

Ancient Shipwrecks and Archaeological Science: Characterising cargo items through stable isotope, organic residue, and DNA analysis



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“Some say an army of horsemen, some of foot-soldiers, some of ships, is the fairest thing on the black earth, but I say it is what one loves.”

Sappho, Barnstone translation

“A big ship’s a big venture, you know.”

Petronius’ Satyricon, Vol. II, Chapter XXIV, Loeb Translation



“And then the boat died.”

Story of the Shipwrecked Sailor, Middle Egyptian Poem, author’s translation

Dedication

This work is dedicated to the memory of Professor Edgar Peltenburg. At the University of Edinburgh, Eddie gave fascinating lectures on the Uluburun and Cape Gelidonya shipwrecks that changed the course of my life. He was a wonderful researcher and a true friend.

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I would like to thank Professor Greger Larson for his supervision, insights, and encouragement throughout my DPhil, Dr Damian Robinson for greenlighting what everyone agreed would be a very ambitious PhD project, and Professor Chris McKenna for helping to see that project through to a successful conclusion.

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project. Last but not least, I would like to thank Martin, Michael, Maisie and Malala for making my last two years at Oxford very memorable, if not a bit surreal.

Overview of Thesis Structure

This integrated thesis is structured in the “Thesis by Publication” format. Here, three pieces of research in journal article format constitute the body of my work. First, an Introduction to the project is provided, with a description of the archaeological sites under study in this research. Next, the three data chapters are presented as discrete pieces of work, with their own Introduction and Conclusion. Finally, a Discussion and Conclusion chapter is presented at the end of the thesis. Signed statements by my co-authors are included under Supplementary Material.

The three data chapters include the following:

Data Chapter 1: “Stable Isotope analysis of waterlogged olive stones from the Mazotos shipwreck.”

Authors: Lisa Briggs and Stella Demesticha.

The research design, laboratory work, analysis of results, and writing was completed by the present author, while Professor Demesticha provided the archaeological samples, new information about ongoing excavations, and proofed the finished article.

Data Chapter 2: “Biomarkers in shipwreck ceramics and sediments: Using residue analysis to explore cargo compositions and the environment of the Uluburun, Mazotos, Kyrenia and Babuljaš shipwrecks.”

Authors: Lisa Briggs, Lea Drieu, Stella Demesticha, Mladen Pesic, Cemal Pulak, Oliver Craig

The research design, sample procurement, laboratory work, interpretation of results, and writing was completed by the present author. Dr Lea Drieu and Professor Oliver Craig have assisted with the interpretation and analysis of the GC-MS data, and the formatting of the chromatograms included in the article.

Data Chapter 3: “DNA from the Wine-Dark Sea: searching for ancient DNA in shipwreck ceramics, cargo items, and environmental samples from the Uluburun, Cape Gelidonya, Kyrenia, Mazotos, and Babuljaš shipwrecks.”

Authors: Lisa Briggs, Roselyn Ware, Mladen Pesic, Cemal Pulak, Stella Demesticha, Robin Allaby, Greger Larson

The research design, sample procurement, laboratory work, analysis of results through bioinformatics, and writing was completed by the present author. Training in ancient DNA laboratory work was provided by Dr Roselyn Ware and Professor Robin Allaby.

As encouraged in the “Thesis by Publication” approach, substantial sections of this thesis have now been published. My literature review on the application of ancient DNA analysis to artefacts from submerged archaeological sites has been published as a peer-reviewed journal article:

Briggs, L., 2020. Ancient DNA Research in Maritime and Underwater Archaeology: Pitfalls, Promise, and Future Directions. *Open Quaternary*, 6(1), p.3. DOI: <http://doi.org/10.5334/oq.71>

Sections of the organic residue analysis work presented here are currently in press, in a forthcoming book on the Kyrenia ship:

Briggs, L., Drieu, L. “Organic residue analysis of ceramic vessels from the Kyrenia shipwreck: searching for past contents.” In L. Swiny and S. Katzev (eds), *The Kyrenia ship: Volume I*, Oxbow Publications, in press.

Research I have conducted on the trade and transportation of whole olives in antiquity, originally conducted as part of Chapter 2 –Stable Isotope Analysis, has now been published as a peer-reviewed book chapter:

Briggs, L., 2020. “The whole story: Exploring the transportation of whole olives in antiquity through shipwreck evidence.” *Proceedings of the Maritime Archaeology Graduate Symposium*, BAR Publications.

Abstract

Finding a way to accurately characterise the contents of cargoes from ancient Mediterranean shipwrecks would provide invaluable insight into economic, agricultural, and social transformations in antiquity. The three forms in which ancient shipwreck cargo items survive in the archaeological record, as macro-remains, visible residues, and molecules invisible to the naked eye, are analysed here in order to better understand what these ships were transporting, and why.

Despite the abundance of preserved organic material found on shipwrecks, the use of stable isotope analysis on waterlogged archaeological plant material remains largely unexplored. Here we present the results from a small, preliminary isotopic study of olive stones recovered from the 4th century B.C.E. Mazotos shipwreck, Cyprus. Analyses of the stable isotopes of carbon and nitrogen are conducted on 20 ancient, waterlogged olive stones from the Mazotos wreck and 10 modern olive stones from Chios, Greece. The extent of lipid retention, and preservation of original isotopic composition is explored. The possibility of determining the geographic origin of the Mazotos olive stones by isotope analysis is assessed.

The majority of amphorae recovered from ancient shipwrecks appear empty on first inspection, or with barely visible residues adhering to the vessel walls. Ceramic vessels thought to have contained either wine or olive oil recovered from multiple Mediterranean shipwrecks and one terrestrial site are analysed through GC-MS to investigate past vessel contents. Assumptions regarding vessel shape as a determining factor in past use are discussed. Environmental samples from shipwreck sites are analysed and compared with results from GC-MS. Results show a prevalence of conifer products in vessels thought to have contained wine. Environmental samples of seafloor sediment and soil from both the shipwrecks and the terrestrial site contain syringic acid, calling into question the utility of relying on syringic acid as a wine biomarker. Molecules from unexpected or unusual contents were detected in several vessels.

The third method by which this research attempts to characterise ancient shipwreck cargo items is through ancient DNA analysis. There is tremendous potential for DNA studies to

resolve long-standing questions in both terrestrial and underwater archaeology. Great optimism for the recovery of ancient DNA (aDNA) from maritime sites has spurred a series of studies claiming to have successfully extracted aDNA from a variety of artefacts recovered from underwater sites including plant remains, human skeletons, and shipwreck amphorae. However, these studies have not adequately addressed the source of the DNA recovered: does it derive from taxa present in the underwater deposition environment or the artefact itself? My research attempts to address this ambiguity by examining the efficacy of extracting aDNA from the ceramic matrix of vessels recovered from five ancient Mediterranean shipwrecks and establishing what DNA can be found in the water column and seafloor sediments that surround these sites. The methods used in this research are designed to enhance current methods, which do not characterise the deposition environment, and utilises shotgun metagenomics to characterise the DNA found on ancient shipwreck sites.

