleosols were pressed into indium foil (see section 4.2 for details) for C NEXAFS spectroscopy, together with pure gold and indium foil as references, (b) the samples for P XANES were compressed into pellets to increase the intensity of X-ray absorbance, and the pellets were attached to a stainless steel holder in place with Kapton tape which has no X-ray absorbance over the P X-ray absorption region, and (c) pellets of samples that were mixed with potassium bromide were ready for synchrotron radiation-based FTIR spectroscopy.

Fig. A2 Sampling for PalEnDNA in paleosols on tephras: (1) soil profile should be excised laterally to remove surface contamination, (2) tools for sampling must be sterile, and (3) protective gear, including gloves, disposable body suit and face mask are required to avoid any extraneous DNA from the sampler. Photo: D.J. Lowe.

Fig. A3 Prof David Lowe documenting site stratigraphy before sampling.

Fig. A4 An array of chemicals/solutions, including water, detergent, ethanol, and disinfectant, used to wash sampling tools to ensure they remained sterile for each use.

Fig. A5 Undertaking sterile fieldwork (photo shows Dr Nic Rawlence) in PalEnDNA research at the Brett Rd site. The Holocene tephra–paleosol section was cleaned back nearly 1 m to expose a fresh face. Sampling was undertaken using sterile equipment from the base upwards to avoid contamination of older samples with younger material (from Rawlence et al., 2014, p. 619).

Appendix B Brief history of vegetation cover and land-use at Brett Road near Lake Rerewhakaaitu

(Compiled by David J. Lowe)

The advent of Polynesians in New Zealand/Aotearoa from c. 1280 AD (e.g. Newnham et al., 1998; Hogg et al., 2003; Wilmshurst et al., 2008, 2011; Anderson, 2015) was followed by anthropogenic burning that rapidly eliminated substantial portions of the forest in the Bay of Plenty and other regions, as described by, for example, Colenso (1894). Such forest was replaced by *Pteridium*-dominated fernland, grassland, and scrubland maintained by repeated burning (McGlone and Wilmshurst, 1999; McGlone et al., 2005; Perry et al., 2012).

The eruption of nearby Mt Tarawera in 1314 ± 12 AD (the Kaharoa eruption) a few decades after Polynesian arrival (Lowe and Pittari, 2014) would have resulted in widespread vegetation destruction and burial in the Lake Rerewhakaaitu area. Early European explorers in the mid-1800s in this area reported mainly scrubland with small patches of forest (Griffiths and Schuler, 1993), and very low intensity sheep grazing reportedly occurred in the area west of Brett Road, which follows the western shoreline of Lake Rerewhakaaitu, from c. 1860 AD until the eruption of Mt Tarawera on 10 June, 1886 (the Tarawera eruption). Fallout of the Tarawera eruptives buried the land surface and destroyed most vegetation proximal to the volcano (including at Brett Rd).

Sparse European settlement then saw the development of widespread exotic grassland west of Brett Road/Lake Rerewhakaaitu by the start of the 20th Century. The areas north and east of Lake Rerewhakaaitu remained as scrubland (mixed endemic and exotic plants) from European times, and were used for military training in the 1920s–1930s (New Zealand artillery units) and intensified in the 1940s (including by New Zealand Tank Brigade and three US Army infantry divisions) (Mossop, 1993), followed by an influx of pastoral farmers from 1953 onwards (Griffiths and Schuler, 1993).

The current surface vegetation at the Brett Rd site is comprised of European grasses, including *Poaceae*, *Fabaceae*, *Asteraceae*, *Ranunculaceae*, *Oxalidaceae*, and *Plantaginaceae.* Such roadside grasses, possibly with exotic broom, have been at the site since at least the early 1980s according to local farmer Mac Pacey (pers. comm., November 2014).

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Appendix C Evaluating the character and preservation of DNA within allophane clusters in buried soils on Holocene tephras, northern New Zealand (refereed conference paper)

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Evaluating the character and preservation of DNA within allophane clusters in buried soils on Holocene tephras, northern New Zealand

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Introduction

Clay minerals possess sorptive capacities for organic and inorganic matter, including DNA (Lorenz and Wackernagel, 1994), and hence reduce the utilization and degradation of organic matter or DNA by microorganisms. Buried allophane-rich soils on tephras (volcanic-ash beds) on the North Island, dated using tephrochronology, provide a valuable paleobiological 'laboratory' for studying the preservation of ancient DNA (aDNA) (Haile et al., 2007). Allophane comprises Al-rich nanocrystalline spherules ~3.5–5 nm in diameter (Fig. 1) with extremely large surface areas (up to 1000 m^2 g^{-1}). Moreover, allophanic soils are strongly associated with organic matter (Parfitt, 2009), and so we hypothesize that allophane also plays an important role for DNA protection within such soils.

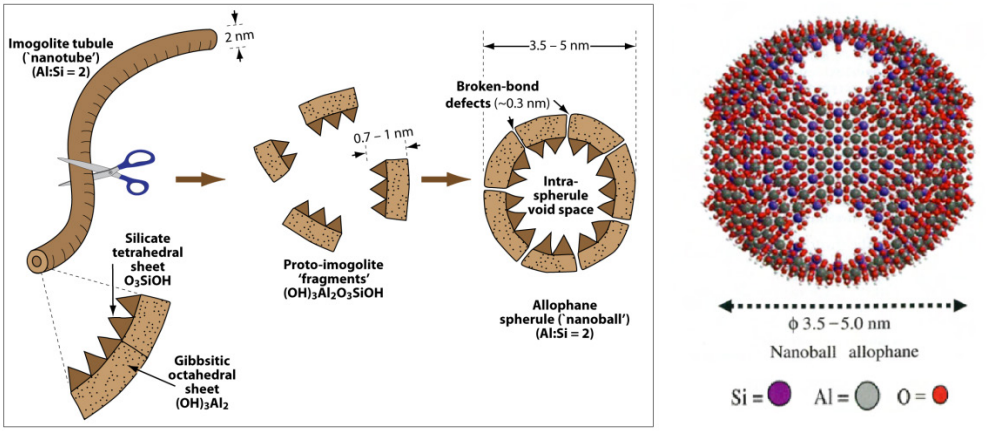

Fig. 1. Nanoscale dimensions and composition of imogolite tubules and allophane spherules (from McDaniel et al., 2012), and (at right) the atomic structure of allophane (from Theng and Yuan, 2008).

Greaves and Wilson (1970) suggested that RNA adsorbed in the central zones of montmorillonites may undergo less attack by microbial enzymes, but the RNA sorbed on surfaces of individual montmorillonite particles is not protected. Their results catalysed our research interest that DNA may be physically protected and preserved in the interspaces between spherules of allophane clustered together as microaggregates as well as being chemically sorbed on the surfaces of allophane spherules. Kahle et al. (2003) showed that coarse and fine clay fractions of illitic soils had different capacities for carbon storage, supporting the hypothesis that allophane clusters of different sizes have various capacities to hold DNA. DNA adsorption isotherms on synthetic and natural allophanes have been completed recently (Saeki et al., 2010), but no one has investigated the influence of allophane cluster

size on DNA adsorption and the exact mechanisms provided by such clusters or microaggregates for adsorption of organic matter.

DNA-clay interaction and analysis

The DNA molecule comprises heterocyclic bases and pentose-phopho-diester backbone (Fig. 2). The distribution, content and structure of DNA in cells have been determined using near-edge X-ray absorption fine structure (NEXAFS) (Zhang et al., 1996; Fujii et al., 2003). Other researchers have also investigated the interactions of DNA with clay minerals, including montmorillonite and kaolinite and other soil colloidal particles using Fourier transform infrared (FTIR) spectroscopy (Cai et al., 2006). Using synchrotron-based NEXAFS, we should be able to determine the electron configuration of the specific atoms (C, N, and P) comprising DNA. To ascertain the real interaction between allophane clusters and DNA (and organic carbon), synchrotron-based techniques provide the ideal approach to clarify the geochemistry of DNA as it occurs in allophanic soils.

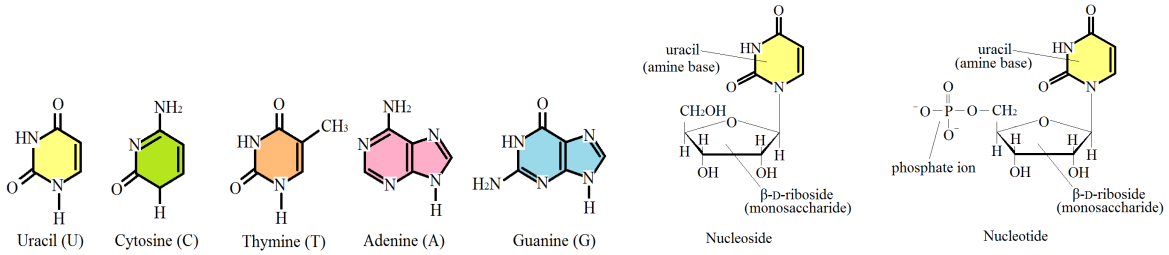

Fig. 2. Five heterocyclic aromatic amine bases most common to nucleic acids and the basic structures of DNA fragments (modified after Brown and Poon, 2005).

Objectives and experimental design

We want to know why and how allophane is able to 'hold' and protect DNA from degradation. The answers are of benefit not only in explaining the development of genetic preservation but also in providing a better understanding of the role of allophanic soils in carbon sequestration. Two objectives of this study are as follows:

1. To evaluate the sorptive capacities for DNA fragments of clusters of allophane (microaggregates) of different sizes and hence to evaluate the physical and chemical adsorption/protection provided by allophane clusters to hold DNA fragments in allophanic soil systems.
2. To determine the fingerprint of DNA bound to allophane clusters using synchrotron-based NEXAFS and to identify ancient DNA within buried soils.

Four sets of allophanic soils from the North Island are to be analysed in this study, three from tephra-derived buried horizons (on rhyolitic Taupo, Whakatane, and Rotoma tephras, deposited c. 1780, 5500, and 9500 cal years ago, respectively) and one from the upper subsoil horizon of the Tirau soil, a Typic Hapludand derived from incremental accumulations of thin, mainly rhyolitic, tephras (Lowe and Palmer, 2005). Clay-size fractions from each sample will be subjected to four physical and chemical

dispersion treatments (hand-inversion, prolonged shaking, zirconium nitrate addition, and pH alteration) to generate allophane clusters/microaggregates with a range of sizes. These allophane clusters will be characterized by measuring their specific surface areas and pore size distributions using nitrogen gas adsorption and laser-sizer analysis. Modern DNA (e.g., salmon-sperm DNA) (Saeki et al., 2010) will be mixed with the allophane cluster fractions of different sizes to determine their DNA sorption capacity. The 'moist' DNA-allophane complexes will be observed using environmental scanning electron microscopy (SEM), because we want to prevent DNA-allophane complexes from transformation during drying. However, dried samples are required for high-vacuum transmission electron microscopy (TEM), and hence air-dried samples without interference will be prepared for TEM analysis. NEXAFS analysis is to be used to examine the air-dried DNA-allophane complexes to obtain the near edge fine structures of C (energy range: 280−300 eV), N (395−425 eV) and P (2140−2180 eV) (1s) transitions comprising the DNA molecules and their surrounding atoms. The X-ray absorption spectra of C, N, and P could be used to enable 'fingerprints' of DNA within allophane clusters, or of DNA fragments in allophanic soil materials, to be recognised. Moreover, speciation of C and N within allophane clusters could be usefully illustrated.

Expected DNA adsorption isotherms and outcomes

We hypothesize that the allophane clusters of different sizes possess differing capacities for DNA adsorption, and that the larger allophane clusters would have more adsorptive sites for DNA (Fig. 3). The small allophane clusters have higher specific surface areas and a higher affinity for DNA adsorption because of chemisorption, and so DNA fragments are easily sorbed on small allophane clusters. Consequently, the adsorptive trend flattens out when available adsorptive sites are saturated (Fig. 3). On the other hand, the large allophane clusters, possessing lower surface areas, have a moderate affinity for DNA as evident in the first part of the adsorption isotherm; nevertheless, large allophane clusters adsorb DNA eventually and have higher sorbed capacity (Fig. 3). We hypothesize that DNA fragments are slowly sorbed into the interspaces (gaps) of the allophane clusters (where they end up being physically 'protected').

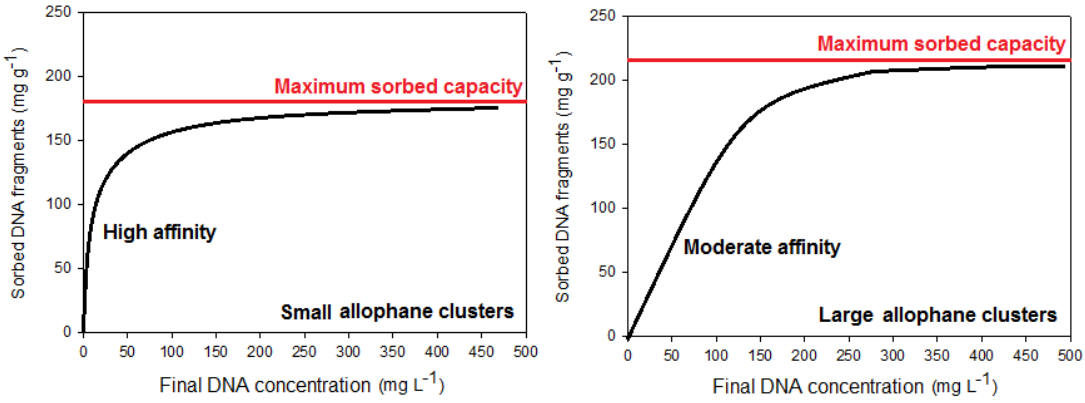

Fig. 3. Predicted DNA adsorption isotherms on small and large allophane clusters (based on findings in Saeki et al., 2010)

Thus, we aim to test this hypothesis to find out if allophane holds DNA (or organic matter) through this 'physical habitation' model – i.e., amidst the interspaces of the allophane clusters – as well as through chemisorption that arises as a consequence of the very large surface area and cation exchange capacity of allophane spherules. It is anticipated that DNA fragments existing in allophanic soils have specific functional groups, and the structures of allophane and the DNA-allophane complex are thus somewhat different. The functional groups and structures of carbonic compounds are able to be identified according to the excited energy (absorption edge) of carbon (Fig. 4). We thus aim to use these differences in functional groups to determine the existence of ancient DNA within buried allophanic soils and to ascertain the DNA-protective ability of such soils.

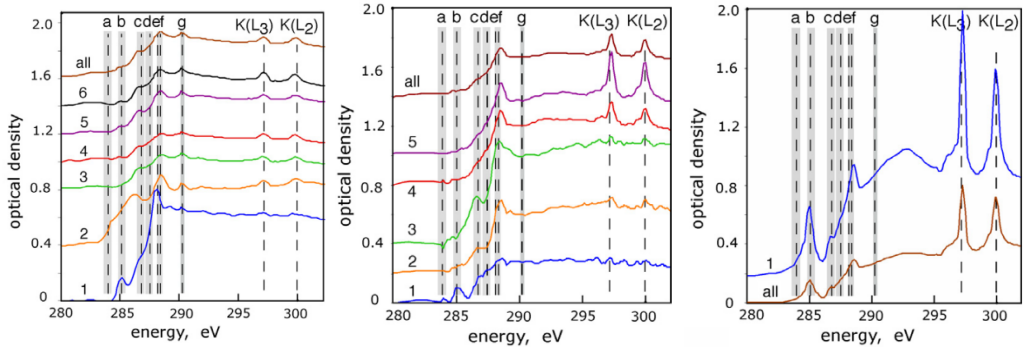

Fig. 4. Carbon NEXAFS spectra obtained within selected areas of microaggregates: (a) quinonic, (b) aromatic, (c) phenolic, (d) aliphatic, (e) peptidic, (f) carboxylic, (g) carbonate/carbonyl functional groups (Wan et al., 2007)

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Appendix D Using palaeoenvironmental DNA to reconstruct past environments: progress and prospects (invited review paper)

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Review

Using palaeoenvironmental DNA to reconstruct past environments: progress and prospects

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ABSTRACT: Palaeoenvironmental DNA (PalEnDNA) is defined as ancient DNA (aDNA) originating from disseminated genetic material within palaeoenvironmental samples. Sources of PalEnDNA include marine and lake sediments, peat, loess, till, ice, permafrost, palaeosols, coprolites, preserved gut contents, dental calculus, tephras, and soils as well as deposits in caves/rockshelters and at archaeological sites. PalEnDNA analysis provides a relatively new tool for Quaternary and archaeological sciences and its applications have included palaeoenvironmental and palaeodietary reconstructions, testing hypotheses regarding megafaunal extinctions, human–environment interactions, taxonomic studies, and studies of DNA damage. Because PalEnDNA samples comprise markedly different materials, and represent wide-ranging depositional and taphonomic contexts, various issues must be addressed to achieve robust, reproducible findings. Such issues include climatic and temporal limitations, the biological origin and state (free versus bound) of PalEnDNA, stratigraphic reliability, sterile sampling, ability to distinguish modern from aDNA signals, DNA damage and PCR amplification, DNA extraction methods, and taxonomic resolution. In this review, we provide a non-specialist introduction to the use of PalEnDNA for Quaternary and archaeological researchers, assess attributes and limitations of this palaeoenvironmental tool, and discuss future prospects of using PalEnDNA to reconstruct past environments. Copyright © 2014 The Authors. *Journal of Quaternary Science* published by John Wiley & Sons Ltd on behalf of Quaternary Research Association

KEYWORDS: ancient DNA; archaeology; environmental DNA; palaeoecology; palaeoenvironmental DNA; PalEnDNA; Quaternary.

Introduction

The use of ancient DNA (aDNA) is becoming an increasingly powerful tool for Quaternary science and archaeology. Since the advent of aDNA-based research a quarter of a century ago (Higuchi *et al.*, 1984; Pääbo, 1985), it has been used to investigate wide-ranging topics, including biogeography (e.g. Mitchell *et al.*, 2014), ecosystem responses to climate change (such as the impact of Holocene climate change on faunal biodiversity: de Bruyn *et al.*, 2011), anthropogenic impact and extinction processes (e.g. Shapiro *et al.*, 2004; Drummond *et al.*, 2005), palaeoenvironments (e.g. Willerslev *et al.*, 2014), human health and disease (e.g. Adler *et al.*, 2013; Krüttli *et al.*, 2014; Metcalf *et al.*, 2014), and human evolution and palaeogenomics (e.g. Green *et al.*, 2010; Meyer *et al.*, 2012; Prüfer *et al.*, 2014). Some of these studies have used discrete materials, such as bone, teeth, leaves and eggshell (Table 1), but aDNA has also been extracted as disseminated genetic material from coprolites, preserved gut contents, dental calculus, sediments (including ice) and soils (Table 2). We term this disseminated genetic material 'palaeoenvironmental DNA' (PalEnDNA). PalEnDNA is becoming more commonly used in attempts to reconstruct past environments (Table 2). However, the first well-known PalEnDNA analysis was only published in 1998 (Poinar *et al.*, 1998) and many issues remain to be addressed to ensure sound and reliable reconstructions of past environments. Most important are the questions relating to the mixed origin(s) of DNA and its state (free versus bound, intracellular versus extracellular) and how these affect DNA extraction efficiency, stratigraphic reliability and the degradation of DNA.

The issues involved with the analysis and interpretation of PalEnDNA are somewhat analogous to those encountered during the early years of radiocarbon (^{14}C) dating and aDNA research. In both these disciplines, methodologies were in their infancy, and replication and self-testing were initially not carried out or made a priority. These deficiencies resulted in the publication of many high-profile claims regarding aDNA that could not be replicated by an independent laboratory. In one such case, Woodward *et al.* (1994) extracted and amplified short (up to 170 bp) fragments of DNA from 80 Ma dinosaur bones. Despite difficulties in identifying a phylogenetic match, Woodward *et al.* (1994) hypothesized the presence of degraded dinosaur DNA. Subsequently, Zischler *et al.* (1995), among others, showed that the putative dinosaur DNA sequences matched human DNA sequences (i.e. human contamination).

In this review, we aim to provide a broad overview of the use of PalEnDNA in Quaternary and archaeological research, to assess attributes and limitations of this palaeoenvironmental

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Table 1. Summary of sample materials used in aDNA research divided into 'discrete' aDNA and 'disseminated' aDNA. The latter forms the basis of most PalEnDNA research.

Specimens	Examples of use
Discrete fossil or subfossil materials	
Bone	Bunce *et al.* (2009)
Skin/muscle	Cooper *et al.* (1992)
Hair	Gilbert *et al.* (2007)
Feathers	Rawlence *et al.* (2009)
Eggshell	Oskam *et al.* (2010)
Insect cuticles	Thomsen *et al.* (2009)
Teeth	Adler *et al.* (2011)
Plants incl. macrofossils and pollen	Gugerli *et al.* (2005)
Discrete environmental materials	
Coprolites	Poinar *et al.* (1998)
Preserved gut contents	van Geel *et al.* (2008)
Dental calculus	Adler *et al.* (2013)
Disseminated environmental materials	
Sediments (excluding ice)	Willerslev *et al.* (2003)
Ice	Willerslev *et al.* (2007)
Soil	Hebsgaard *et al.* (2009)
Tephra	Haile *et al.* (2007)

tool and to discuss future prospects of using PalEnDNA to reconstruct past environments. To achieve these aims, our review focuses on the following areas:

1. Definitions of aDNA and PalEnDNA
2. Progress in the use of PalEnDNA for Quaternary and archaeological research
3. Climatic and temporal limitations of PalEnDNA-based research
4. Further issues with PalEnDNA-based research
5. Recommendations for future PalEnDNA-based research
6. Prospects for the use of PalEnDNA for palaeoenvironmental reconstruction.

Defining aDNA and PalEnDNA

aDNA is defined as highly degraded, fragmented and chemically modified DNA extracted from historical, archaeological and fossil remains (Fig. 1). Typically, aDNA samples are discrete materials, such as fossil samples of bones, eggshells, feathers or plant remains (e.g. see Gugerli *et al.*, 2005) in contrast to 'environmental' samples (see below). In this definition, aDNA is not defined by age alone but can include archival samples containing degraded DNA. Typically, the size of aDNA fragments is limited to <300 bp, compared with the multiple kilobases standard for modern DNA, unless extraordinary preservation circumstances exist. These circumstances generally feature cold and dry conditions (Orlando *et al.*, 2013) or samples derived from geologically very young materials (e.g. less than around 1000 years old: Rawlence and Cooper, 2013). Most aDNA studies have focused on mitochondrial DNA (mtDNA) for animals, or chloroplast DNA (cDNA) for plants, because these circular genomes exist in multiple copies per cell. In contrast, a single copy nuclear DNA (nDNA) locus has only two copies per cell (inherited from each parent). Consequently, the probability of DNA preservation is generally assumed to be greater for mtDNA and cDNA loci because of their high abundance compared with that of nDNA. As for fragment size, the preservation of large amounts of nDNA is generally limited to situations where highly favourable circumstances exist for preservation (Orlando *et al.*, 2013).

Environmental samples used in aDNA research are those obtained from various sedimentary deposits, including marine and lacustrine sediments, peat, loess, glacial till, ice and frozen materials (permafrost) as well as tephras (loose pyroclastic deposits of explosive volcanic origin including volcanic ash: Lowe, 2011), deposits in caves and rockshelters and archaeological sites, soils, buried soils (palaeosols), coprolites, preserved gut contents and dental calculus (Figs 2 and 3). The optimal materials for sampling and extraction are permafrost (e.g. Lydolph *et al.*, 2005), recently frozen material (e.g. Gould *et al.*, 2010) or desiccated remains (e.g. Wood *et al.*, 2013a, b) mainly because of the exceptional preservation of aDNA provided by such conditions/materials. However, there is potential for many other materials to provide information about the past via aDNA analysis.

The aDNA within environmental samples originates from multiple sources, which can have a significant impact on DNA extraction efficiency (Haile, 2012). The DNA potentially originates from plant material (rootlets, seeds, leaves, pollen), animal waste products (faeces, urine), material that has been shed (skin, hair, feathers, nails) and invertebrate remains (Willerslev *et al.*, 2003; Lydolph *et al.*, 2005; Andersen *et al.*, 2012; Yoccoz *et al.*, 2012). In coprolites, DNA can comprise the defaecator's intestinal biota (e.g. bacteria, parasites), dietary components and environmental sources that arise after deposition (e.g. bacterial and fungal growth) (Goldberg *et al.*, 2009; Jenkins *et al.*, 2012; Tito *et al.*, 2012; Wood *et al.*, 2012a, b, c, 2013a, b).

Environmental samples can be lithologically, mineralogically, chemically and biologically variable, containing disseminated, non-discrete material, such as sediment or soil (rather than samples of bone, for example), and represent a wide range of depositional and taphonomic contexts. We advocate the portmanteau abbreviation 'PalEnDNA' to replace several previous terms used for palaeoenvironmental DNA (Fig. 2). Here, PalEnDNA is defined as aDNA originating from disseminated genetic material within palaeoenvironmental samples, and the analysis of PalEnDNA is an exploratory subdiscipline of broader aDNA studies (Fig. 2). Although technically discrete material in one sense, PalEnDNA from coprolites, preserved gut contents and dental calculus originates ultimately from multiple sources. The term 'environmental DNA' (or eDNA) in the context of isolating aDNA from environmental samples (Hebsgaard *et al.*, 2005; see also Bohmann *et al.*, 2014) is not appropriate because eDNA includes modern DNA. 'Sedimentary ancient DNA' (or sedaDNA) (Haile *et al.*, 2009) is used widely and applies to DNA isolated from sedimentary deposits, but this term technically cannot include samples extracted from tephras (which are volcanic/pyroclastic deposits), soils (which form on a wide range of lithologies in addition to sedimentary deposits) or coprolites. 'Lake sediment DNA' (lake sedDNA) obviously applies only to lake sediments (Giguet-Covex *et al.*, 2014). Similarly, 'dirt DNA' (Willerslev and Cooper, 2005; Hebsgaard *et al.*, 2009) applies only to soil-derived aDNA (moreover, the term 'dirt' is a pejorative and non-scientific word meaning excrement). Finally, the use of the term 'fossil DNA' by Coolen and Gibson (2009) with respect to lacustrine sediments is too restricted and easily confused with aDNA isolated from Quaternary fossil material, such as bone. PalEnDNA encompasses all of these sample types.

Progress in the use of PalEnDNA in Quaternary and archaeological research

Since the first publication reporting reproducible PalEnDNA (Poinar *et al.*, 1998, who isolated ground sloth and plant aDNA from a coprolite dated to ca. 20 000 ^{14}C a BP), an increasing number of Quaternary and archaeological studies have incorporated PalEnDNA analyses (Table 2). These

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Table 2. A range of PalEnDNA-related publications*. Studies have been organized according to general research area and sample type.

Deposit or material	Target taxa	Age range	Examples (references)
PALAEOECOLOGY: PALAEOENVIRONMENTAL RECONSTRUCTION			
Peat	Plants	155 ka	Suyama *et al.* (1996, 2008)
Permafrost	Bacteria, fungi, bryophytes, plants, invertebrates (insects), vertebrates (mammals, birds)	2 – < 600 ka	Shi *et al.* (1997), Willerslev *et al.* (2003, 2004), Lydolph *et al.* (2005), Vishnivetskaya *et al.* (2006), Johnson *et al.* (2007), Sønstebø *et al.* (2010), Arnold *et al.* (2011), D'Costa *et al.* (2011), Kuzmina *et al.* (2011), Boessenkool *et al.* (2012), Epp *et al.* (2012), Willerslev *et al.* (2014)
Ice	Fungi, protists, plants, invertebrates (insects)	0.3 – < 800 ka	Willerslev *et al.* (1999, 2007), Ma *et al.* (2000)
Lacustrine	Diatoms, plants, invertebrates (crustaceans, copepods)	13 cal ka–modern	Limburg and Weider (2002), Bissett *et al.* (2005), Parducci *et al.* (2005, 2013), Marková *et al.* (2006), Bennett and Parducci (2006), Anderson-Carpenter *et al.* (2011), Xu *et al.* (2011), Magyari *et al.* (2011), Stoof-Leichsenring *et al.* (2012), Gugerli *et al.* (2013), Pedersen *et al.* (2013), Boessenkool *et al.* (2014)
Cave deposit	Plants, invertebrates (insects), vertebrates (mammals, birds)	10.8–0.6 ^{14}C ka	Hofreiter *et al.* (2003b), Willerslev *et al.* (2003), Thomsen *et al.* (2009), Haouchar et al. (2014)
Marine	Invertebrates (foramnifera, radiolarian), plants	≤45 ka	Paffetti *et al.* (2007), Lejzerowicz *et al.* (2013)
Glacial (fluviogravel and moraine)	Plants	4.5–5.2 cal ka	Gould *et al.* (2010)
Soil	Plants, vertebrates (mammals, birds)	5.5 cal ka–modern	Anderson *et al.* (2012), Jørgensen *et al.* (2012b), Wilmshurst *et al.* (2014)
Rodent midden	Plants, vertebrates	10.1 ^{14}C ka	Kuch *et al.* (2002)
Silt, sand, organic deposit (silt-soaked)	Plants		Jørgensen et al. (2012a)
PALAEOECOLOGY: MEGAFAUNAL EXTINCTION			
Permafrost	Vertebrates (mammals, birds)	7.6–11.76 ^{14}C ka	Haile *et al.* (2009)
PALAEOECOLOGY: PALAEODIETARY RECONSTRUCTION			
Coprolite	Plant, invertebrates (parasites), vertebrates (mammals, birds)	32–0.6 ^{14}C ka	Poinar *et al.* (1998, 2001), Hofreiter *et al.* (2000, 2003a), Reinhard *et al.* (2008), Wood *et al.* (2008, 2012a,b,c, 2013a,b)
Intestinal contents	Plants, vertebrates (mammals)	41.9–5.1 ^{14}C ka	Rollo *et al.* (2002), van Geel *et al.* (2008, 2011, 2012, 2014)
Dental calculus (calcified plaque)	Bacteria	Mesolithic, Neolithic, Bronze Age, Medieval	Adler *et al.* (2013), De la Fuente *et al.* (2013), Metcalf *et al.* (2014), Warinner *et al.* (2014)
PALAEOECOLOGY: TAXONOMY			
Coprolite	Vertebrates (mammals)	Late Pleistocene to <3 ^{14}C ka	Poinar *et al.* (2003), Bunce *et al.* (2009), Campos *et al.* (2010)
Hairs in coprolite	Vertebrates (mammals)	13 ka	Clack *et al.* (2012a,b)
ARCHAEOLOGICAL HISTORY			
Cave deposit	Plants, vertebrates (mammals, birds)	12.3 ^{14}C ka	Gilbert *et al.* (2008, 2009), Goldberg *et al.* (2009), Poinar *et al.* (2009), Rasmussen *et al.* (2009), Jenkins *et al.* (2012)
Lacustrine	Bacteria, vertebrates (mammals, fish)	ca. 1.7 cal ka	Matisoo-Smith *et al.* (2008), Madeja *et al.* (2010), Giguet-Covex *et al.* (2014)
Soil	Vertebrates (mammals)	0.9–0.39 ^{14}C ka	Hebsgaard *et al.* (2009)
Gravel, sand, gyttja, peat	Bacteria	10–2.9 ^{14}C ka	Madeja *et al.* (2009)
Coprolites	Bacteria, invertebrates (parasites), vertebrates (mammals, birds)	12.3 ^{14}C ka – 0.6 cal ka	Loreille *et al.* (2001), Iniquez *et al.* (2003a, b, 2006), Kemp *et al.* (2006), Luciani *et al.* (2006), Gilbert *et al.* (2008, 2009), Leles *et al.* (2008), Goldberg *et al.* (2009), Poinar *et al.* (2009), Rasmussen *et al.* (2009), Speller *et al.* (2010), Jenkins *et al.* (2012), Tito *et al.* (2012)
DNA DAMAGE			
Permafrost	All taxa, bacteria	600–10 cal ka	Mitchell *et al.* (2005), Hansen *et al.* (2006)

*A more comprehensive list is given in supporting information Table S1.