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Chapter 1 - Introduction

1.1 The promise, and perils, of maritime trade

In a satire written almost 2,000 years ago and set in the Roman world, at the height of imperial opulence, a dinner is described at the home of a fictional freedman named Trimalchio. The author of this piece, Petronius, worked at the court of the notorious Roman emperor Nero, as 'arbiter of elegance and dictator of fashion' (Firebaugh, 2006: 1) and as such, was no stranger to the finer things in life. The dinner he describes in his satire, the *Satyricon*, is the epitome of conspicuous consumption: the guests are given pedicures, plied with 100-year-old wine, and offered decadent delicacies. The host, Trimalchio, describes to his guests how he first inherited a fortune, but then sought to acquire even greater wealth:

But nobody's ever satisfied with what he's got, so I embarked in business. I won't keep you long in suspense; I built five ships and loaded them with wine--worth its weight in gold, it was then--and sent them to Rome. You'd think I'd ordered it so, for every last one of them foundered; it's a fact, no fairy tale about it, and Neptune swallowed thirty million sesterces in one day! You don't think I lost my pep, do you? By Hercules, no! That was only an appetizer for me, just as if nothing at all had happened. I built other and bigger ships, better found, too, so no one could say I wasn't game. A big ship's a big venture, you know. I loaded them up with wine again, bacon, beans, Capuan perfumes, and slaves: Fortunata [Trimalchio's wife] did the right thing in this affair, too, for she sold every piece of jewellery and all her clothes into the bargain and put a hundred gold pieces in my hand. They were the nest-egg of my fortune. A thing's soon done when the gods will it; I cleared ten million sesterces by that voyage, all velvet, and bought in all the estates that had belonged to my patron, right away. I built myself a house and bought cattle to resell, and whatever I touched grew just like a honeycomb (Petronius' *Satyricon*, Vol. II, Chapter XXIV, Loeb Translation).

This passage offers interesting insight into sea-borne trade and the acquisition of wealth in the ancient world. As a satire, the author would endeavour to employ certain tropes that would be instantly familiar to readers, the first of which is the cliché of acquiring great wealth through maritime trade. The second is the risk inherent in maritime trade. The Mediterranean, while effectively tideless with calm waters and clear visibility for most of the summer, can in fact present a variety of difficulties and hazards to the ancient mariner (Parker, 1992, p. 1). It is the

fact that Trimalchio was not financially ruined forever after losing five ships and 30 million sesterces that is meant to be unusual to his dinner guests, not that the ships were lost in the first place. Third, the mixed composition of the cargo is of note. We often assume ancient ships carried a cargo composed entirely of wine, but it could be argued that a mixed cargo represents a diversity of investments that could further minimise risk in a fluctuating marketplace, to which Trimalchio refers when he states the cost of wine has fallen in his lifetime. By diversifying a cargo of wine by adding bacon, beans and Capuan perfumes, Trimalchio appears to be hedging his bets.

Rather than the homogeneous cargo of wine conveyed on his first, and sadly unfortunate venture, the second trip transported wine, but also multifarious agricultural products, as well as human cargo. While this is admittedly a fictional account of maritime trade, the description of a diverse cargo is portrayed as neither strange nor unfamiliar to Trimalchio's guests. While investigating ancient shipwreck sites, it is useful to recall elements of Trimalchio's boastful speech: more than the remains immediately visible on the seafloor, it may be possible to recover molecular evidence for the hypothetical beans, bacon, wine and perfume transported on board. Given the diverse nature of goods transported on ships in antiquity, it is necessary to determine what scientific methods may be most helpful in exploring the remains of ancient shipwreck cargoes. One method may provide insights that another method may not. First, we must reflect on why it is necessary to characterise ancient shipwreck cargoes to begin with.

Coupled with what ancient authors such as Petronius have written about sea-borne trade, the archaeological remains of ancient merchant ships wrecked at sea allows us to study the physical remains of commerce and trade. Fortunes were acquired, or lost, through maritime trade. In the unfortunate cases of the shipwrecks studied in this thesis, these lost ships may well represent the dashed hopes for wealth and glory of an ancient investor(s) who did not have the vast funds of Petronius' fictional Trimalchio. No industry or activity practiced in the ancient world held the potential for rapidly making a profit more than long-distance maritime trade. Arguably, ancient shipwrecks, as the physical remains of long-distance maritime trade, are the best sources of evidence we have for the genesis of complex economic phenomena in the ancient

world. The artefacts they hold may be capable of providing insight into these fundamental developments in human history.

This work utilises stable isotope, organic residue, and DNA analysis to investigate cargo items from five ancient shipwrecks, in the hope that we can learn a great deal more about what these ships carried, and why. Artefacts analysed include objects from the Bronze Age Cape Gelidonya and Uluburun shipwrecks of Turkey (Bass, 1967; Pulak, 1998), the Classical and Hellenistic era Kyrenia and Mazotos shipwrecks of Cyprus (Demesticha, 2011; Katzev, 1969; Swiny and Katzev, 1973), and the Late Roman Babuljaš shipwreck of Croatia (publications pending). Some of the methods used in this thesis are novel in their application to shipwreck artefacts: included here is the first study that attempts to use stable isotope analysis on waterlogged botanical remains from a shipwreck, and the first application of metagenomic DNA analysis to ancient shipwreck artefacts and their associated environmental samples. A third method used here, organic residue analysis through Gas Chromatography / Mass Spectrometry (GC-MS), has long been applied to shipwreck amphorae (Formenti et al., 1978), and serves as a complimentary approach to the novel use of metagenomic analysis of ceramics from submerged sites. This work builds on the enduring symbiotic relationship between maritime archaeology and archaeological science.

For many decades the study of ancient shipwrecks, harbours, and sunken cities has offered tantalising clues to migration, mobility, trade, and urban development in human history. Artefacts recovered from shipwreck sites in particular have proved to be fertile material for experimental research into various avenues of archaeological science: early examples of C¹⁴ dating utilised wood from a shipwreck (Ralph, 1967), one of the first applications of organic residue analysis to ancient ceramics involved shipwreck amphorae (Formenti et al., 1978), and a study that reported the first successful extraction of DNA from bone involved a pig bone from the *Mary Rose* shipwreck (Hagelberg and Clegg, 1991). In recent years, the investigation of submerged prehistoric sites of ever greater antiquity has become a unifying focus across a wide range of disciplines (Sturt et al., 2018), thus raising the profile of underwater archaeology internationally and highlighting the potential for underwater archaeological sites to reveal aspects of ever earlier periods of the human past.

Despite the long convergence of archaeological science and underwater archaeology, it remains necessary to explore the merits of various scientific methods used to investigate organic archaeological remains, and how their application to artefacts from underwater sites may be either helped or hindered by unique aspects of the underwater deposition environment. Here, three separate techniques of scientific investigation are applied to artefacts recovered from five ancient Mediterranean shipwrecks, in an effort to better understand how effective these methods are in characterising shipwreck artefacts, what potential these techniques hold, and what limitations still exist in their application.

This chapter reviews the use of stable isotope, organic residue, and ancient DNA analysis in archaeological science, explores the application of these methods to artefacts from underwater contexts, and considers what aspects of the marine environment may present challenges during their utilisation in underwater archaeological research. While prehistoric landscapes are touched upon, it is ancient shipwrecks and the artefacts recovered from them that forms the focus of this research.

First, the nature of ancient shipwreck sites is explored, with special reference to their perceived status as unchanging ‘time-capsules,’ and how we can infer aspects of maritime trade from shipwreck evidence. Next, unique aspects of the marine deposition environment are considered. Finally, a review is given of how stable isotope, organic residue, and ancient DNA analysis has been, or can be, applied to artefacts from underwater archaeological sites.

1.1.1 Shipwrecks as archaeological sites

From the outset it would appear that archaeological sites on land lack the simplicity frequently observed on ancient shipwreck sites. On terrestrial sites hundreds, if not thousands, of years of habitation can leave a hodgepodge of architectural remains, refuse, domestic goods, imported finery, and charred agricultural remains. Layers of anthropogenic activity form archaeological

‘stratigraphy,’ and understanding the formation of this stratigraphy is a fundamental facet of archaeological research (Renfrew and Bahn, 2000). In fact, perhaps the greatest challenge faced by a terrestrial archaeologist is teasing out the complex stratigraphic relationships that exist among these diverse layers of human occupation, and establishing to what period of occupation, or by what human activity, the various objects, artefacts, or stratigraphic feature belongs. The complexity of a long-inhabited terrestrial site caused Matthew Johnson to state ‘you will never see a “trade” or “subsistence” subsystem in the archaeological record’ (Johnson, 1999).

The nature of ancient shipwreck sites gives us pause to reconsider this statement. For what is a ship, laden with goods, making its way to distant lands, if not a ‘trade subsystem’? For Adams, it is the fact that the artefacts recovered from ancient shipwrecks remain in their ‘context of use’ (Adams, 2001) which allows greater insight into the relationships among objects in such an assemblage. In contrast to a midden, a water-well, or urban drains where objects can be disposed of or lost over the course of centuries, the discrete nature of an ancient shipwreck context means there is no doubt the collection of artefacts found on such a site were deliberately assembled, placed on board a ship, and perished at the same moment. While the purpose of artefacts found on an ancient shipwreck may be unclear (cargo or provisions? Trade good or personal effects of the crew?), it would seem that their contextual relationship is not.

Everything found on an ancient shipwreck has one thing in common: regardless of artefact type, place of origin, or utility, these objects were all deliberately included together in the hold of one ship, as the demand for these goods, the price they could fetch, the prestige they could endow, or their usefulness on board, was greater in a geographical location across water from where they were produced or loaded on to the ship. As such, ancient shipwrecks are the physical remains of a trade process in action and allow us to study elusive trade systems dating from thousands of years ago.

However, the reality of site formation dynamics on ancient shipwrecks are far more complex than they would first appear. If unfavourable currents, winds, or hazards consistently occur in the same area then several ships may wreck in that perilous place, causing more complicated

stratigraphy. In the case of the Plemmirio wreck, Sicily, a ship from around 200 C.E. settled on top of, and adjacent to, the remains of a 6th century B.C.E. ship, and which in turn was partially covered by artefacts from Byzantine wrecks (Gibbins, 2001). Muckelroy has discussed this phenomenon, calling it a 'smear of shipwrecks' (Muckelroy, 1978). This phenomenon has recently been observed off the Greek islands of Fourni, where over 58 shipwrecks from the Classical to Medieval periods have been discovered in close proximity to one another (Triandafyllou and Tsakiri, 2018).

Broadly, the archaeological context of a shipwreck implies the objects recovered are of contemporaneous production, which imbues the assemblage with heightened value (Gibbins, 2001, p. 315). However, ships themselves may come with their own 'stratigraphy', including residues of past cargoes, modifications to the hull, and layers of ballast which in some cases may extend back for more than a century (Adams, 2001, p. 297). On the Gnalič shipwreck, a 16th century merchant ship which sank off of Gnalič, Croatia, the gravel ballast contained worn fragments of Roman pottery, which predated the wreck by over 1,000 years (personal observation). The gravelly ballast stones had been originally collected in the 16th century from a rocky beach where the remains of a Roman villa had imparted ancient pottery to the shoreline (Rossi 2019, personal communication). Apart from ballast or timbers, personal objects or cargo items may also have been antique at the time of sailing. A cylinder seal found on the Cape Gelidonya shipwreck pre-dates the wrecking event by several centuries and was likely an 'heirloom' or 'antique' at the time it was placed onto the ship (Buchholz, 1967). The infamous Batavia shipwreck which sank in 1629 was carrying a cameo created circa 312 C.E. that was considered to be one of the most valuable items aboard a ship filled with gold, silver and other precious items destined for the Eastern headquarters of the Dutch East Indian Company (Dash, 2002). These examples serve to illustrate the complexity of a shipwreck assemblage, which may not always display the 'contemporaneous production' suggested by Gibbins (2001, p. 315).

Contemporaneous or not, the assemblage itself may be disturbed between the wrecking event, and discovery. Cargo items from wrecks in shallow waters may be salvaged, even in antiquity, and the salvors may originate from a very different society than the one that fostered the

building of the ship (Adams, 2001, p. 297), further complicating the site formation processes. A fascinating example of this comes from the famed Madrague de Giens shipwreck, found in the late 1960's off the southern coast of France. Amongst the cargo of amphorae, a conspicuous number of stones were scattered throughout the site, causing excavators to wonder whether these stones formed part of the cargo, or were ballast stones from the ship in very unusual places. After geological analysis of these stones had been undertaken, it transpired that these were local stones from the Giens peninsula, and further investigation showed that free-diving looters had used these stones to plunge more effectively to the bottom, collected amphora, and left the stones behind as they swam to the surface with their looted artefacts (Tchernia, 1987).

Despite a depth of 18 – 20 metres, it appears that not only had free-divers been able to loot items from the Madrague de Giens, but they had added a modern layer of objects to the assemblage, complicated the interpretation of the site. The line between salvage and looting may remain in the eye of the beholder, but either activity by SCUBA divers can further undermine the 'time-capsule' aspect of ancient shipwrecks. The presence of modern humans SCUBA diving, free-diving, or simply moored above underwater archaeological sites can increase the potential for macro-remains and molecules to enter into the archaeological record, which can undermine our attempts to use archaeological science to determine what ancient ships carried in the past. As a final example of unexpected site formation dynamics, on an ancient shipwreck site in the Mediterranean, the excavators noticed strange objects slowly falling through the water column and settling on the site as while they excavated. Upon closer inspection, these were the remains of fried chicken wings, and once the archaeologists surfaced it transpired that colleagues had brought buckets of fried chicken to their team on the research vessel moored above the site. Luckily, an archaeological scientist with an appreciation for study of faunal remains, evidence for animal fats, and ancient DNA was present, and a 'do-not-throw-chicken-bones-off-the-boat' policy was quickly established. While it may not be possible to protect the integrity of underwater archaeological sites from exogenous contamination, we can certainly try.