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studies mainly focused on palaeoecology and archaeology (e.g. Speller *et al.*, 2010; Lejzerowicz *et al.*, 2013), with recent but limited work on tephras (Haile *et al.*, 2007) and soils (e.g. Andersen *et al.*, 2012; Jørgensen *et al.*, 2012b; Wilmshurst *et al.*, 2014).

Palaeoecology

Palaeoecological research using PalEnDNA has included examination of bacteria, animals and plants to reconstruct past environments and evaluate ecosystem change through time (Table 2). Willerslev *et al.* (2007) isolated PalEnDNA from silt-rich ice at the base of the Dye-3 drill core through the Greenland ice sheet (dated to 450–800 ka), and showed that central Greenland supported a diverse flora and fauna, including conifers and butterflies, during a major ice retreat phase – perhaps during Marine Oxygen Isotope Stage 11 (Alley *et al.*, 2010) – before it was subsequently covered in ice more than 2 km in thickness. More recently, Gould *et al.* (2010) extracted PalEnDNA from frozen plant material uncovered by receding glaciers in south-eastern Peru. The material, found at 5200 m elevation and dated to 4500–5200 cal a BP, represents the ice-free vegetation in the area before climatic conditions changed in the mid-Holocene, allowing the extension of glaciers and formation of an ice cap. The study showed that the pre-glacial vegetation was characteristic of wetland environments, which occur predominantly at warmer lower elevations today. Similarly, D'Costa *et al.* (2011) isolated ancient bacterial DNA from frozen sediments from the Dawson City area, Yukon Territory, aged ca. 30 000 cal a BP, and they characterized genes conferring antibiotic resistance to confirm that antibiotic resistance is a natural phenomenon in ecosystems that pre-dates the modern selective pressure of clinical antibiotic use (see also Metcalf *et al.*, 2014; Warinner *et al.*, 2014).

PalEnDNA from coprolites and preserved gut contents has been used to reconstruct palaeodiets of extinct fauna, particularly woolly mammoth, bison and the moa, a large, flightless New Zealand ratite bird (van Geel *et al.*, 2008, 2012, 2014; Wood *et al.*, 2008, 2012a, b, 2013a, b). PalEnDNA from coprolites also contains information about parasite faunas, including those of extinct species. For example, Wood *et al.* (2013a) isolated parasite aDNA and eggs from moa coprolites to show that when moa were hunted to extinction by Polynesians/early Maori, potentially a suite of cryptic co-extinctions in the fossil record was attainable. This research also revealed that parasite abundance could be mapped geographically and that it was largest in lowland moa species, which were at greater population densities.

Archaeology

aDNA has been used in a variety of archaeological contexts but the use of PalEnDNA is an underdeveloped but emerging area of archaeological research. Currently, PalEnDNA has been used to examine broad archaeological topics, such as past human and farming activities including animal domestication (Hebsgaard *et al.*, 2009; Speller *et al.*, 2010; Giguet-Covex *et al.*, 2014), ancient human health (Leles *et al.*, 2008; Tito *et al.*, 2012; Adler *et al.*, 2013; Warinner *et al.*, 2014), whether it is possible to use bacterial indicators of 'human presence' (Matisoo-Smith *et al.*, 2008; Madeja *et al.*, 2009, 2010, cf. the use of faecal sterol molecular markers by D'Anjou *et al.*, 2012) and the settlement chronology of the Americas (Gilbert *et al.*, 2008; Jenkins *et al.*, 2012) (Table 2). Further research could utilize PalEnDNA (in the absence of an archaeological record) to examine the introduction of food crops and animals and to evaluate the environmental impacts of humans.

Hebsgaard *et al.* (2009) analysed a soil profile from a field adjacent to a Norse farm in Greenland dated to 1000–1400 AD to investigate past farming activities and to track the relative proportions of cattle, sheep and goat aDNA at the site. The study showed that cattle decreased in relative abundance between 1180 and 1260 AD, reflecting abandonment of the farm but occasional use by other Norse farmers for livestock grazing. Similarly, Speller *et al.* (2010) investigated turkey domestication using aDNA from bones and PalEnDNA from coprolites sourced from archaeological sites in the south-western USA dating from 200 BC to 1800 AD. Their study showed that turkeys were domesticated twice in North America before European contact.

Tito *et al.* (2012) used PalEnDNA from human coprolites (sourced from archaeological sites in south-western USA, Mexico, and Chile) to examine the ancestral human distal gut microbial community assemblage and human health, and examined several approaches to address issues with post-depositional changes in microbial content. Similarly, dental calculus (calcified plaque) on the teeth of ancient humans has also been used to examine changes in the human oral

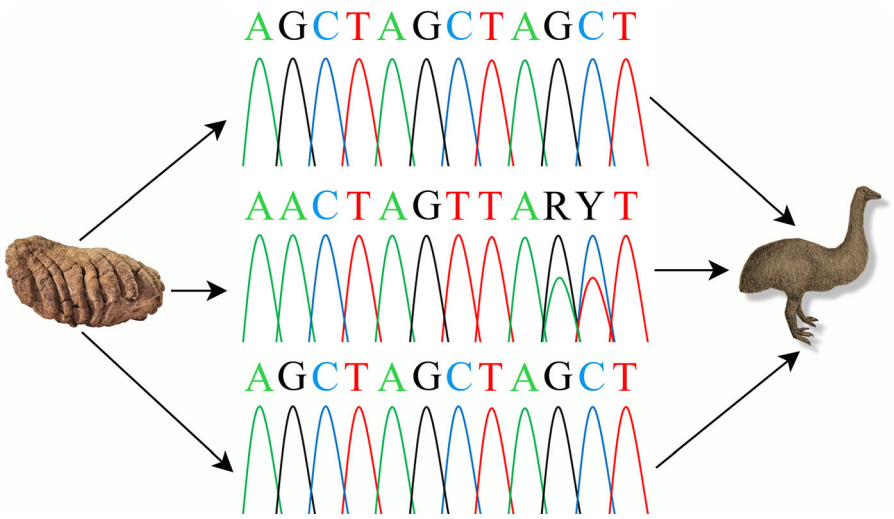

Figure 1. Schematic diagram of ancient DNA (aDNA) sequences obtained from a moa (Aves: Dinornithiformes) coprolite. The three sequences represent the same mitochondrial DNA (mtDNA) region used to identify moa coprolites to species. Because there are multiple mtDNA genomes per cell, each fragment has been independently amplified from a different genome. The peaks represent one of the four DNA bases, adenine (A), guanine (G), cytosine (C) and thymine (T). aDNA is highly degraded, fragmented and chemically modified. One common type of DNA damage is miscoding lesions, which are post-mortem base pair modifications leading to the incorporation of incorrect bases during DNA amplification. The middle sequence has characteristic miscoding lesions (unlike the upper and lower DNA sequences) represented by G–A and C–T transitions or by the ambiguous bases R (G or A) and Y (C or T). Ambiguous bases result from varying levels of DNA damage on each of the mitochondrial genomes.

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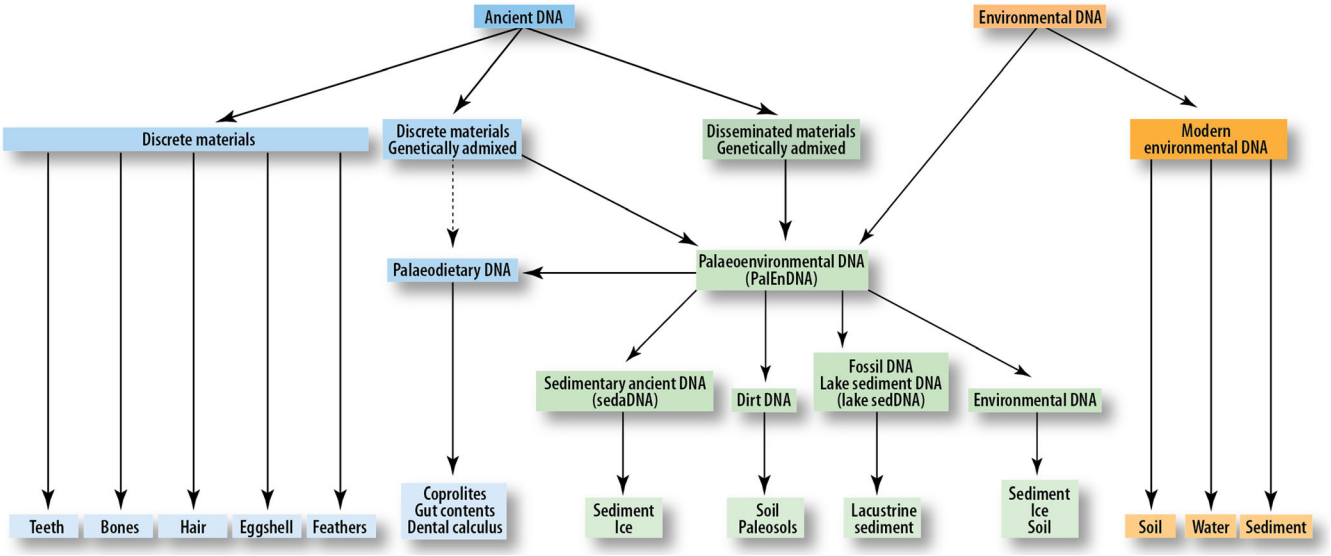

Figure 2. Hierarchy and relationships of the main descriptive terms and sampling materials for PalEnDNA research in comparison with terms used for modern environmental DNA research and aDNA research focusing on discrete samples. We suggest that some terms used previously, including dirt DNA, environmental DNA (in the context of aDNA derived from sediment), sedimentary ancient DNA, lake sediment DNA and fossil DNA, are ambiguous or unnecessary and should be abandoned, and 'palaeoenvironmental DNA' (PalEnDNA) used instead. Studies on coprolites, preserved gut contents and dental calculus fall under PalEnDNA but, as effectively 'discrete' rather than 'disseminated' materials per se, they additionally occupy an intermediary position (see also Table 1).

Figure 3. Examples of deposits that potentially contain PalEnDNA. Clockwise from top left: permafrost comprising frozen organic-rich loess and ice wedges (Yukon Territory, Canada); three split cores of organic-rich lake sediment containing well-preserved tephra layers dating from ca. 15 600 to ca. 7000 cal a BP (NI, New Zealand) (photo from Lowe, 1988, p. 133, with permission from Taylor and Francis; age on layer labelled Mamaku Ash is ca. 8000 cal a BP); moa coprolite (SI, New Zealand); preserved dental calculus (denoted by arrow) on ancient human molar tooth (photo courtesy of Julien Soubrier and Laura Weyrich, ACAD); multisequal soil and palaeosols developed on five Holocene tephra beds (NI, New Zealand) (see McDaniel *et al.*, 2012); and peat, with a thin, mid-Holocene tephra layer showing as a white layer (NI, New Zealand) (photo from Alloway *et al.*, 2013, p. 288, with permission from Elsevier).

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microbiome and diet over time, and host immunity (Adler *et al.*, 2013; Metcalf *et al.*, 2014; Warinner *et al.*, 2014). Dental calculus holds a major advantage for microbial study because the bacterial DNA is calcified in place before death (e.g. Fig. 3), minimizing subsequent taphonomic alteration of community signals that complicate coprolite analysis. Both the coprolite and the dental calculus studies suggested that ancestral human microbial communities are a close match to those from modern rural or hunter-gatherer communities, and that cosmopolitan lifestyles have led to significant changes in the human microbiome.

The use of PalEnDNA from sediments as an indicator of human presence is controversial (e.g. Matisoo-Smith *et al.*, 2008; Madeja *et al.*, 2009, 2010). PalEnDNA from lake sediments sampled from Round Lake, northern New Zealand (Matisoo-Smith *et al.*, 2008), revealed bacterial sequences below the Taupo tephra (dated at 232 ± 10 AD: Hogg *et al.*, 2012) that were a 98–99% match to human faecal bacteria (e.g. *Prevotella* spp.). From this match, the authors suggested human presence in New Zealand before the currently accepted earliest settlement date of ca. 1280 AD (Higham *et al.*, 1999; Hogg *et al.*, 2003; Wilmshurst *et al.*, 2008; Lowe, 2011). However, as admitted by Matisoo-Smith *et al.* (2008), *Prevotella* can also be found in animals' and insects' guts that utilize anaerobic digestion, thereby compromising its use as an indicator of human presence unless species-level identifications are possible.

Studies on tephras and soils

Although several aDNA studies have utilized tephras as an age-equivalent dating method through tephrochronology (e.g. Chan *et al.*, 2005; D'Costa *et al.*, 2011; Letts *et al.*, 2012), so far only Haile *et al.* (2007) have reported PalEnDNA from tephra deposits. Their study found moa DNA within two discrete tephra layers in two adjacent New Zealand caves, which Haile *et al.* (2007) suggested originated either from trampling of faeces or feathers, or the incorporation into the tephras of previously deposited aDNA-bearing cave sediment (Fig. 4).

Under some circumstances the deposition of tephra can increase the probability of fossil preservation. Pyroclastic flows are emplaced at high temperatures (ca. 150–700 °C) (e.g. Banks and Hoblitt, 1981; McClelland *et al.*, 2004; Hudspith *et al.*, 2010), rendering such deposits temporarily sterile (e.g. Smith, 1985; Clarkson *et al.*, 1988; VanderHoek and Nelson, 2007). Burial through rapidly accumulating tephra-fall, especially in proximal localities, is also likely to increase the preservation potential because any biological material is quickly isolated from biogeochemical processes active at the land surface (Ponnamperuma *et al.*, 1967; Fridriksson, 1987; Allen *et al.*, 2005). Consequently, it has been hypothesized that tephra, where present, could provide sterile control layers between deposits containing PalEnDNA – for example, Haile *et al.* (2007) assumed there would be no aDNA within tephra layers. However, soils developed on tephra deposits tend to promote excessively long root growth (e.g. Lowe and Palmer, 2005; Hewitt, 2010; McDaniel *et al.*, 2012) that penetrate through layers, and the assumption of sterility is thus questionable.