The growing popularity of recreational SCUBA diving can be linked directly to the potential for looting or general disturbance by humans of underwater archaeological sites to occur, as

these sites were previously inaccessible. Even to the untrained eye, a mound of hundreds of amphorae standing proud on the seafloor is incongruous with the natural Mediterranean seascape, making ancient shipwrecks easily identifiable (Frost, 1963; Muckelroy, 1978; Parker, 1992). To what extent closed-shape ceramics such as shipwreck amphorae remain protected from exogenous molecules and macro-remains must be established. Central to any discussion on ancient shipwrecks, the nature of cargoes, and long-distance maritime trade, are these humble ceramic transport containers, referred to in the Classical and later periods as ‘amphorae,’ and in the Bronze Age as ‘jars.’ Understanding what the amphorae and jars transported aboard ancient ships contained on their last voyage can provide invaluable insight into the motivations for the journey, the ports visited, the agricultural products produced in the hinterland of these ports, and the social systems in place that ushered these goods from the soil to the sea.

1.1.2 Maritime trade, amphorae contents, and shipwreck evidence

A wide variety of artefact types recovered from shipwrecks have been explored to infer aspects mobility and ancient maritime trade, including stone anchors (Frost, 1963), archaeobotanical remains (Haldane, 1993), metal ingots (Muhly et al., 1977), and cargoes of stone (Russell, 2013). However, it is vital to recall that only relatively durable cargo items, or those covered by seafloor sediments, are likely to survive among ancient shipwreck assemblages, with textiles, baskets of goods, and wooden hull structure unlikely to survive in the archaeological record if these elements of the ship remain above anoxic sea muds. As with the stone anchors, metal ingots, and architectural stone cargoes examined in the studies above, shipwreck amphorae do not decay on ancient shipwreck sites, even when they remain exposed in the water column.

More conspicuous than concreted metal ingots, stone anchors, or architectural stone cargoes, ceramic transport amphorae constitute the major part of most ancient shipwreck assemblages that survive in the archaeological record, and form the bulk of archaeological remains by which

ancient shipwrecks sites are discovered in the Mediterranean (Frost, 1963; Muckelroy, 1978; Parker, 1992b) and studied to illuminate aspects of ancient trade (Wilson, 2011). Resistant to decomposition and highly durable, amphorae settle into mounds on the seafloor as the wooden hull structure of the ship rots away, allowing for discovery by SCUBA divers (Wilson, 2011, p. 37), and insights into the nature of the cargo the ship carried. Evidence from ancient shipwrecks is of crucial importance to our understanding of the development and modalities of long-distance trade in antiquity (Robinson and Wilson, 2011), and is vital to produce any accurate reconstruction of ancient economies.

Attempts to quantify the volume of maritime trade throughout different time periods in Mediterranean history had previously been based on the number of shipwrecks from different time periods (Parker, 1992). As piles of amphorae are so conspicuous on the seafloor, there is an inherent bias in using amphorae-laden wrecks to quantify trade. These quantifications may in fact simply chart amphora use (Horden and Purcell, 2000), and what had first appeared to be a dearth of trade in the 2nd century C.E. may in fact be the results of the increased use of wooden barrels in maritime trade (Wilson, 2011, p. 37), a type of container that would not survive readily in the archaeological record to be quantified. Despite the replacement of amphorae with barrels in later periods, for approximately two millennia ceramic transport containers were the preferred vessels for transporting goods. What goods they contained, however, may not be clear.

As pottery constitutes a central class of artefacts preserved in the archaeological record, this important material must be ‘appropriately interrogated’ (Renfrew, 1972). These interrogations continue to grow and evolve as new archaeological material comes to light. Long before the invention of SCUBA, early studies of amphorae excavated from Monte Testaccio in Italy (Dressel, 1899) and the Athenian agora (Grace, 1961, 1946, 1934), often assigned a specific cargo type to an amphorae of a given shape and geographic origin, aided substantially by the study of stamped amphora handles or *tituli picti* which provided insight into the origin of these vessels, the past vessel contents and their ancient producers. Distinctive elements of shape were identified and catalogued: the angle of amphorae handles, the presence or absence of a bulge in the neck, the shape of the toe, all could be used to distinguish vessels from a given

geographical location (Whitbread, 1995). If a given product was noted from a certain region of amphorae production, a close association between geographic origin and past vessel contents developed. Long after the early work of Dressel, Grace, and others, the idea that amphorae shape can be used to establish function and past vessel contents has endured (Koehler, 1995; Lawall and Lund, 2011).

As amphorae are the predominant feature on ancient Mediterranean shipwrecks, as discussed above, their already-important status in the archaeology record takes on new significance on shipwreck sites. In fact, amphorae are so closely associated with maritime trade and overseas shipping that some have called for the renaming of these vessel to 'Maritime Transport Containers' or MTCs (Knapp and Demesticha, 2017). Others have argued that commercial amphorae represent the earliest example of consumer packaging (Twede, 2002), as certain shapes came to represent a given product, and to some degree, the quality of that product. Attempts to use the distribution of ceramic amphorae to quantify aspects of ancient trade certainly have merit, and as more pottery is discovered the strength of these statistically models will grow (Tomber, 1993). However, this is predicated on the idea we can safely assume what amphorae contained. If this approach is taken during the survey of an ancient shipwreck site, it would appear that as soon as a positive identification has been made of the shape and geographic origin of the majority of the amphorae, one could safely state the nature of the cargo contained within these jars in the past. The products assumed to have been contained in amphorae are often limited to three commodities: wine, olive oil, and fish sauce. However, the reality of amphora use may not have been so straightforward.

The nature of cargo assemblages in terms of homogeneity or heterogeneity will inevitably have oscillated between various time periods, and is likely a reflection of numerous factors including the methods of maritime trade (cabotage or emporia), the power of central administrative authorities to direct agricultural pursuits, the levying of taxes, the shifting tastes of consumers, the reliability of sea-borne trade, and fluctuating market prices. The diverse cargoes seen on Late Bronze Age shipwrecks such as the Uluburun, or Cape Gelidonya, may not be seen in the massive, and seemingly homogenous shipments of wine from Italy to Gaul as evidenced on the Roman wrecks of Albenga and Madrague de Giens. However, recent work has highlighted the

normality of mixed cargoes, by examining 54 Eastern Mediterranean shipwrecks from the Roman Period that demonstrate how the ebb and flow of network connectivity could have a profound influence on the goods transported by maritime trade (Leidwanger, 2017; Leidwanger et al., 2015). For the Western Mediterranean, Rice (2016) has highlighted how the relative heterogeneity of a cargo has been used to argue for either a tramping model of maritime trade, also known as cabotage or the ‘Brownian model’ (Morley, 2007), or a direct emporium model which envisions large centres of redistribution (Arnaud, 2005; Wilson, 2009) (Rice, 2016). These debates are germane to the present research in that the crux of these arguments rely on the *heterogeneity* of shipwreck cargoes. It is argued here that judgements are made all too quickly regarding the true nature of what is contained within the amphorae found on ancient shipwrecks, and therefore how homogeneous or heterogenous the cargo actually was on any given ship.