Few studies have explicitly examined PalEnDNA from soils or palaeosols (Table 2) (e.g. Hebsgaard *et al.*, 2009; Jørgensen *et al.*, 2012b; Yoccoz *et al.*, 2012; Wilmshurst *et al.*, 2014). Jørgensen *et al.* (2012b) analysed PalEnDNA extracted from soil developed on nunataks (rock outcrops) in southern Greenland, together with modern and historical botanical survey data, to examine changes in plant species composition since the Holocene Thermal Maximum (ca. 5500 cal a BP). The research showed that significant changes in species composition occurred with anthropogenic climate change in contrast to previous episodes of natural climate change. In an investigation on pollen and aDNA in soil cores from an offshore island, Tawhiti Rahi, the largest of the Poor Knights Islands group in northern New Zealand, Wilmshurst *et al.* (2014) found that even though better taxonomic resolution was possible with pollen analysis compared with short fragments of aDNA, the aDNA provided a very useful complementary dataset by confirming the local presence of certain taxa. Moreover, Wilmshurst *et al.* (2014) found no sign of post-depositional

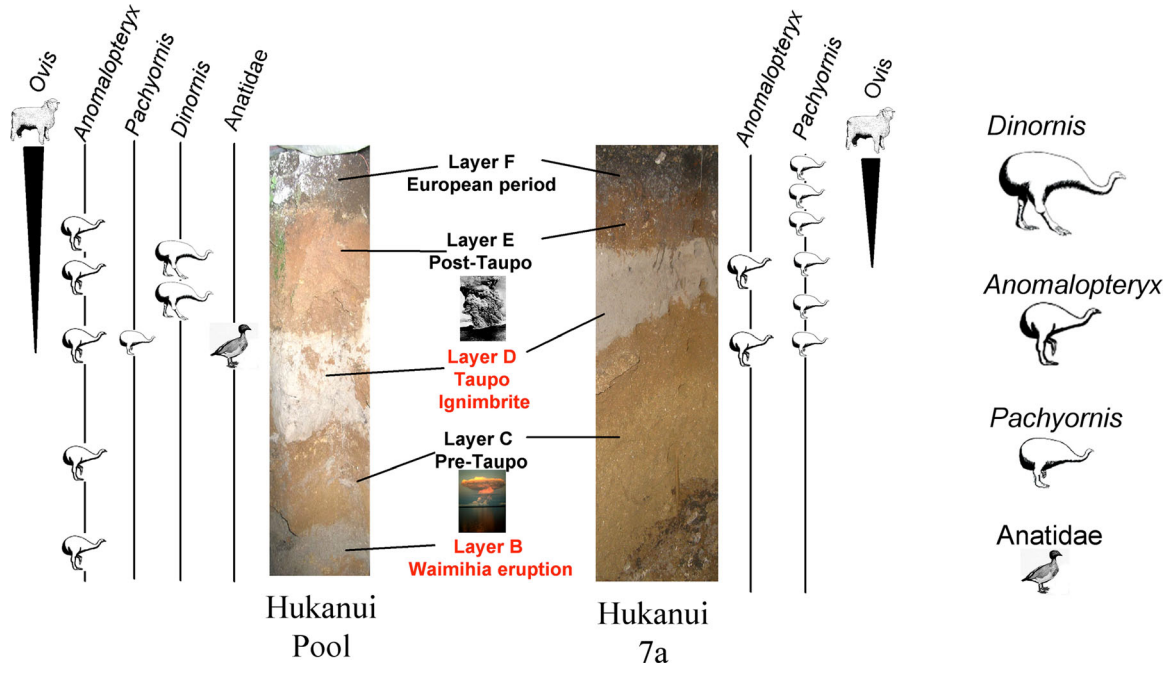

Figure 4. Evidence for DNA leaching in two different stratigraphic sections (approximately 300 m apart) at Hukanui cave sites, eastern North Island, New Zealand. The two named tephras are layer D, Taupo ignimbrite (erupted 232 ± 10 AD) (Hogg *et al.*, 2012) and layer B, Waimihia tephra (erupted 3401 ± 108 cal ka BP) (Lowe *et al.*, 2013). Reproduced from Haile *et al.* (2007, p. 984, with permission from Oxford University Press).

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reworking and mixing with modern material in pre-human samples.

Despite these studies showing the utility of soils and palaeosols for PalEnDNA analysis, such materials are usually only examined from permafrost or cave/rockshelter settings (e.g. Lydolph *et al.*, 2005; Haile *et al.*, 2007; Johnson *et al.*, 2007) rather than from extensive soils from open (non-frozen) landscapes. By nature, soils comprise a highly complex and varied ecosystem (Churchman and Lowe, 2012) and can be grouped at the highest taxonomic level into orders (with numerous sub-orders, groups, and lower taxa) (e.g. Ahrens and Arnold, 2012) defined by characteristics such as different amounts and types of clay minerals, which potentially affect DNA binding capacity, DNA preservation and DNA extraction efficiency (Lloyd-Jones and Hunter, 2001; Herrera and Cockell, 2007; Young *et al.*, 2014). For example, modifications to DNA extraction methods are commonly required for soils with high concentrations of humic acids and certain types of clay minerals (e.g. allophane, a nanocrystalline aluminosilicate clay with extremely high surface areas and variable charge: Churchman and Lowe, 2012) that promote phosphate, and therefore potentially DNA, adsorption (e.g. Herrera and Cockell, 2007; Rai *et al.*, 2010; Huang *et al.*, 2014).

Climatic and temporal limits of PalEnDNA-based research

The theoretical limit of aDNA survival under ideal conditions, such as in permafrost and ice, is ca. 1 million years (Lindahl, 1993a, b; Allentoft *et al.*, 2012). However, the current empirical limit is up to 400–800 ka for PalEnDNA from ice and permafrost (Willerslev *et al.*, 2007) and around the same age for bone (Orlando *et al.*, 2013). In general, DNA preservation is site specific and heavily influenced by the thermal history of the material (Lindahl, 1993a, b; Smith *et al.*, 2001, 2003; Sawyer *et al.*, 2012). The highest success rate for PalEnDNA isolation is achieved with specimens from frozen (Gould *et al.*, 2010), arid, or temperate areas rather than hot and humid areas (Poinar *et al.*, 1998; Thomsen *et al.*, 2009; Haouchar *et al.*, 2014). The current upper ages for replicable results from warm to cool, wet sites (including those containing lacustrine and marine deposits) are ca. 10 000 and ca. 45 000 years, respectively (Bissett *et al.*, 2005; Paffetti *et al.*, 2007; Anderson-Carpenter *et al.*, 2011; Lejzerowicz *et al.*, 2013), compared with ca. 32 000 years for dry sites (Poinar *et al.*, 1998). Hot and humid locations do not favour DNA preservation, although small amounts may still be isolated if the microsite conditions are suitable (Larson *et al.*, 2007) and appropriate precautions against exogenous contamination are taken (Thomson *et al.*, 2014a,b). New *in vitro* DNA repair mechanisms and single-stranded genomic library methods have the potential to increase the empirical limit of DNA detection to include mid-Quaternary remains (see Meyer *et al.*, 2012).

Issues with PalEnDNA-based research

DNA extraction

PalEnDNA can exist as either intracellular DNA or extracellular DNA. Extracellular DNA can be either free from the matrix (non-metabolized and metabolized via invertebrates and bacteria), or bound to humic acids, minerals (including clay), proteins and sugars (Alvarez *et al.*, 1998; Poinar *et al.*, 1998; Arnold *et al.*, 2011). The form of PalEnDNA within a sample may have a significant effect on DNA extraction efficiency. Humic acids and some clay minerals (e.g. allophane) have a strong binding capacity for DNA, therefore inhibiting recovery of DNA from such materials (Alvarez *et al.*, 1998; Saeki *et al.*, 2010; Huang *et al.*, 2012, 2014). Saeki *et al.* (2010) showed that <2% of adsorbed DNA could be released from humic acids. There has been limited testing of commonly used PalEnDNA extraction methods to determine the relative efficiency and taxonomic biases (e.g. Willerslev *et al.*, 2003; Xu *et al.*, 2011; Haile, 2012; Kuch and Poinar, 2012; Taberlet *et al.*, 2012b). van Geel *et al.* (2012) showed that fast commercial and slower in-house extraction methods of mammoth intestinal contents resulted in a different but complementary taxonomic coverage for plant species. In contrast to PalEnDNA, it is well known within the soil microbiological field that different DNA extraction methods can result in an over- or under-representation of specific bacterial phyla, with extraction efficiency and taxonomic coverage forming important aspects of experimental design (e.g. Holmsgaard *et al.*, 2011; Knauth *et al.*, 2013; Young *et al.*, 2014).

Geographical origin

It is generally assumed that PalEnDNA reflects a local signal, with animals required to be physically present to leave genetic traces, and the physical conditions of a site (e.g. size of cave entrance), organism behaviour and physiology (e.g. urine content, biomass) dictating which species are able to be detected (Lydolph *et al.*, 2005; Haile *et al.*, 2007; Andersen *et al.*, 2012). Lydolph *et al.* (2005) isolated ancient fungal DNA from Siberian permafrost, and showed that mammalian coprophilous fungi were present in samples containing mammalian DNA, suggesting that the PalEnDNA originated from animal faeces and was local in origin. Haile *et al.* (2007) showed that the DNA of small moa was only found in small rockshelters, whereas DNA of moa of all sizes was found in large rockshelters, also suggesting a local origin in this instance. Andersen *et al.* (2012) found similar results to those of Lydolph *et al.* (2005) and Haile *et al.* (2007) in European zoological parks. Animal DNA distribution was governed by behaviour and use (e.g. trails and latrine sites), and was absent from sites not frequented. For plants, Yoccoz *et al.* (2012) showed that plant DNA from sediment and soil reflected the taxonomic diversity of the local underground plant biomass (i.e. roots).

Recently, it has been argued that regional processes may also be responsible for some PalEnDNA. For example, a significant component of PalEnDNA can originate from long-distance dispersal (e.g. wind-blown pollen) and post-depositional reworking (Arnold *et al.*, 2011; Reyes *et al.*, 2011). Jørgensen *et al.* (2012a) found that although plant macrofossils (and the aDNA extracted from them) represented a local signal, pollen represented a regional and reworked signal. Ancient DNA can be isolated from individual pollen grains (Parducci *et al.*, 2013), meaning regional and reworked pollen signals could influence palaeonenvironmental reconstructions based on PalEnDNA. In reality, the geographical origin of PalEnDNA is probably site- and taxon-specific, and PalEnDNA is likely to reflect a mixture of local, regional and reworked signals.

Stratigraphic reliability

Stratigraphic reliability can be compromised by post-depositional reworking and DNA leaching or migration, and can cause intermixing of modern and ancient genetic signals and seriously affect the robustness of palaeoenvironmental

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reconstructions. Such intermixing can occur through the movement of water, active bacterial or fungal growth (e.g. Tito *et al.*, 2012), and bioturbation, especially in marine and lacustrine sediments and in soils.

Post-depositional reworking occurs when material from one stratigraphic layer or position is incorporated into another layer or position of a different age. This reworking has been shown to occur during glacial–interglacial transitions and in soils via pedogenesis (including multiple processes, such as leaching of solutes, migration of clay or organic matter in suspension, and soil mixing by biota or by shrink–swell or freeze–thaw processes), through mass movement events on slopes, or by mixing of materials from the act of coring itself. Reworking may partly explain differences between taxonomic assemblages reconstructed from plant macrofossil, pollen and PalEnDNA (Arnold *et al.*, 2011; Reyes *et al.*, 2011; Jørgensen *et al.*, 2012a). Post-depositional reworking poses considerable problems for PalEnDNA analyses when deposits without good stratigraphic control are chosen for analysis. For example, Haile *et al.* (2009) isolated mammoth and horse aDNA from palaeosols developed on loess adjacent to the Yukon River, Alaska, dated to ca. 10 500 cal a BP which was 3000–5000 cal years younger, and 8 m higher, in a stratigraphic succession than the last fossilized instances of these species in the Alaskan–Yukon region. Haile *et al.* (2009) therefore concluded that there was no upward reworking at the site because of the absence of mammoth and horse aDNA in control samples taken from adjacent sites. However, it is difficult to rule out reworking of older upstream deposits in settings such as a floodplain. In a separate study, Arnold *et al.* (2011) isolated mammoth aDNA from permafrost dating to ca. 5000 cal a BP, long after mammoth became extinct in the region. Dating of permafrost sediments containing mammoth aDNA using both ^{14}C for organic fractions and optically stimulated luminescence (OSL) for inorganic fractions showed significant mismatches between the ages obtained (^{14}C: ca. 19 000–5000 cal a BP; OSL: mean age 5700 a BP). These results suggest that extensive reworking can occur even in some permafrost situations, and reinforces the point that an understanding of geomorphological or pedogenic processes, and stratigraphic control, is critical for successful PalEnDNA research.

DNA leaching and migration (hereafter referred to as DNA leaching) occur when stratigraphically younger DNA is transported through a sequence into older layers or vice versa (Fig. 4). DNA leaching may be partly a function of animal behaviour (e.g. nature of latrine sites, population density), physiology (e.g. mammalian versus avian, urine volume and form), the amount of net water movement (e.g. degree of soil saturation at a latrine site) and soil properties (e.g. grain-size distribution, pore size distribution, hydraulic conductivity: Andersen *et al.*, 2012). The extent of DNA leaching is heavily debated (e.g. Gilbert *et al.*, 2008; Rasmussen *et al.*, 2009; cf. Poinar *et al.*, 2009) and its prevalence is unknown. Currently, DNA leaching has been documented with mammalian-derived DNA in seasonally wet sites and possibly coprolites (Haile *et al.*, 2007; Andersen *et al.*, 2012; Jenkins *et al.*, 2012). Haile *et al.* (2007) found that in temperate New Zealand caves, modern sheep DNA had leached into pre-Polynesian layers containing extinct avian species. In contrast, Hebsgaard *et al.* (2009) found no evidence for DNA leaching in a wet open site in Greenland, suggesting this phenomenon may not be a universal concern in seasonally wet sites in cold environments.

In contrast to wet sites, it has been hypothesized there is no DNA leaching in permafrost, recently frozen sediments, in some dry cave deposits, or in saturated marine or lacustrine sediments (Willerslev *et al.*, 2004; Lydolph *et al.*, 2005; Hansen *et al.*, 2006; Gilbert *et al.*, 2008; Anderson-Carpenter *et al.*, 2011; Jenkins *et al.*, 2012; Giguet-Covex *et al.*, 2014). However, DNA leaching must be tested critically in each site and situation to enable it to be ruled out or otherwise (e.g. Haile *et al.*, 2007; Jenkins *et al.*, 2012; Giguet-Covex *et al.*, 2014).