One example would be ‘wine amphorae.’ Far from only containing wine, so-called wine amphorae found on ancient shipwrecks have contained organic items including butchered beef bones (Carlson, 2003), butchered pork (Bruni, 2000), almonds (Swiny and Katzev, 1973), whole olives (Demesticha, 2011; Haldane, 1993; Santamaria, 1995), orpiment (Bass, 1995), pomegranates (Haldane, 1993) and pitch (Carlson, 2003). Inorganic items found in amphorae include glass beads (Pulak, 1998), copper coins (Sharvit et al., publication pending) and broken pieces of metal statuary (Galili et al., publication pending). Thus, in order to create accurate models of maritime trade, we must understand the relative homogeneity or heterogeneity of cargoes, and in order to do that, we must understand what amphorae carried aboard ships actually contained. Attempting to reconstruct aspects of ancient trade by assuming unfailing homogeneity of exports from a given island or city with a unique amphorae shape has drawbacks. This can lead to false assumptions regarding the goods amphorae contained, and the volume of trade in these goods (Dallongeville et al., 2011).

While macro-remains can be preserved in an underwater environment, the opposite could be said for past amphorae contents that are in a liquid or soluble state. Stoppered amphorae like those recovered from the Madrague de Giens shipwreck (Formenti et al., 1978, p. 95), and Albenga shipwreck (Arobba et al., 2014) are exceptional; the norm is to find unstoppered

amphorae with no visible remains inside. Wine, olive oil, and fish sauce are the ancient Mediterranean products most often associated with amphorae (Pecci et al., 2013, p. 109), and these liquid commodities do not typically leave behind macro-remains. In such cases, molecular evidence is needed to be sure what these vessels contained. Yet, just as shipwrecks may have their own stratigraphy that forms over the course of the life of a ship, so too could ceramic transport containers preserve palimpsests of residue in their porous walls. Widespread evidence for the reuse of pottery in the ancient world (Peña, 2007), gives us pause to consider whether the macro-remains or molecular evidence we find in an amphora represents its first, intermediate, or last use. It seems that in order to characterise a seemingly heterogeneous cargo of similar amphorae on an ancient shipwreck it is necessary to go beyond simply noting the shape and geographic origin of the ceramic transport containers found there.

Compounding the myriad issues surrounding the use of amphorae on ancient ships is evidence that amphorae were occasionally transported empty, with the intention that they would be sold and reused at their destination. It has been previously stated that there is little evidence from shipwrecks that amphorae were transported empty (Parker, 1992), yet according to a contract dated to 176 C.E. and preserved on papyrus from Roman Egypt, 200 Caesarion jars were to be sold and transported empty, by ship, to their buyer (Adams, 2017). With this in mind, it is worth considering the value of both archaeological evidence and documentary evidence in our search for what ancient amphorae truly contained. An amphora that appears empty on the seafloor may have been empty when loaded on the ship, further complicating our attempts to determine past vessel contents. It appears that sometimes these amphorae contained nothing at all.

Adding to this conundrum is the issue that certain amphorae contents will simply be better preserved in the underwater environment than others. The dynamic marine environment will inevitably interact with amphorae and the items they contain on biological, chemical, and molecular levels. In some instances, the marine environment may increase preservation, and in others it may accelerate decay.

1.1.3 Aspects of the marine environment

In order to explore how the marine environment may affect the artefacts recovered from ancient shipwrecks, it is necessary to examine the nature of seawater, and the creatures that live within it. Seawater consists of a solvent (water) and a complex mixture of particulate matter, gas bubbles, and chemical constituents (Florian, 1987). The principal ions present as chemical constituents are presented in the Table 1.

Table 1. Relative amounts of dominant ions which make up 99.5% of the dissolved chemical constituents in seawater. After Florian 1987, 3.

Ion	Amount in water at 35‰ salinity (g/kg)
Cl ⁻	19.35
Na ⁺	10.76
SO ₄ ²⁻	2.71
Mg ²⁺	1.29
Ca ²⁺	0.41
K ⁺	0.39

As is shown above, chlorine (Cl) and sodium (Na) are the most prominent ions in seawater. Due to the relatively porous nature of the pottery and organic remains analysed here, it is likely that levels of Cl and Na are elevated in these artefacts, with detection of low levels of SO (sulphide), Mg (magnesium), Ca (calcium) and K (potassium) also possible.

Previous studies on Rhodian amphorae recovered from ancient shipwreck contexts suggest that ratios of trace elements in the ceramic fabric may be altered due to deposition in the marine environment (Hein and Kilikoglou, 2017). A study involving dozens of Rhodian amphorae found that concentrations of Cs (caesium) and Rb (rubidium) were conspicuously low in the two Rhodian amphorae samples recovered from shipwreck sites, causing the authors to

speculate that these elements had leached out into the seawater (Hein and Kilikoglou, 2017, p. 568). As ratios of trace elements have been shown to be useful in determining the provenance of ceramic artefacts, the so called 'provenance postulate' (Weigand et al., 1977), it is important to recognise that these ratios may be altered in the marine environment, potentially undermining the utility of trace element analysis in determining the origin of ceramic artefacts recovered from ancient shipwrecks.

In addition to interacting with trace element composition, the marine environment may also have an effect on the isotopic ratios found in organic material deposited there. The stable isotope analysis of carbon in organic archaeological material relies on establishing the $\delta^{13}\text{C}$ value for the tissues analysed, yet the $\delta^{13}\text{C}$ value for marine organic matter is established during the photosynthetic fixing of CO_2 or bicarbonate by phytoplankton within the marine environment (Hansell and Carlson, 2002). As traces of marine organic matter may adhere to archaeological materials it is necessary to consider how this may affect the isotopic ratios found for waterlogged organics from marine sites (Figure 1 -2).



Figure 1. Evidence of biofouling on a Samian amphora sherd from the Kyrenia shipwreck.

Ancient wrecks create artificial reefs that attract marine organisms and provide protection for juvenile fish. Biofouling, the term given to instances in which marine organisms adhere to man-made objects in the sea, could have an effect on the molecular composition of shipwreck artefacts.



Figure 2. Biofouling on Kyrenia shipwreck sherd at 20x magnification.

A vast and diverse collection of marine creatures can form a discrete ecosystem on shipwrecks. In a limited survey of fourteen shipwrecks, that excluded microscopic organisms and borers, 306 different species were identified including diatoms, numerous types of algae, hydroids, sponges, coelenterates, seaworms, flatworms, tubeworms, nudibranchs, diverse species of barnacle, gastropods, pelecypods, tunicates and echinoderms, among others (Florian, 1987, p. 14). The extent to which this biofouling can contribute to change in the chemical and molecular composition of shipwreck artefacts must be considered during characterisation of this material.

Seawater is a solvent and deposition within it likely has implications for organic residue analysis of shipwreck ceramics. Two compounds used to identify traces of wine, tartaric and syringic acid, are extremely water-soluble and prone to leaching (Barnard et al., 2011; Pecci et al., 2013; Romanus et al., 2009), which may mean that in underwater deposition environments, even biomarkers for wine that had previously penetrated into the ceramic matrix could be lost into the natural solvent of the water column.