DNA leaching can also occur when environmental DNA 'leaches' into coprolites from the surrounding matrix (Jenkins *et al.*, 2012). Jenkins *et al.* (2012) excavated a Camelidae coprolite from Paisley Cave, Oregon, with a macrofossil age of 12 125 ± 30 ^{14}C a BP, which was found to have a water-soluble fraction age of 11 315 ± 25 ^{14}C a BP, a difference of around 800 ^{14}C years. Three human coprolites were excavated in close proximity to the Camelidae coprolite but these had concordant macrofossil and water-soluble fraction ^{14}C ages. Jenkins *et al.* (2012) suggested that DNA leaching had occurred in the Camelidae coprolite but that any DNA leaching was limited spatially, stratigraphically and in volume, and that any wetting events (to enable dissolution and leaching to occur) were rare.

DNA damage

PalEnDNA preservation is not uniform across the environment and is heavily influenced by geological age, climate and depositional site characteristics. In addition, PalEnDNA is degraded by hydrolytic and oxidative attack, and bacterial metabolism. As a result, aDNA is heavily fragmented, degraded and chemically modified (Fig. 1). Four general types of damage can occur: (i) fragmentation, (ii) abasic sites (missing DNA bases), (iii) crosslinking (condensation reactions between DNA and proteins or sugars) and (iv) miscoding lesions (base pair modifications leading to the incorporation of incorrect bases during DNA amplification) (Fig. 1) (Pääbo *et al.*, 2004; see also Rizzi *et al.*, 2012). Fragmentation, abasic sites and crosslinking inhibit the amplification of aDNA, whereas miscoding lesions result in erroneous sequences that can have a significant impact on taxonomic resolution.

Although these types of damage have been well characterized for homogeneous samples, such as bone (e.g. Brotherton *et al.*, 2007), data relating to DNA damage in heterogeneous palaeoenvironmental samples are limited. The available studies suggest that PalEnDNA from permafrost is significantly more damaged than DNA from marine sediments of similar age, with crosslinking and miscoding lesions potentially a dominant type of damage (Hansen *et al.*, 2006; Orlando *et al.*, 2013). In contrast, Corinaldesi *et al.* (2008) found that in marine sediments enzyme activity that degrades DNA was high compared with processes that lead to abasic site damage. Anderson-Carpenter *et al.* (2011) suggested that miscoding lesions occur rapidly after deposition of plant material in lacustrine settings, whereas Wood *et al.* (2012a) showed that miscoding lesions were present in highly conserved plant cDNA *rbcL* sequences isolated from coprolites. However, given the absence of individual (discrete) specimens in PalEnDNA, it is difficult to tell the difference between taxonomic diversity and miscoding lesions, which may lead to an overestimation of taxonomic diversity. To overcome such difficulties, results can be replicated or analysis can be limited only to sequences that match 100% to reference sequences (e.g. Willerslev *et al.*, 2007). However, these methods are not foolproof because a 100% match could still be generated with a sequence containing miscoding lesions. Additionally, the use of computer programs that can account for DNA damage during data analysis are available (e.g. Munch *et al.*, 2008a, b; Taberlet *et al.*, 2012a).

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Recommendations for future PalEnDNA-based research

Several recommendations concerning field and laboratory work, and data analysis, are provided here to guide researchers in the use of PalEnDNA and to help ensure palaeoecological reconstructions are robust (Fig. 5). It is important to note that potential complexities vary at every field site, and hence requirements need to be addressed individually according to local conditions. It is also important to appreciate that even when a genetic result passes all suggested tests, the default hypothesis should still be that it could have resulted from contamination, as is true for all criteria of authenticity used for aDNA (e.g. Cooper and Poinar, 2000; cf. Gilbert *et al.*, 2005).

Fieldwork

Site selection and stratigraphy

Careful site selection and reliable stratigraphy are essential because post-depositional reworking and DNA leaching can reduce the robustness of palaeoenvironmental reconstructions. Arnold *et al.* (2011) recommended that high-energy environments and those with very rapid deposition should normally be avoided. Rather, low-energy environments, where DNA is perhaps more likely to remain intracellular, should be targeted. To control for DNA leaching, sites with effectively no net water movement, such as permafrost, recently frozen sites, lacustrine or marine sediments, and some dry cave deposits, provide possibilities to minimize these processes. Even at sites such as these, past hydrological conditions may have resulted in DNA leaching, and hence over long periods the potential for this phenomenon is usually regarded as high unless demonstrated otherwise.

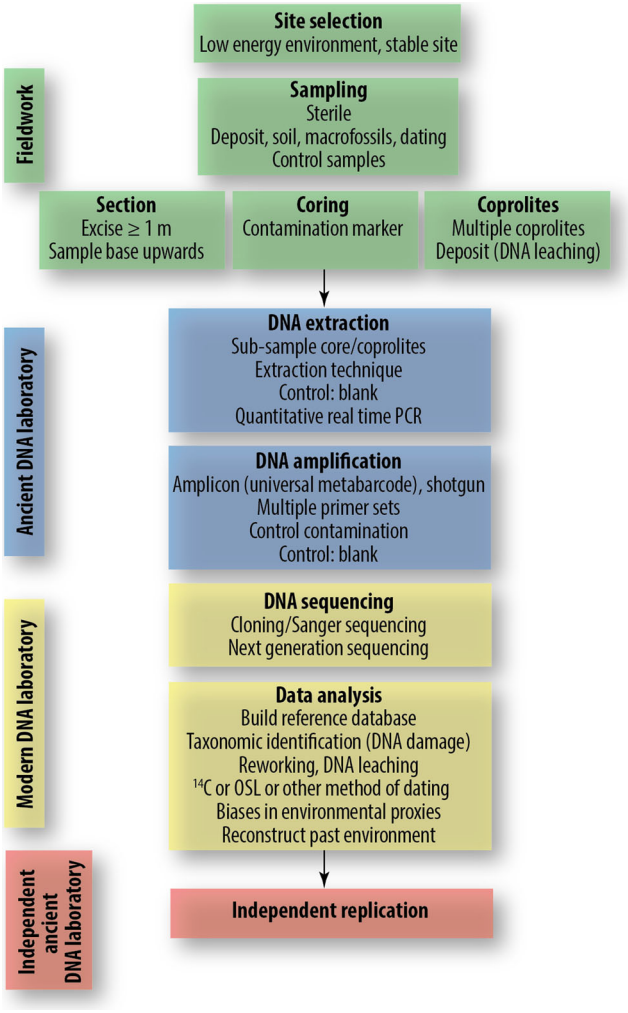

Figure 5. Recommended PalEnDNA-based research workflow summary to help ensure robust palaeoecological reconstructions are obtainable.

Detailed examination of the stratigraphic features of deposits at an exposure or in an excavation, and associated fieldwork over a wider region, can provide an indication of stratigraphic reliability because the identification of a consistent pattern indicates a degree of replication. Tephrostratigraphy (where available) can also be used to assess stratigraphic integrity, but not necessarily the reliability of the PalEnDNA record with regard to post-depositional reworking and DNA leaching. D'Costa *et al.* (2011) sampled aDNA sequences from Late Pleistocene permafrost sediments (mainly frozen loess) immediately overlain by a geochemically distinctive, thick tephra layer (up to 80 cm in thickness), the Dawson tephra aged ca. 30 000 cal a BP, at Bear Creek east of Dawson City in the Yukon Territory. The presence of the intact tephra layer and cryostratigraphic features demonstrated that the permafrost had not thawed since the time of deposition and so, in the absence of fluid leaching, the site represented an ideal source of uncontaminated and, through tephrochronology, securely dated aDNA (D'Costa *et al.*, 2011).

Additional control samples should be taken for plant macrofossil and pollen analysis, and OSL and ^{14}C dating (if in range), or other methods where appropriate, such as (U−Th)/He or U−Pb dating (e.g. Danišík *et al.*, 2012; Sirocko *et al.*, 2013; Coffey *et al.*, 2014), to help assess the stratigraphic reliability of PalEnDNA.

Recent studies have shown that PalEnDNA, macrofossils and microfossils have complementary overlapping datasets, potentially allowing distinction between local, regional and re-worked signals in some sites (Jørgensen *et al.*, 2012a). OSL dating of quartz grains within sediment can potentially date the inorganic, and hence bound, DNA fraction. In contrast, ^{14}C dating can potentially date the organic and unbound DNA fraction. The same sort of split applies to macrofossil versus water-soluble-fraction ^{14}C dates on coprolites (Jenkins *et al.*, 2012). Mismatched reliable dates would suggest that post-depositional reworking has occurred, as was the case with mammoth aDNA dating to 5000 cal a BP in permafrost (Arnold *et al.*, 2011), or that there is some problem with dating procedures as demonstrated, for example, with the application of OSL on loess–tephra sequences in New Zealand and north-west Canada (Lowe *et al.*, 2010; Demuro *et al.*, 2013). For bulk sediment and soil, radiocarbon dates should generally be viewed as range finders rather than precise (or accurate) age estimates. The only study where PalEnDNA has been directly dated is that of Willerslev *et al.* (2007), where silty ice at the base of a Greenland ice core containing PalEnDNA was dated using a combination of ^{10}Be/^{36}Cl isotope ratios, single-grain luminescence measurements, amino acid racemization combined with basal ice temperature modelling, and phylogenetic tree-branch length estimates for the age of the PalEnDNA sequences. For studies utilizing coprolites, samples of associated deposits should be collected and analysed in conjunction with those of the coprolites to search for signs of potential DNA leaching (e.g. Jenkins *et al.*, 2012). However, this practice is not applicable if a significant amount of the deposit is composed of disaggregated coprolites (e.g. Poinar *et al.*, 2001; Willerslev *et al.*, 2003; Hofreiter *et al.*, 2003b; Haile *et al.*, 2007; Wood *et al.*, 2008, 2012a) because there will be overlapping genetic

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signals arising from both the coprolites and the associated deposits.

Modern control samples, including analyses of water and surface deposits, can be used to test for the presence of aDNA, which could indicate post-depositional reworking (Haile *et al.*, 2009). Ancient control samples, including intervening purportedly 'sterile' layers (e.g. tephra deposits) between stratigraphic layers of interest, should also be included to test for the presence of DNA leaching and post-depositional reworking.

Sterile sampling

Results from PalEnDNA research, and aDNA research in general, are highly prone to errors because of contamination from exogenous modern DNA. As noted earlier, there are numerous examples of studies where contamination has led to erroneous results, such as those involving amber-preserved insects (Cano *et al.*, 1992; Austin *et al.*, 1997), Permian salt crystals (Vreeland *et al.*, 2000; Park *et al.*, 2009) and even some Neanderthal fossils (Green *et al.*, 2006, 2009; Wall and Kim, 2007). Contamination can arise contemporaneously with the geological processes that resulted in the incorporation of DNA into a deposit, during post-depositional reworking or leaching (as discussed previously), during sampling, and during laboratory work. To minimize the risk of contamination in the field, several precautions should be taken (Willerslev *et al.*, 2004). Sampling should normally be conducted in a sterile manner, which involves the wearing of protective clothing, such as gloves, facemask or a body suit, and using sterilized equipment (Fig. 6). When undertaking coring, a recognizable genetic tracer can be used to determine how far exogenous contamination can penetrate the retrieved core (Willerslev *et al.*, 2003, 2007; Hebsgaard *et al.*, 2009; D'Costa *et al.*, 2011). When sampling sections (e.g. road cuttings) or pit faces, the sampling surface ideally should be incised at least 1 m to minimize the possibility of sampling deposits containing modern DNA (Willerslev *et al.*, 2004), although the depth of incision remains arbitrary and untested. Sampling of sections (outcrops) should also be conducted from the base upwards to avoid contamination of older layers by younger materials (Haile *et al.*, 2009). Regardless of sampling technique, parallel (duplicate) samples should be taken to examine intra-site variation, and from nearby sites to examine inter-site variation. Importantly, for coprolites, Wood *et al.* (2012a) showed that multiple samples from the same deposit should be analysed to help ensure palaeodietary reconstructions are not distorted by single defaecation events or seasonal bias. For instance, James and Burney (1997) examined the diet of the extinct moa-nalos from Hawaii, and found that pollen analysis of the coprolites indicated deposition in spring alone.

Laboratory work

Because of the degraded nature of PalEnDNA, and the ease with which samples can be contaminated, the DNA extractions and polymerase chain reaction (PCR) setup – the method used to amplify aDNA to workable concentrations – must be conducted in a physically isolated, dedicated aDNA laboratory with large numbers of controls and independent replication where necessary (see Cooper and Poinar, 2000). Unlike radiocarbon dating, there are very few commercial aDNA laboratories (e.g. Lakehead University Paleo-DNA Laboratory), and hence Quaternary and archaeological researchers wanting to incorporate aDNA into research projects will need to collaborate with staff at existing laboratories. Table S2 (supporting information) provides a list of some of the aDNA laboratories around the world in which PalEnDNA research has been conducted within the last 5 years.

Figure 6. Undertaking sterile fieldwork in PalEnDNA research. The Holocene tephra–palaeosol section was cleaned back nearly 1 m to expose a fresh face for sampling, which has been conducted using sterile equipment. Sampling was undertaken from the base upwards to avoid contamination of older samples with younger material. Note the protective gear, including gloves, disposable body suit and face mask.

Subsampling

When working with sediment cores, the outside 1–3 cm should be removed because this may have been exposed to exogenous contamination in the field during coring (Willerslev *et al.*, 2004). For previously collected cores without a contaminant tracer, this trimming procedure is recommended to ensure 'sterile' samples are obtained for PalEnDNA analysis. Sub-samples should be taken from the centre of the core, and from the base to the top of the core, to avoid contamination of older layers by younger DNA. This protocol also applies to coprolites, where the outer layer should be irradiated by UV light and removed so that only 'sterile' inner materials are sampled (Wood *et al.*, 2012a).

DNA extraction

A key target of PalEnDNA research is an efficient DNA extraction method regarding DNA concentration, fragment size, purity (e.g. absence of humic acids and tannins that can inhibit the amplification of aDNA) and taxonomic coverage (see van Geel *et al.*, 2012), with reproducible differences between sites, deposits, and origin and form of DNA (Young *et al.*, 2014). Currently, PalEnDNA extraction methods are neither efficient nor consistent across these categories, and only limited testing of their efficacy and consistency has been performed (e.g. van Geel *et al.*, 2012; Wales *et al.*, 2014).

There are relatively few commonly used DNA extraction methodologies that are specific for environmental DNA (including PalEnDNA). These include the use of kits that are commercially available (e.g. MoBio, Norgen, Macherey & Nagel), and specialized within-laboratory methods (Willerslev *et al.*, 2003; Haile, 2012; Kuch and Poinar, 2012; Taberlet *et al.*, 2012b). These methods have been trialled on a relatively limited number of samples and are generally based around the targeting of intracellular DNA, although Taberlet *et al.* (2012b) targeted extracellular DNA using a phosphate buffer. The wide range of materials analysed in PalEnDNA research, and their depositional and taphonomic contexts,

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will have a significant impact on extraction efficiency. For example, allophane can bind 95–99% of available DNA, with <2% of DNA in an extractable form (Saeki *et al.*, 2010). Humic acids, which are representative of organic matter in soils, also have a strong binding affinity for DNA with a 2% release rate (Saeki *et al.*, 2010). For research questions comparing taxonomic diversity between samples, a consistent DNA extraction method will be sufficient. However, when the aim is to explore the taxonomic diversity within a single sample, the most efficient DNA extraction method, or a combination of multiple extraction methods, would help to maximize taxonomic coverage (e.g. van Geel *et al.*, 2012). Differing taxonomic coverage between plant macrofossils and PalEnDNA indicates that the commonly used extraction methods are not particularly efficient at extracting all DNA from a sample (e.g. Wood *et al.*, 2012a).

Library preparation

PCR uses primers specific to genetic markers to amplify aDNA to workable concentrations. PalEnDNA samples are nearly always genetically admixed, and potentially contain ancient and modern inputs. Thus, the choice of genetic marker will depend on the target of interest, the required taxonomic resolution and the extent of DNA degradation. There are two approaches to obtaining PalEnDNA data from environmental samples: amplicon sequencing and shotgun sequencing. Amplicon sequencing uses 'universal' metabarcode genetic markers. These metabarcodes are highly variable, allowing taxonomic discrimination, but they are flanked by regions conserved across multiple taxa. Commonly used metabarcodes in PalEnDNA research are listed in Table S3. However, many metabarcodes have resolution problems. The short plant *rbcL* (h1a/h2a) and *trnL* (p6-loop) metabarcodes for aDNA can only be resolved to the family–order level (and, more rarely, to genus or species) because of the small fragment size (Willerslev *et al.*, 2003; Taberlet *et al.*, 2007; Sønstebø *et al.*, 2010) (cf. longer *rbcL* and *trnL* metabarcodes can resolve to genus and species level, but are not usually suitable for aDNA research because of their size). van Geel *et al.* (2012) recommended that multiple, increasingly specific, genetic markers are used in a tiered approach to avoid biases towards different plant taxa. The use of universal metabarcodes also increases the chances of amplifying exogenous contamination. It is possible to avoid specific types of contaminating sequences through the use of blocking primers, which were originally developed to prevent the amplification of defaecator DNA in dietary analysis of stomach contents (Vestheim and Jarman, 2008; Rasmussen *et al.*, 2009; Boessenkool *et al.*, 2012; Calvignac-Spencer *et al.*, 2013), improving the detection of rare DNA sequences. Boessenkool *et al.* (2012) used blocking primers to prevent the amplification of human DNA from permafrost samples, allowing the amplification of aDNA specifically from rare extinct mammalian taxa, including woolly rhino. An additional approach includes sequencing negative extraction and PCR controls, followed by the removal of DNA sequences found in the negative controls from the PalEnDNA sequence datasets.

In contrast to targeted amplicon sequencing, shotgun sequencing can be used to provide a random survey of PalEnDNA within a sample, regardless of taxa and genetic marker (Tringe *et al.*, 2005). The results, however, will be dominated by bacterial and human DNA sequences unless approaches are taken to block their amplification.

The amount of DNA sequence data that can be obtained from PalEnDNA samples has taken a technological leap forward over the past decade. Next-generation sequencing (NGS) provides orders-of-magnitude greater amounts of sequence data than traditional (Sanger) methods, and has brought about a revolution in aDNA research to the extent that entire genomes of extinct hominins can now be reconstructed (e.g. Meyer *et al.*, 2012). The power of the approach lies in the vast numbers of sequences that are generated in parallel, providing significantly greater sequencing coverage and depth. These in turn allow a detailed analysis of DNA damage, and hence NGS can provide a valuable complement to the replication of results. Consequently, there is a significant (growing) demand for bioinformatics tools to analyse such quantities of data (Knapp and Hofreiter, 2010).

Data analysis

Reference sequence database and taxonomic identification

For all taxa, the construction of a reference sequence database to facilitate taxonomic resolution is usually essential. Gould *et al.* (2010) could only identify 50% of plant *trnL* sequences to a specific taxon because of the small size of the *trnL* metabarcode and the number of available reference sequences on GenBank, a comprehensive public database of nucleotide sequences and supporting bibliographic and biological annotation (see www.ncbi.nlm.nih.gov/genbank/). Local databases can be created to include only the reference sequences of interest, which will reduce the computational power required for data analysis. For example, Sønstebø *et al.* (2010) constructed a database of complete plant *trnL* sequences from 842 modern Arctic species, which has been used in subsequent studies by Boessenkool *et al.* (2012) and Jørgensen *et al.* (2012a). Curated online databases are also available for PalEnDNA analysis, including GREENGENES for bacterial 16S rRNA gene sequences, UNITS for fungal internal transcribed spacer (ITS) sequences, and SILVA for 18S rRNA gene sequences.

Several phylogenetic methods have been developed to determine the taxonomic affinity of PalEnDNA sequences but a full description is beyond the scope of this review. Briefly, the methods can be divided into comparisons against reference databases (e.g. GenBank) or phylogenetic analyses, such as Bayesian-based approaches (e.g. Haile *et al.*, 2007; Wood *et al.*, 2012a, 2013a, b), including those that can take into account DNA damage, especially miscoding lesions and how they affect taxonomic identification (e.g. Munch *et al.*, 2008a, b).

Complementary nature of PalEnDNA and biases of different environmental proxies

Multidisciplinary and multiproxy approaches to Quaternary and archaeological research have been shown to improve palaeoenvironmental reconstructions (e.g. Newnham *et al.*, 1995; Birks and Birks, 2006; Birks *et al.*, 2010; Jørgensen *et al.*, 2012a; Wood *et al.*, 2012a; Parducci *et al.*, 2013; van Geel *et al.*, 2014). aDNA analyses, including PalEnDNA studies, should not be viewed as replacing more traditional techniques, such as studies of plant macrofossils and pollen, but rather as a complementary tool generating both overlapping and separate results (e.g. Wood *et al.*, 2012a; Pedersen *et al.*, 2013; Boessenkool *et al.*, 2014). For example, comparisons of PalEnDNA analysis with pollen records from the same sediment cores from two volcanic crater sites in the Albertine Rift, eastern Africa, showed that plant diversity determined from aDNA analyses improved vegetation reconstructions based on pollen records by revealing additional

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taxa and by enhancing taxonomic resolution (Boessenkool *et al.*, 2014). Furthermore, the two measures together (PalEnDNA and pollen) enabled vegetation changes at different geographical scales to be distinguished, with PalEnDNA mainly reflecting local vegetation, whereas pollen represented a wider provenance area.

Similarly, Parducci *et al.* (2013), working on lake sediments, concluded that the use of metabarcoding provided a complementary, but not an alternative, tool to pollen analysis for investigating past flora. In addition, metabarcoding can provide a local signal from the vegetation in the absence of (sub)fossil evidence, but has limited capacity to detect all taxa, regardless of their abundance. Parducci *et al.* (2013) suggested that metabarcoding should be followed by pollen analysis and the use of species-specific primers to provide the most comprehensive signal from the environment (see also van Geel *et al.*, 2012, and commentary by Gugerli *et al.*, 2013). In another multi-faceted study, Wood *et al* (2012a) combined analysis of PalEnDNA, pollen and plant macrofossils from coprolites to reconstruct the habits and habitats of New Zealand's extinct upland moa comprehensively (Fig. 7), and Wood *et al.* (2013b) used a parallel approach to reconstruct ecological niche partitioning among four sympatric species of moa in the Dart River Valley, South Island, New Zealand. In these studies, a high abundance of Poaceae was detected from pollen analysis but only single Poaceae DNA sequences were detected, suggesting that Poaceae pollen was incidentally ingested while feeding.

Plant macrofossils represent a local signal (potentially more regional in some environmental settings, such as those involving alluvial deposits) but they are affected to some degree by taphonomic biases. Hard parts such as seeds, wood and leaf cuticles preserve better than soft parts such as fruit and flowers in coprolites (Wood, 2007). Pollen represents a local to regional signal but can be devalued by taxonomic resolution issues (Anderson-Carpenter *et al.*, 2011). For example, *Coprosma* and Poaceae pollen in New Zealand can only be identified to genus and family level, respectively, despite each group being relatively species-diverse. Pollen analyses also suffer from quantification problems with differences in pollen production and dispersal variability between species biasing palaeovegetation reconstructions made from pollen-count data (Wood *et al.*, 2012a). PalEnDNA has further biases associated with differential extraction efficiencies (van Geel *et al.*, 2012; Wales *et al.*, 2014) and taxonomic resolution. Importantly, DNA-based species identifications are not quantitative either, partly because of the DNA extraction method or because of the sequence coverage from NGS. Additional proxies that could be included are geographical distribution data and historical botanical checklists (Hofreiter *et al.*, 2003a; Jørgensen *et al.*, 2012a; Wood *et al.*, 2012a; Wilmshurst *et al.*, 2014).

Replication

A key aspect of aDNA research is replication, either internally (within the laboratory) or externally (by another laboratory). Willerslev *et al.* (2007) considered PalEnDNA sequences to be genuine (reliable) only after they were independently replicated and a 100% match to reference sequences was attained. However, independent replication is commonly not used, encouraged by a misconception that the sequencing depth generated by NGS approaches is a suitable substitute for contamination tests. This approach was shown when Green *et al.* (2006) published 1 million base pairs of Neanderthal nuclear DNA using NGS, but subsequently 10–78% of the data were suggested to be contamination from modern human DNA (Wall and Kim, 2007; Green *et al.*, 2009).

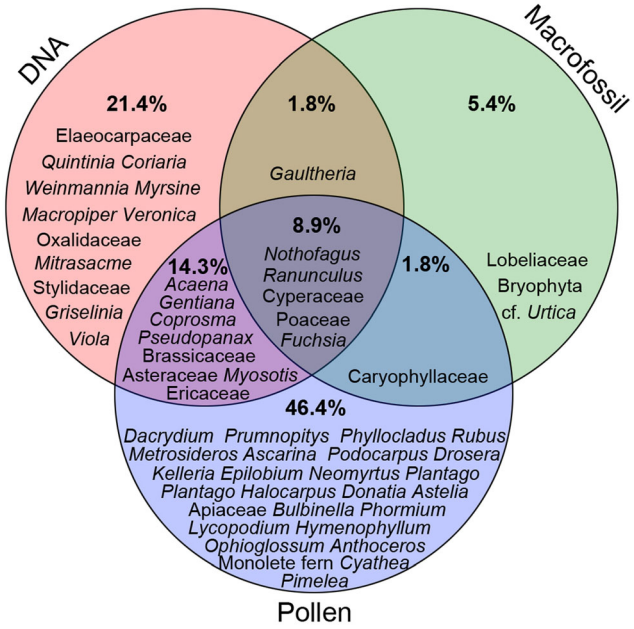

Figure 7. Plant taxa detected in eight upland moa coprolites using three different diet proxies (aDNA, plant macrofossils, pollen). Each proxy revealed plant taxa not detected by the others, reinforcing the conclusion that the proxies are complementary and that a multiproxy approach is needed for gaining maximum palaeodietary information (Jørgensen *et al.*, 2012a; Wood *et al.*, 2012a). Figure from Wood *et al.* (2012a, p. 10).

For PalEnDNA, putative taxa with low abundance and heterogeneous distributions, especially in independent samples from the same layer or deposit, may not be replicated because of drop-out (failure to amplify) and stochastic variation (Willerslev *et al.*, 2007; Haile *et al.*, 2009). In lieu of independent replication, macrofossil- and pollen-derived data may provide an additional means of verification (Wood *et al.*, 2012a; Fig. 7).

Prospects: using PalEnDNA for Quaternary palaeoenvironmental reconstruction

PalEnDNA, defined as aDNA extracted from disseminated (non-discrete) genetic material from environments of the past, forms the basis of an emerging and exciting sub-discipline of aDNA research. The combination of PalEnDNA studies with the analysis of plant macrofossils and pollen and other proxies is providing a powerful means to reconstruct past environments more comprehensively (e.g. Jørgensen *et al.*, 2012a; Boessenkool *et al.*, 2014). PalEnDNA researchers are also beginning to obtain a greater understanding of both the power and the limitations of the technique (e.g. Munch *et al.*, 2008a,b; Boessenkool *et al.*, 2012; van Geel *et al.*, 2012), meaning that more robust reconstructions are possible. However, scientists undertaking Quaternary and archaeological studies who want to utilize the PalEnDNA technique need to plan prudently and to evaluate field procedures and sample collection techniques, as noted earlier.

For PalEnDNA-based research to become a more firmly established technique, and for its value as a tool for palaeoenvironmental reconstruction to be properly assessed, the issues discussed in this review need to be addressed, including the form of PalEnDNA, and the efficiency and taxonomic coverage of DNA extraction methods. The field would benefit

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from an in-depth understanding of DNA damage and degradation rates in PalEnDNA, and procedures to distinguish between true taxonomic diversity and miscoding lesions. Laboratory and analytical methods to determine the level of mixing of ancient and modern DNA in samples also need developing.

The incorporation of PalEnDNA into Quaternary and archaeological research programmes requires careful planning of research questions and of field and laboratory work – rather than simply being an extra 'add-on' to conventional stratigraphic studies – and will require modifications to existing fieldwork protocols. To advance the field of PalEnDNA-based research, metadata relating to the type of deposit, or soils, such as stratigraphy, soil horizonation, mineralogy and chemical properties (e.g. pH) may need to be included. Multiple proxies, replicable stratigraphies and reliable dating methods can be used to help determine the stratigraphic reliability of PalEnDNA assays and to increase taxonomic resolution in conjunction with the construction of DNA sequence reference databases. Publication of failures for given DNA extraction methods is also critical so that success/failure rates can be calculated for given deposits or soils, environments and time periods.

In conclusion, the field of PalEnDNA research remains young and the reliability of signals extracted from a wide variety of sedimentary or volcanic deposits, soils and palaeosols for palaeoecological or archaeological research still needs to be fully assessed. However, the potential is considerable, and PalEnDNA-based research is set to grow rapidly. It will be important for Quaternary and archaeological researchers, together with geochronologists and various other specialists, such as bioinformaticians, to be actively involved in guiding and developing the field.

Supporting Information

Additional supporting information can be found in the online version of this article:

Table S1. A more comprehensive list of PalEnDNA publications to supplement Table 2 in the main article.
Table S2. A selection of ancient DNA laboratories that have published PalEnDNA research within the last 5 years.
Table S3. Universal metabarcoding genetic markers and PCR primers used in PalEnDNA-based research.

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Abbreviations. ACAD, Australian Centre for Ancient DNA; aDNA, ancient DNA; NGS, next-generation sequencing; NI, North Island; OSL, optically stimulated luminescence; PalEnDNA, palaeoenvironmental DNA; PCR, polymerase chain reaction; SI, South Island.

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JQS 2740. Supporting information, Tables S1 to S3

Table S1. A more comprehensive list of PalEnDNA publications to supplement Table 2 in the main article.

Table S2. A selection of ancient DNA laboratories that have published PalEnDNA research within the last 5 years.

Table S3. Universal metabarcoding genetic markers and PCR primers used in PalEnDNA-based research.

References for Tables S1-S3 are listed in the main article in *Journal of Quaternary Science* (2014), vol. 29 (7) pp. 610-626.

Table S1. A more comprehensive list of PalEnDNA publications to supplement Table 2 in the main article. Studies have been organised chronologically within general Quaternary research areas. Taxa in **bold type** were identified morphologically.