It is also necessary to consider the composition of seafloor sediment, and the potential chemical alterations that deposition in the seafloor sediment / water column interface may induce. Seafloor sediment is composed of organic and inorganic material that enters the water column through fresh water run and shoreline erosion, suspended particulate matter which settles on

the seafloor, deceased remains of plankton, marine organisms, micro-organisms such as bacteria, and solids created from dissolved loads precipitating out of their liquid state through chemical precipitation (Florian, 1987, p. 8). A multitude of the organisms found in seafloor sediment may impart their DNA to this matter as it accumulates around ancient shipwreck sites. Recent applications of metagenomics on archaeological material have focused on environmental DNA recovered from submerged landscape sites and inland lake environments which may hold genetic information about the local environment in the past (Niemeyer et al., 2017; Sjögren et al., 2017; Smith et al., 2015). Such applications may also be possible for seafloor sediments collected from shipwreck sites.

Of the various site formation processes that must occur for the remains of a ship to be preserved, it is the rapid burial under sediments that will have the most profound influence on whether currents, marine-borers, salvage attempts, aquatic plants, and marine animals will have the opportunity to disturb a wreck site (Stewart, 1999). Many factors contribute to the composition, and accumulation, of seafloor sediment including the water content of the interstitial space, the particle size, and degree of compaction at a given locality, and these physical characteristics influence the chemical reactions that occur in the seafloor sediment of a given area (Florian, 1987, p. 8). Local levels of rainfall, proximity to an estuary environment, and the quality and quantity of soils and sediments on proximal landmasses will all be contributing factors in the sedimentation rate of the local seafloor.

Depth and salinity will also influence what organisms are found in seafloor sediment: amounts of dissolved O_2 decrease as salinity increases (Menzel and Ryther, 1968), so marine organisms that are reliant on this O_2 , and would consume archaeological material in shallower sites with lower salinity, may not be present on deeper sites. Recent work in the Black Sea has demonstrated the extraordinary level of preservation possible for ancient shipwrecks in areas of high salinity and low dissolved O_2 (Davis et al., 2018).

The shipwreck artefacts analysed here were all collected from partially buried areas of the sites that occur at a depth of 50 metres or less in the Mediterranean Sea. At this depth, the

seawater/sediment interface is a dynamic area with the highest levels of bacteria in the marine environment, shifting from aerobic bacteria at the upper, oxygen rich levels, to facultative bacteria when oxygen levels begin to wane at lower levels, finally culminating in anaerobic bacteria in the anoxic sediment regions (Florian, 1987, p. 15). Preservation of organic cargo macro-remains is best achieved lower down in the seafloor sediment where aerobic bacteria are excluded. Yet at these greater depths in the sediment new issues may arise, including higher levels of sulphur.

Initial analysis of several of the shipwreck ceramics including in this research revealed evidence of sulphur. Through examination by Scanning Electron Microscopy (SEM) of a cooking pot from the Kyrenia shipwreck framboidal pyrites were revealed to have formed within the ceramic (Figure 3).

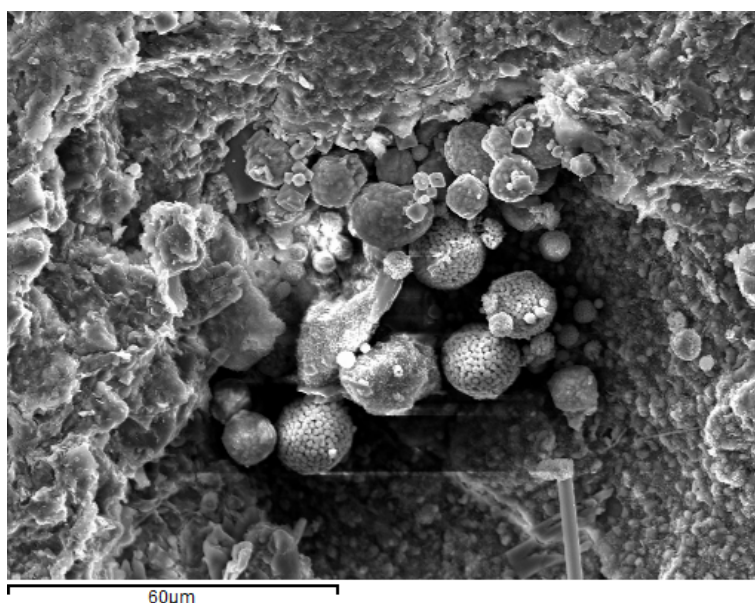


Figure 3. Framboidal pyrite in cooking pot P137, Kyrenia shipwreck.

The interaction of carbon dioxide, iron sulphide and hydrogen sulphide can result in organic sulphur compounds in seafloor sediments (Heinen and Lauwers, 1996). Framboidal pyrite is an iron and sulphur compound composed of iron monosulfide microcrystals that can form in anoxic marine environments (Wilkin and Barnes, 1997), and are called after the French *frambois* for their distinctive appearance which is similar to raspberries (Figure 4).

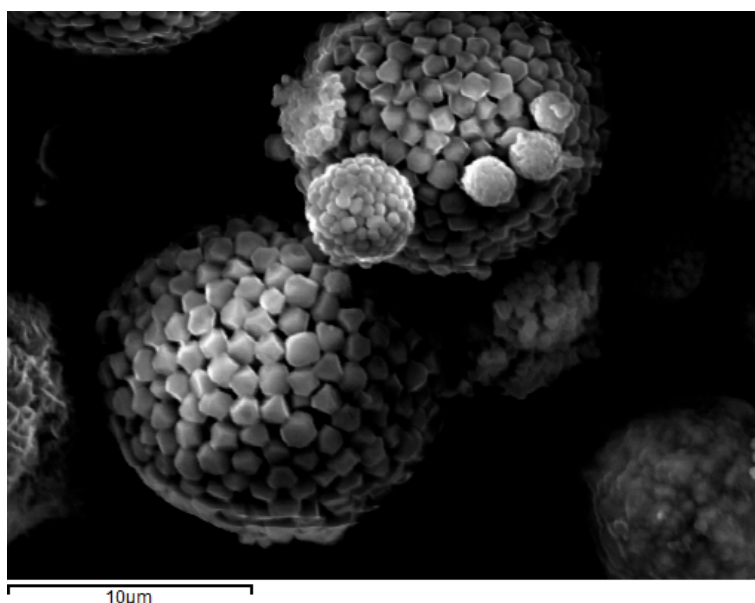


Figure 4. SEM image of framboidal pyrite on cooking pot P137, Kyrenia shipwreck.

The formation of framboidal pyrite will depend on the level of sulphur in the local seafloor sediment, as this will affect the amount of sulphur imparted to archaeological material. High levels of sulphur can complicate the conservation of shipwreck wood (Fors, 2008) and can damage GC-MS columns used in the organic residue analysis of archaeological material.

As a further example of the dynamic nature of the marine environment effecting ancient shipwreck artefacts, there is evidence that certain marine species interact with the artefacts on ancient shipwrecks. Many species of fish make their homes in amphorae; some even decorate the interior. On several ancient shipwreck sites octopuses have been observed making their homes in ancient amphorae, collecting other artefacts from the wreck, and transporting them into their home. This phenomenon was observed decades ago (e.g. Frost, 1963), and has been noted as occurring on numerous sites, including the site of the Egadi sea battles from circa 241 B.C.E. (Tusa and Royal, 2012), but has not been properly investigated. Octopus behaviour could have implications for our interpretation of ‘vessel contents,’ as some objects found inside a shipwreck amphora may have been due to a quirky behavioural aspect of cephalopods, rather than empirical evidence for past vessel contents. And it is empirical evidence that is needed in order to make declarative statements about what shipwreck amphorae transported, anything else is just a ‘best guess’.