DEPOSIT OR MATERIAL	TAXA	AGE	LOCATION	REFERENCE
PALAEOECOLOGY: PALAEOENVIRONMENTAL RECONSTRUCTION				
Peat deposit	Plant (*Pincea abies*)	155 ka	Japan	Suyama *et al.* (1996)
Permafrost (fluvial)	Viable bacteria	2 ^{14}C ka–3 Ma	Kolyma-Indigirka lowland, Siberia, Russia	Shi *et al*. (1997)
Ice	Fungi, protists, plants	2–4 ^{14}C ka	Greenland	Willerslev *et al*. (1999)
Ice	Fungi	0.3–140 ka	Greenland	Ma *et al.* (2000)
Coprolite/rodent midden	Rodent (*Phyllotis limatus*), mammal, birds, plants	10.1 ^{14}C ka	Atacama Desert, Chile	Kuch *et al*. (2002)
Lacustrine	Microcrustacean (*Daphnia* sp.)	Modern–0.2 ^{14}C ka	Belauer See, Germany	Limburg and Weider (2002)
Cave deposit	Sloth, condor, ringtail cat, rodent, human	10.8 ^{14}C ka	Arizona, USA	Hofreiter *et al*. (2003b)
Cave deposit, sand	Birds, plants	0.6–3 ^{14}C ka	New Zealand	Willerslev *et al*. (2003)
Permafrost (tundra soil, fluvial/lacustrine, unknown)	Vertebrates, plants	10.4 ^{14}C ka–400 ka	Lena-Kolyma River region, Sibera, Russia	Willerslev *et al*. (2003)
Permafrost (unknown)	Bacteria	< 400–600 ka	Northeast Siberia, Russia	Willerslev *et al*. (2004)
Lacustrine	Copepod	0–9.9 cal ka	Antarctica	Bissett *et al*. (2005)
Permafrost (tundra soil, fluvial/lacustrine, unknown)	Fungi	<400 ka	Lena-Kolyma River region, Sibera, Russia	Lydolph *et al*. (2005)
Lacustrine	Microcrustacean (*Daphnia* sp.)	Unknown	High Tatra Mountains, Western Carpathians, Europe	Markova *et al*. (2006)
Lacustrine	Scots pine	0.1–10 ka	Holtjarnen, Sweden	Parducci *et al*. (2005); Bennett and Parducci (2006)
Cave deposit (soil, tephra)	Moa, sheep, duck, insect, plants	< 3.2 ^{14}C ka	New Zealand	Haile *et al.* (2007); Thomsen *et al*. (2009)
Permafrost (marine, lacustrine, alluvium, palaeosol)	Bacteria	5 ka–3 Ma*	Kolyma lowland, Siberia, Russia	Vishnivetskay *et al*. (2006)
Marine	Plants (*Fagus* sp.)	ca. 45 ka	Venice	Paffetti *et al*. (2007)
Permafrost (tundra soil, fluvial/lacustrine, ice rich/silty sediment, unknown)	Bacteria, plants	< 400 ka	Lena-Kolyma River region, Mayn River, Southern Chukotka, Khomus-Yuryakh River, Sibera, Russia	Johnson *et al*. (2007)
Ice (silt-rich)	Insects, plants	< 800 ka	Greenland	Willerslev *et al.* (2007)
Peat deposit	Peat moss (*Sphagnum fascum*)	Modern–0.45 ka	Norway	Suyama *et al*. (2008)
Glacial (fluviogravel and moraine)	Plants	4.5–5.2 cal ka	Peru	Gould *et al.* (2010)
Permafrost (ice rich, silty sediment)	Plants	15.8–22.9 ^{14}C ka	Mayn River, Southern Chukotka, Siberia, Russia	Sonstebo *et al*. (2010)
Lacustrine	Plants	< 4.6 ^{14}C ka	Great Lakes, North America	Anderson Carpenter *et al*. (2011)
Permafrost (peat, clay, silt, sand)	Mammals	4.47–50.6 ^{14}C ka	Taimyr Peninsula and north-central Siberia, Russia	Arnold *et al*. (2011)

Lacustrine, marine	Copepods	Unknown	Antarctica	Xu *et al.* (2011)
Permafrost	Bacteria	30 cal ka	Alaska	D'Costa *et al*. (2011)
Soil	Mammals, birds	Modern	Norway	Anderson *et al*. (2012)
Permafrost (silt, fine sand)	Mammals (incl. woolly rhino)	15.8–>50 ^{14}C ka	Main River, Southern Chukotka, Duvanny Yar, Kolyma River, Siberia, Russia	Kuzmina *et al*. (2011); Boessenkool *et al*. (2012)
Lacustrine	Norway spruce	10.7–13 cal ka	Retezat Mountains, South Carpathians, Europe	Magyari *et al*. (2011)
Silt, sand, organic deposit (silt-soaked)	Plants	12–45 ^{14}C ka	Taymir Peninsula, Siberia, Russia	Jorgensen *et al*. (2012a)
Soil, sediment	Plants	Modern–5.5 cal ka	J.A.D. Jemsen's Nunataks, Greenland	Jorgensen *et al*. (2012b)
Permafrost (silt, fine sand)	Plants, bryophytes, fungi, beetles, birds	15.8–> 50 ^{14}C ka	Main River, Southern Chukotka, Duvanny Yar, Kolyma River, Siberia, Russia	Epp *et al*. (2012)
Lacustrine	Diatoms	0–0.2 ka	Lake Naivasha, Kenya	Stoof-Leichsenring *et al.* (2012)
Lacustrine	Plants	0.342–9.52 cal ka	Lake Comarum, Greenland	Pendersen *et al*. (2013)
Cave deposit	Mammal, marsupial, reptile, plant	6.8–>20 ka	Kelly Hill Caves, Kangaroo Island, South Australia	Haouchar *et al*. (2014)
Lacustrine, mire	Plants	1980 AD–2790 BC	Mt. Gahinga, Mt. Muhavura, Albertine Rift, East Africa	Boessenkool *et al*. (2014)
Lacustrine	Mammals (*Bos, Ovis, Equus*), plants (*Pinus, Alnus*)	Present – ca. 4000 BC	Lake Anterne, France	Giguet-Covex *et al*. (2014)
Permafrost (various), coprolites, gut contents	Plants	Present – 50 ka	Arctic region	Willerslev *et al*. (2014)
Soil	Plants	Present–ca. 236 AD	Tawhiti Rahi, Poor Knights Islands, New Zealand	Wilmshurst *et al*. (2014)
PALAEOECOLOGY: MEGAFAUNAL EXTINCTION				
Permafrost (loess, palaeosol)	Mammals (especially mammoth, horse)	7.6–11.76 ^{14}C ka	Steven's Village, Yukon Flats, Alaska	Haile *et al*. (2009)
PALAEOECOLOGY: PALAEODIETARY RECONSTRUCTION				
Coprolite	Shasta ground sloth, plants	19.8 ^{14}C ka	Nevada, USA	Poinar *et al*. (1998)
Coprolite	Various extinct species	10–32 ^{14}C ka	SW USA, Chile	Poinar *et al*. (1998)
Coprolite	Shasta ground sloth, plants	11–28.5 ^{14}C ka	Nevada, USA	Hofreiter *et al*. (2000)
Coprolite	Human, mammal, plants	2.1–2.2 ^{14}C ka	Texas, USA	Poinar *et al*. (2001); Reinhard *et al*. (2008)
Intestinal contents	Mammal, plants	5.1–5.2 cal ka	Italy	Rollo *et al*. (2002)
Coprolite	Cuchillo Cura ground sloth, plants	14.6 ^{14}C ka	Argentina	Hofreiter *et al*. (2003a)
Coprolite	**Mammoth**, plants	18.5 ^{14}C ka	Yakutia, Siberia, Russia	van Geel *et al*. (2008)
Coprolite	Moa, plants, parasites	0.6-1 ^{14}C ka	New Zealand	Wood *et al*. (2008, 2012a, 2012b, 2013a, 2013b)
Coprolite	**Mammoth**, plants, fungi	12.3 ^{14}C ka	Cape Blossom, Alaska, USA	van Geel *et al*. (2011)
Intestinal contents	**Mammoth**, plants, **fungi**	41.9 ^{14}C ka	Yamal Peninsula, northwest Siberia, Russia	van Geel *et al*. (2012)

Coprolite	Kakapo, **plants**	< 3 ^{14}C ka	New Zealand	Wood *et al*. (2012c)
Intestinal contents	Bison	10.5 cal ka BP	Yakutia, Siberia, Russia	Van Geel *et al*. (2014)
ARCHAEOLOGICAL HISTORY				
Cave deposit	Human, mammal, birds, plants	12.3 ^{14}C ka	Oregon, USA	Gilbert *et al*. (2008, 2009); Goldberg *et al*. (2009); Poinar *et al*. (2009); Rasmussen *et al*. (2009); Jenkins *et al*. (2012)
Lacustrine	'Human', rat, fish, bacteria**	ca. 1700 cal a BP	New Zealand	Matisoo-Smith *et al*. (2008)
Palaeosol (anthropogenic)	Human, mouse, reindeer, sheep, goat, cattle	0.39–0.9 ^{14}C ka	Greenland	Hebsgaard *et al*. (2009)
Gyttja, gravel, sand, detritus, moss peat, herbaceous peat	Bacteria	2.8–10 ka	Poland	Madeja *et al.* (2009)
Lacustrine	Bacteria	2.9–0.6 ka	Poland	Madeja *et al*. (2010)
Coprolite	Parasite (*Ascaris* sp.)	0.6 cal ka	Belgium	Loreille *et al*. (2001)
Coprolite	Human intestinal parasites	< 7 ka	Brazil, Chile	Iniquez *et al*. (2003a)
Coprolite	Human, Pinworm	4110 BC–900 AD	Chile, USA	Iniquez *et al*. (2003b)
Coprolite	Pinworm	4110 BC–900 AD	Chile, USA	Iniquez *et al.* (2006)
Coprolite	Human	0.7–2 ka	California, USA	Kemp *et al.* (2006)
Coprolite	Intestinal bacteria (incl. *Haemophylus parainfluenzae*)	980–1170 AD	Peru	Luciani *et al*. (2006)
Coprolite	Human, mammal, birds, plants	12.3 ^{14}C ka	Oregon, USA	Gilbert *et al*. (2008, 2009); Goldberg *et al*. (2009); Poinar *et al*. (2009); Rasmussen *et al*. (2009); Jenkins *et al*. (2012)
Coprolite	Human, parasite (*Ascaris* sp.)	0.95–8.8 ^{14}C ka	Brazil, Chile, Peru	Leles *et al*. (2008)
Coprolite	Turkey	200 BC–1800 AD	USA	Speller *et al*. (2010)
Coprolite	Human	1.4–ca. 8 ka	USA, Mexico, Chile	Tito *et al*. (2012)
Dental calculus	Human, bacteria	Mesolithic, Neolithic, Bronze Age, Medieval	Poland, Germany, England	Adler *et al.* (2013)
Dental calculus	Human, bacteria, virus, mammals, plants	Medieval	Dalheim, Germany	Metcalf *et al*. (2014); Warriner *et al*. (2014);
DNA DAMAGE				
Permafrost (unknown)	All taxa, bacteria	10–600 cal ka	Region between Lena and Kolyma River, Siberia, Russia	Mitchell *et al*. (2005); Hansen *et al.* (2006)
TAXONOMY				
Coprolite	Shasta ground sloth	> 10 ^{14}C ka	Nevada, USA	Poinar *et al*. (2003)

Coprolite	Moa	< 3 ^{14}C ka	New Zealand	Bunce *et al*. (2009)
Coprolite	Harrington's mountain goat	11.2–14.4 ^{14}C ka, Late Pleistocene	Grand Canyon, USA	Campos *et al*. (2010)
Hairs in coprolite	Darwin's ground sloth (*Mylodon darwinii*)	13 ka	Milodon Cave, Chile	Clack *et al*. (2012a, 2012b)

*We question the veracity of this older age limit

**Cloned bacterial DNA sequences from sediments aged ca. 1700 cal a BP produced a 98% similarity match with human gut bacterial genus *Prevotella*. This genus, however, is found in a range of animal (including insect) guts and so postulated evidence for possible human presence is equivocal (see text of main article).

Table S2. A selection of ancient DNA laboratories that have published PalEnDNA research within the last 5 years.

North America	
Paleo-DNA Laboratory	Lakehead University, Thunder Bay, Ontario, Canada
McMaster Ancient DNA Centre	Department of Anthropology and Biology, McMaster University, Hamilton, Ontario, Canada
Department of Archaeology	Simon Fraser University, Burnaby, Canada
Department of Anthropology	University of Oklahoma, Norman, USA
New Zealand	
Landcare Research Long-Term Ecology Lab	Landcare Research, Lincoln (Canterbury)
Otago Zoology Palaeogenetics Lab	Department of Zoology, University of Otago, Dunedin
Australia	
Australian Centre for Ancient DNA	School of Earth and Environmental Science, University of Adelaide, SA
Trace and Environmental DNA Laboratory	Curtin University, Perth, WA
Europe	
School of Biological Sciences	Royal Hollaway University of London, Egham, UK
Molecular Taxonomy Laboratory	Natural History Museum of Budapest, Hungary
Evolutionary Biology Centre	Department of Ecology and Genetics, Uppsala University, Sweden
Institute of Botany	Department of Palaeobotany, Jagiellonian University, Krakow, Poland
Institute of Palaeobiology	Polish Academy of Sciences, Warsaw, Poland
National Centre for Biosystematics	Natural History Museum, University of Oslo, Norway
Centre for Geogenetics	Natural History Museum of Denmark, University of Copenhagen, Denmark

Table S3. Universal metabarcoding genetic markers and PCR primers used in PalEnDNA-based research.

Taxon	Forward primer	Sequence (5`-3`)	Reverse primer	Sequence (5`-3`)	Marker	Size (bp)	Reference
Universal primers (eukaryotes)							
Eukaryotes	BMBC-R	GTACACACCGCCCGTCG	NS8	TCCGCAGGTTCACCTACGGA	18S	125	Lydolph *et al*. (2005)
Eukaryotes	SR7R	AGTTAAAAAGCTCGTAGTTG	SR5	GTGCCCTTCCGTCAATT	18S	510	Lydolph *et al*. (2005)
Eukaryotes	Uni1	TCCCCAACAAACTAGGAGG	Uni2	ACTGGTTGTCTCCAATTCA	cyt b	178	Matisoo-Smith *et al*. (2008)
Protists, fungi and higher plants							
Protists, fungi and higher plants	NS8	TCCGCAGGTTCACCTACGGA	nu-SSU-1627-31-r	GRACACACCGCCCGT	18S	160	Willerslev *et al*. (2007)
Diatoms	Diat rbcL 705F	AACAGGTGAAGTTAAAGGTTCATAYTT	Diat rbcL 808R	TGTAACCCATAACTAAATCGATCAT	rbcL	103	Stoof-Leichsenring *et al.* (2012)
Diatoms	Diat rbcL 708F	AGGTGAAGTTAAAGGTTCATACTTDAA	Diat rbcL 802R	CCCATAACTAAATCGATCATAAYRAT	rbcL	94	Stoof-Leichsenring *et al*. (2012)
Fungi	KW3	TCCAGCTCCAATAGCGTATA	SL43	GAACCACACGTCCTATTC	18S	210	Lydolph *et al*. (2005)
Fungi	ITS5	GGAAGTAAAAGTCGTAACAAGG	5.8S fungi	CAAGAGATCCGTTGTTGAAAGTT	ITS1	50-100	Epp *et al.* (2012)
Bryophytes	Bryo P6F	GATTCAGGGAAACTTAGGTTG	Bryo P6R	CCATTGAGTCTCTGCACC	trnL	50-100	Epp *et al.* (2012)
Plants							
Plants	rbcL h1aF	GGCAGCATTCCGAGTAACTCCTC	Hp2R	CGTCCTTTGTAACGATCAAG	rbcL	120-130	Willerslev *et al*. (2003)
Plants	rbcLZ1	ATGTCACCACAAACAGAGACTAAAGCAAGT	rbcL h1aR	GAGGAGTTACTCGGAATGCTGCC	rbcL	132	Poinar *et al.* (2001)
Plants	rbcL h1aF	GGCAGCATTCCGAGTAACTCCTC	Hp2R	CGTCCTTTGTAACGATCAAG	rbcL	137	Hofreiter *et al.* (2000)
Plants	Z1aF	ATGTCACCACCAACAGAGACTAAAGC	19bR	CTTCTTCAGGTGGAACTCCAG	rbcL	157	Hofreiter *et al.* (2000)
Plants	rbcLZ1	ATGTCACCACAAACAGAGACTAAAGCAAGT	19bR	CTTCTTCAGGTGGAACTCCAG	rbcL	157	Poinar *et al.* (2001)
Plants	rbcLZ1	ATGTCACCACAAACAGAGACTAAAGCAAGT	rbcL19	AGATTCCGCAGCCACTGCAGCCCCTGCTTC	rbcL	183	Poinar *et al.* (2001)
Plants	rbcL h1aF	GGCAGCATTCCGAGTAACTCCTC	rbcL h1aR	GAGGAGTTACTCGGAATGCTGCC	rbcL	?	Poinar *et al.* (1998)
Plants	Z1aF	ATGTCACCACCAACAGAGACTAAAGC	Hp2R	CGTCCTTTGTAACGATCAAG	rbcL	?	Hofreiter *et al.* (2000)
Plants	trnLg	GGGCAATCCTGAGCCAA	trnLh	TTGAGTCTCTGCACCTATC	trnL	59-100	Taberlet *et al*. (2007)
Plants	trnLc	CGAAATCGGTAGACGCTACG	trnLd	GGGGATAGAGGGACTTGAAC	trnL	ca. 450	Taberlet *et al*. (2007)
Plants	trnLg	GGGCAATCCTGAGCCAA	*TrnL*gh	CCATTGAGTCTCTGCACCTATC	trnL	83-103	Haile *et al*. (2007)
Plants	trnLg	GGGCAATCCTGAGCCAA	Plant_trnL_49863R	GGGGATAGAGGGACTTGAAC	trnL	438	Willerslev *et al*. (2007)
Insects/Invertebrates							
Insects	InsCOIf1	TTATGCTATATTANCTATTGG	InsCOIr1	GTAAAGTAAGCTCGTGTATC	COI	97	Willerslev *et al*. (2007)
Insects	InsCOIR	GTAAAGTAAGCTCGTGTATC	Ins3L	AAAGAAACATTTGGAGCTTTAGGA	COI	125	Thomsen *et al.* (2009)
Insects	Ins3R	TCCTGTTGGAACAGCAATAAT	Ins3L	AAAGAAACATTTGGAGCTTTAGGA	COI	159	Thomsen *et al.* (2009)
Coleoptera	Coleop 16Sc	TGCAAAGGTAGCATAATMATTAG	Coleop 16Sd	TCCATAGGGTCTTCTCGTC	16S	50-100	Epp *et al*. (2012)
Enchytraeidae	Ench 12Sa	GCTGCACTTTGACTTGAC	Ench 12Sc	AGCCTGTGTACTGCTGTC	12S	50-100	Epp *et al*. (2012)
Intestinal parasites							
Ascaridoidea	Asc8	ATACATGCACCAAAGCTCCG	Asc9	GCTATAGTTATTCAGAGTCACC	18S	99	Lorielle *et al*. (2001)

Ascaridoidea	Asc6	CGAACGGCTCATTACAACAG	Asc7	TCTAATAGATGCGCTCGTC	18S	123	Lorielle *et al*. (2001)
Ascaridoidea	Asc10	CCATGCATGTCTAAGTTCAA	Asc11	CARAAAWTCGGAGCTTTGGT	18S	147	Lorielle *et al*. (2001)
Nematodes	Nem18SF	ATTCCGATAACGARCGAGAC	Nem18SR	CCGCTKRTCCCTCTAAGAAGT	18S	40-120	Wood *et al*. (2013a)
Nematodes	Nem18SlongF	CAGGGCAAGTCTGGTGCCAGCAGC	Nem18SlongR	GACTTTCGTTCTTGATTAATGAA	18S	350-400	Wood *et al*. (2013a)
Ascaris	Asc1	GTTAGGTTACCGTCTAGTAAGG	Asc2	CACTCAAAAAGGCCAAAGCACC	cyt b	142	Lorielle *et al*. (2001)
Vertebrates							
Vertebrates	12ss	AATTTCGTGCCAGCCACCGCGGTCA	12st	AAGCTGTTGCTAGTAGTACTCTGGC	12S	?153-273	Poinar *et al*. (1998)
Vertebrates	12sd	TAAAGGACTTGGCGGTGCTTCAC	12sn	CCATTTCATAGGCTACACCTTGACC	12S	?153-273	Poinar *et al*. (1998)
Vertebrates	12shp	GCACAATTATTACTCATAAGC	12sb	TGACTGCAGAGGGTGACGGGCGGTGTGT	12S	?153-273	Poinar *et al*. (1998)
Vertebrates	12s ll	GCATAACTATTACCCATAAGTA	12sb	TGACTGCAGAGGGTGACGGGCGGTGTG T	12S	?153-273	Hofreiter *et al*. (2003a)
Vertebrates	12sa	CTGGGATTAGATACCCCACTAT	12Sm	GAGGATGGCGGTATATAGGCTG	12S	205	Kuch *et al*. (2002)
Vertebrates	16SNS1	CCTCCGAACGACTATGCGCCCA	16S7	TTGCGCTGTTATCCCTAGGGTAACT	16S	?35	Hofreiter *et al*. (2000)
Vertebrates	16S6	TTTCGGTTGGGGCGACCTCGGAG	16S7	TTGCGCTGTTATCCCTAGGGTAACT	16S	141	Poinar *et al*. (2001)
Mammals							
Mammals	16SA&M Fv2	TCACTATTTTGCNACATAGA	16SA&M Rv2	CCCCGAAACCAGACGAGCTA	16S	70	Rassmussen *et al*. (2009)
Mammals	16Smam3	TGGGGTGACCTCGGAGAAY	16Smam4	TCAACGGAMCAAGTTACCCTA	16S	78	Haile *et al*. (2009)
Mammals	16Smam1	CGGTTGGGGTGACCTCGGA	16Smam2	GCTGTTATCCCTAGGGTAACT	16S	120	Willerslev *et al*. (2003)
Human blocking probe	Human block1	GAGCTACCTAAGAACAGCTA	Human block2	TTTGCTACATAGACGGGTGT	16S	n/a	Rasmussen *et al*. (2009)
Human blocking probe		TTCTCGTCTTGCTGTGTCATGCC			16S	n/a	Epp *et al*. (2012)
Mammals	12Sa	CTGGGATTAGATACCCCACTAT	12So	GTCGATTATAGGACAGGTTCCTCTA	12S	151	Poinar *et al*. (2001)
Human	H9bpA	ATGCTAAGTTAGCTTTACAG	H9bpB	ACAGTTTCATGCCCATCGTC	tRNA-Lys	121	Matisso-Smith *et al*. (2008)
Mammals	L2638	CCTCAGGATAGCTGGCGCTCT	2639H	TCTAATCATTCGCTTTACCGGAT	28S	74	Poinar *et al*. (2002)
Birds							
Bird	12SHf	CCTTGACCTGTCTTGTTAGC	12SKr	CCTACATACCGCCGTCGCCAG	12S	85	Willerslev *et al*. (2007)
Bird	12SF5	CTAACAAGACAGGTCAAGGTAT	12SR4	CCTATTTTACTGCTAAATCCG	12S	125	Oskam *et al*. (2010)
Bird	12SE	CCCACCTAGAGGAGCCTGTTC	12SH	CCTTGACCTGTCTTGTTAGC	12S	153	Haile *et al*. (2007)
Bird	12Sa	CTGGGATTAGATACCCCACTA T	12Sh	CCTTGACCTGTCTTGTTAGC	12S	250	Oskam *et al*. (2010)
Bird	Aves 12Sa	GATTAGATACCCCACTATGC	Aves 12Sc	GTTTTAAGCGTTTGTGCTCG	12S	50-100	Epp *et al*. (2012)

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Nature of contribution by PhD candidate	Designed and conducted experiment, and analyzed data, including creation of figures used for this work. Wrote the manuscript/thesis chapter.
Extent of contribution by PhD candidate (%)	85%

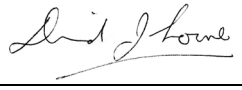
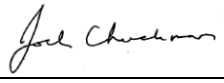
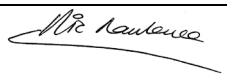
CO-AUTHORS

Name	Nature of Contribution
David J. Lowe	Project conception; provided substantial comments and editing of numerous versions of the manuscript and figures used for this work.
G. Jock Churchman	Project conception and experiment design; provided substantial comments and editing of versions of the manuscript.
Louis A. Schipper	Provided substantial comments and editing of versions of the manuscript.
Nicolas J. Rawlence	Provided comments and editing of versions of the manuscript.
Alan Cooper	Provided comments and editing of versions of the manuscript.

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

Name	Signature	Date
David J. Lowe		10 Aug 2015
G. Jock Churchman		14 Aug 2015
Louis A. Schipper		18-8-15
Nicolas J. Rawlence		14 Aug 2015
Alan Cooper		12 Aug 2015

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As it stands in the submitted thesis, chapter 4 has been submitted to *Quaternary Science Reviews*. The title of the manuscript is the same as the title of the chapter: Origin and preservation of soil organic matter in clay fractions of allophanic paleosols on Holocene tephras in New Zealand characterized using C NEXAFS spectroscopy.

Nature of contribution by PhD candidate	Sampling, designed and conducted experiments; data analysis, including creation of figures used for this work. Wrote the manuscript/thesis chapter.
Extent of contribution by PhD candidate (%)	80%

CO-AUTHORS

Name	Nature of Contribution
David J. Lowe	Project conception; sampling; provided substantial comments; providing substantial comments and contributions on the text regarding the volcanism in North Island of New Zealand; editing of numerous versions of the manuscript.
G. Jock Churchman	Project conception and fundamental experimental design; provided comments.
Louis A. Schipper	Provided substantial comments.
Tsan-Yao Chen	Provided fundamental technical support on synchrotron radiation work; provided comments.
Nicolas J. Rawlence	Sampling; provided comments.

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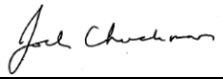
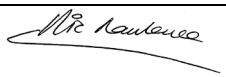
Name	Signature	Date
David J. Lowe		10 Aug 2015
G. Jock Churchman		14 Aug 2015
Louis A. Schipper		18-8-15
Tsan-Yao Chen		12 Aug 2015
Nicolas J. Rawlence		14 Aug 2015

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As it stands in the submitted thesis, chapter 5 has been published in: Huang, YT, Lowe, DJ, Churchman, GJ, Schipper, LA, Cursons, R, Zhang, H, Chen, TY, Cooper, A. 2016. DNA adsorption by nanocrystalline allophane spherules and nanoaggregates, and implications for carbon sequestration in Andisols. *Applied Clay Science* 120, 40–50.

Nature of contribution by PhD candidate	Designed and conducted experiments, and analysed data, including creation of figures used for this work. Wrote the manuscript/thesis chapter.
Extent of contribution by PhD candidate (%)	90%

CO-AUTHORS

Name	Nature of Contribution
David J. Lowe	Project conception; provided substantial comments and editing of numerous versions of the manuscript.
G. Jock Churchman	Project conception and fundamental experiment design; provided comments and editing of versions of the manuscript.
Louis A. Schipper	Provided comments and editing of versions of the manuscript.
Ray Cursons	Provided comments and editing of versions of the manuscript.
Heng Zhang	Provided comments and editing of versions of the manuscript.
Tsan-Yao Chen	Provided fundamental technical support on synchrotron radiation work; provided comments and editing of versions of the manuscript.
Alan Cooper	Provided comments and editing of versions of the manuscript.

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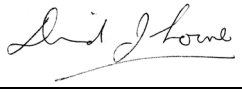
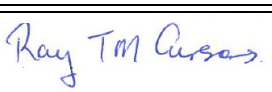
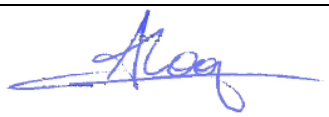
David J. Lowe		10 Aug 2015
G. Jock Churchman		14 Aug 2015
Louis A. Schipper		18-8-15
Ray Cursons		18-8-2015

Heng Zhang		12 Aug 2015
Tsan-Yao Chen		12 Aug 2015
Alan Cooper		12 Aug 2015

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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work. As it stands in the submitted thesis, chapter 6 has been submitted to *Geoderma*. The title of the manuscript is the same as the title of the thesis chapter: A new method to extract and purify DNA from allophanic soils and paleosols, and potential for paleoenvironmental reconstruction and other applications. The title and text may change upon revisions.

Nature of contribution by PhD candidate	Designed and conducted experiments, and analysed data, including creation of figures used for this work. Wrote the manuscript/thesis chapter.
Extent of contribution by PhD candidate (%)	80%

CO-AUTHORS

Name	Nature of Contribution
David J. Lowe	Project conception and sample collection; provided substantial comments and editing of numerous versions of the manuscript.
Heng Zhang	Conducted experiment; collaboration on data interpretation; provided substantial comments.
Ray Cursons	Experimental design and supervision/guidance; collaboration on data interpretation; provided substantial comments and editing of numerous versions of the manuscript.
G. Jock Churchman	Project conception; provided comments and editing of versions of the manuscript.
Jennifer M. Young	Provided comments and editing of versions of the manuscript.
Louis A. Schipper	Provided comments and editing of versions of the manuscript.
Nicolas J. Rawlence	Provided comments and editing of versions of the manuscript.
Jamie R. Wood	Collaboration on data interpretation; provided comments and editing of versions of the manuscript.
Alan Cooper	Project conception; provided substantial comments.

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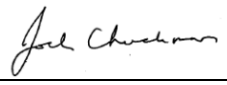
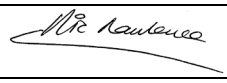
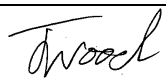
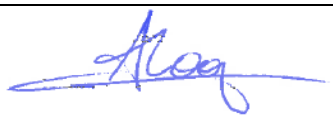
Name	Signature	Date
David J. Lowe	David J Lowe	10 Aug 2015
Heng Zhang	Heng Zhang	12 Aug 2015
Ray Cursons	Ray TM Cursons	18-8-2015

G. Jock Churchman		14 Aug 2015
Jannifer M. Young		12-AUG-15
Louis A. Schipper		18-8-15
Nicolas J. Rawlence		14 Aug 2015
Jamie R. Wood		12 Aug 2015
Alan Cooper		12 Aug 2015