1.2 Exploring past vessel contents

Richard Evershed saw the contents of vessel recovered from archaeological sites as occurring in three states: i) actual contents (preserved and visible to the naked eye), ii) surface residues visible on the interior surface of the vessel, but amorphous, and impractical to characterise without chemical analysis, and iii) ‘absorbed residues preserved within the vessel wall, invisible to the naked eye’ (Evershed, 2008, p. 904). In most archaeological contexts, it is likely that the frequency of these finds is a direct inversion of their size, with the macro remains of actual vessel contents being the least frequent and the invisible residues inside vessel walls being the most frequent. This is due to the fact that macro-remains and their associated greater surface area offer a ready food supply for bacteria and other microorganisms, while absorbed residues are protected by a ceramic substrate.

Following the categories defined by Evershed, this thesis investigates shipwreck amphorae by attempting to characterise vessel contents that have occurred in these three states – the macro-remains of whole olives, residues that are barely visible on amphorae walls, and DNA, which is entirely invisible to the naked eye.

1.2.1 Isotope analysis of macro-remains

By far the simplest way of characterising the contents of a transport vessel recovered from an underwater context is to empty it, and, if preserved, examine the contents. The visible organic remains on archaeological sites are variously called ‘macrofossils’ (Dillon Gorham and Bryant, 2001), ‘ecofacts’ and ‘macro-remains’ (Kelly and Tomas, 2011). In this thesis, the term macro-remains will be used. The possibility for the recovery of exceptionally well-preserved organic remains is a feature of underwater archaeology (Bowens, 2009), with both submerged

settlement sites and shipwrecks yielding organic remains that would otherwise have perished on terrestrial sites.

In this research, stable isotope analysis is used to investigate a type of macro-remain that is frequently found on shipwreck sites (Bound and Vallintine, 1983; Demesticha, 2011; Frey, 1982; Haldane, 1993; Lanza, 2011; Santamaria, 1995) but has been largely ignored in the literature on archaeobotanical remains: the whole olive. While the production, shipping, and trade in olive oil in the ancient world is much discussed (e.g. Foxhall, 2007; Mattingly, 1988; Warncok, 2007), the consumption and trade of whole olives has not been adequately explored given the importance of this species of tree in the Mediterranean region. In order to provide context for the work presented in this thesis research, which constitutes the first use of stable isotope analysis on waterlogged botanical remains from an ancient shipwreck, a review is presented of the use of isotope analysis to investigate the geographic origins and cultivation conditions of archaeobotanical remains.

1.2.1.1 Carbon and nitrogen isotope analysis of charred and desiccated plant remains

There are many ways to investigate botanical remains from archaeological sites, but one method that is well established for artefacts recovered from terrestrial sites is analysis of the stable isotopes of carbon (^{12}C and ^{13}C) and nitrogen (^{14}N and ^{15}N). Differing levels of water, sunlight, and temperature have an effect on the ratio of $^{12}\text{C}/^{13}\text{C}$ in plant material, expressed as $\delta^{13}\text{C}$, while the ratio of $^{14}\text{N}/^{15}\text{N}$, expressed as $\delta^{15}\text{N}$, can further indicate aspects of the soil and the amount of water available during the growth of a plant (Brown and Brown, 2011, p. 84), and whether or not nitrogen-rich manure was used during a plant's cultivation (Bogaard et al., 2013; Kanstrup et al., 2011; Styring et al., 2014). This type of analysis has been used to investigate the geographic origin and the cultivation conditions of preserved agricultural products from both archaeological sites (Bogaard and Outram, 2013; Fiorentino et al., 2015)

and in the modern olive oil industry (Angerosa et al., 1999; Gumus et al., 2017; Portarena et al., 2014).

It is predominantly charred plant remains and phytoliths that are analysed in studies of organic archaeological remains (Fiorentino et al., 2012; Harvey and Fuller, 2005; Hillman, 1981; Jones, 1996, 1984; Lightfoot and Stevens, 2012; Styring et al., 2017; van Der Veen, 2007). An early study of the isotopic composition of archaeological plant remains argued that charred plant remains were less susceptible to alteration in the deposition environment than other types of preserved archaeobotanical remains (e.g. desiccated or waterlogged) and were therefore more likely to preserve their original isotopic ratios (Deniro and Hastorf, 1985). This influential paper, combined with the abundance of charred plant remains on terrestrial archaeological sites, may explain the focus on charred remains in archaeological studies of the stable isotopes of ancient plant material (Metcalf and Mead, 2019).

Experimental charring of modern plant material seemed to support DeNiro and Hastorf's (1985) argument, as studies showed very little shift in $\delta^{13}\text{C}$ value as a result of the charring process (Fiorentino et al., 2015; Poole et al., 2002). Recent work has questioned DeNiro and Hastorf's influential conclusion that the isotope composition of non-charred archaeobotanical material would be significantly diagenetically altered (1985), and results from this recent study found that desiccated plant remains from the Pleistocene – Holocene transition (circa 13,000 - 7,000 before present) were consistent with good preservation, including a noted difference between the isotopic values found for leaves versus seeds, C_3 plants versus CAM plants, and environmental preferences by species (maple versus oak) (Metcalf and Meade, 2019, p. 22).

Previous investigations into diagenetic alterations in desiccated plant material indicated that in hot, arid environments Maillard reactions occur in plant remains, producing volatiles that in temperate climates would be released during the decomposition of the organic matter, but in hot and arid environments are trapped within the desiccated plant remains (Evershed et al., 1997). While desiccated archaeological plant remains which had undergone Maillard reactions mostly preserved their original polysaccharide and protein composition (Bland et al., 1998), it

remains unclear what level of fractionation occurs in plant remains that were desiccated at temperatures lower than those found in Egypt (Metcalf and Meade, 2019, p. 6), an area from which an abundance of desiccated plant material has been studied (Evershed et al., 1997; Veen, 1996).

While it has been established that both charred plant remains (Fraser et al., 2013; Styring et al., 2014) and desiccated plant remains (Evershed et al., 1997; Metcalf and Mead, 2019) can be successfully analysed by stable isotope analysis, the effects of waterlogging by seawater on the isotopic composition of archaeobotanical remains has not yet been determined. As waterlogged agricultural products from submerged landscapes and shipwreck sites are subject to a different preservation bias than charred material (Colledge and Conolly, 2014) or desiccated plant material, and do not rely on proximity to fire, the remains from the Mazotos shipwreck may offer new insights into past agricultural pursuits. Shipwrecks are neither production sites, nor domestic areas, and thus provide an entirely different context for the packaging, shipping, trade and consumption of agricultural products. Furthermore, as the mechanisms by which organic remains are preserved through water logging are different to that of charring or desiccation, it is probable that diagenetic alterations in the isotopic ratios of waterlogged material will differ from their charred or desiccated counterparts.

While the archaeological literature abounds with articles that confidently utilise carbon and nitrogen isotope analysis to determine geographic origins, harvesting practices, and storage schemes in the past (Fiorentino et al., 2012; Heaton et al., 2009; Lightfoot and Stevens, 2012), it is worth noting that studies which utilise modern plant material find a significant variation in the isotopic values of plants grown in a single, established location. One study found that levels of CO₂ were significantly elevated near the forest floor up to a height of 3 metres, and that the average leaf $\delta^{13}\text{C}$ for the four understory plants sampled from the area below 3 metres showed a wide range of variation in their average leaf values of 4‰ (Berry et al., 1997, p. 499). More recently, a similar result was found for grasses of the same species grown in shade at the edge of a woodland, and in full sunlight, with a difference in values up to 5‰ (Bonafini et al., 2013). If plants of the same species, grown in the same geographical location, can display values that vary up to 4‰ and 5‰ simply due to their height (above or below 3 metres) or their

position (in shade or in sunlight) this could significantly undermine our ability to confidently use the stable isotopes of carbon and nitrogen in to pinpoint geographical origin, or interannual harvests in archaeological material.

Further complicating the picture, it appears that genotype within the same species may also affect the measures levels of carbon and nitrogen isotopes. A study that analysed the genotype to environment interaction among ponderosa pine trees (*Pinus ponderosa*) showed that genotype may be a strong contributing factor in $\delta^{13}\text{C}$ values, in addition to geographic location (Cregg et al., 2000). Comparison with modern sample sets such as these can provide tremendous insights into the potential for wide variation found in archaeological remains, and the difficulty in interpreting what this variation means for our understanding of past agricultural practices.

1.2.1.2 Oxygen isotope analysis and precipitation

There are three stable isotopes of oxygen that occur in nature: ^{16}O , ^{17}O , and ^{18}O , with ^{16}O occurring the most frequently (Brown and Brown, 2011, p. 86). The ratio of $^{18}\text{O}/^{16}\text{O}$ is expressed as $\delta^{18}\text{O}$, and the variability of this ratio in rainwater is determined by altitude, latitude, temperature at meteoric precipitation, and distance from the sea (Brown and Brown, 2011, p. 86). Based on this geographic variability, oxygen isotopes can be used to infer geographic origin, as the $^{18}\text{O}/^{16}\text{O}$ ratio in local water sources can fix within mineralised tissues in animals and humans hydrated by a local water source. Oxygen isotope analysis has been used to pinpoint geographic origin and climatic variation in archaeological remains (Evans et al., 2012; White et al., 1998), however, it may not have the same resolution and geographic specificity as strontium isotope analysis (Brown and Brown, 2011, p. 86), which is discussed below. Several studies have utilised a combined approach of oxygen isotope analysis with either carbon (Angerosa et al., 1999), or strontium (Benson et al., 2006), which may be able to pinpoint the origins of plant remains with greater geographical accuracy.

Oxygen isotope analysis was considered as a method for this research, but as the laboratory standard by which we measure the ratios of oxygen isotopes is the Standard Mean of Ocean Water (SMOW), archaeological remains which have been saturated by ocean water for millennia may be more likely to yield results closer to the laboratory standard than their original isotopic ratios. It remains unclear whether or not ocean water, and its specific $\delta^{18}\text{O}$ value, would contaminate waterlogged olive stones. Mineralised material such as stone, tooth enamel, or bone apatite seem less likely to be contaminated, but more porous material such as olive stones that are fully saturated with ocean water may well have their $\delta^{18}\text{O}$ values compromised by the ocean water in which they have remained for over two millennia. Due to the ambiguity surrounding to what extent $\delta^{18}\text{O}$ values for waterlogged plant remains may reflect the Standard Mean of Ocean Water (SMOW), this method has been excluded from the isotope analysis conducted here.

1.2.1.3 Strontium isotope analysis and geographic origin

Strontium (Sr) isotope analysis has been effectively used to infer the regional origin and patterns of migration for faunal remains (Britton et al., 2011, 2009; Gignoux et al., 2019), and human remains (Bentley, 2006; Evans et al., 2006; Snoeck et al., 2018). Strontium has four natural isotopes: ^{84}Sr , ^{86}Sr , ^{87}Sr , and ^{88}Sr , with the latter being the most commonly occurring isotope at 82.58% of the total for Sr isotopes worldwide (Brown and Brown, 2011, p. 87). The ratios of these strontium isotopes (expressed as the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio) depend on the parent geology of an area, as varying amounts of strontium leach from local rocks into drinking water and soils. As plants and animals consume this water, or higher-level predators consume the plants and animals hydrated by these local water sources, strontium is fixed within the calcium-rich elements of skeletal remains including bone apatite and tooth enamel. This method has also been used to investigate the geographic origin of archaeological plant remains including timber used in domestic structures (English et al., 2001; Reynolds et al., 2005) maize (Benson, 2012),

willow and tule baskets (Benson et al., 2006), and cedar hull structure components from ancient watercraft (Rich et al., 2016a). While shown to be effective in determining origins and mobility for humans, animals, and plant remains from terrestrial archaeological sites, questions have been raised regarding how suitable this method is for determining the geographic origin of plant remains from marine archaeological sites.

First, strontium isotope analysis is most often applied to mineralised material such as bone apatite or tooth enamel. In the case of waterlogged plant remains that are not mineralised, it is possible that the values found are more likely to reflect the local water sources present in the underwater deposition environment, rather than the $^{87}\text{Sr}/^{86}\text{Sr}$ isotope values found where the plant was grown. In arid conditions, highly desiccated plant remains like architectural timbers (English et al. 2001; Reynolds et al. 2005), maize (Benson, 2012), and the willow and tule baskets (Benson et al., 2006) cited above are inevitably less susceptible to contamination from the modern and local water of their deposition environment.

When strontium isotope analysis was attempted on waterlogged cedar from ancient watercraft (Rich et al., 2016a, 2016b), results seemed to suggest diverse origins for the cedar; some cedar timbers showed a similarity to cedar from the Troodos mountain range in Cyprus, some from Lebanon, while timbers from the Uluburun shipwreck yielded $^{87}\text{Sr}/^{86}\text{Sr}$ signatures that did not match to any known cedar source (Rich et al., 2016b, p. 520). However, during experiments on the efficacy of removing exogenous Sr from wool textiles buried in both terrestrial soils and marine muds, it was shown that two experimental methods including exposure to N_2 gas and extraction by organic solvents appeared to successfully remove environmental Sr from textiles buried in terrestrial soils, but neither of these methods successfully removed environmental Sr from the wool textiles that had been buried in a marine context (Von Holstein et al., 2015). These results challenged earlier findings which used $^{87}\text{Sr}/^{86}\text{Sr}$ analysis of waterlogged wool textiles to argue for long-distance trade in textiles in the Iron Age (Frei et al., 2009). Further, the work of Von Holstein et al. (2015) calls into question the findings of Rich et al. (2016a; 2016b) by undermining the viability of successfully removing exogenous Sr from waterlogged archaeological samples.

At the heart of this issue is the fact that the strontium isotope value for ocean water is globally consistent at 0.7092 (Whipkey et al., 2000), while the $^{87}\text{Sr}/^{86}\text{Sr}$ value for terrestrial geosystems falls between 0.720 to 0.703 depending on the age of the local geological formation (Brown and Brown, 2011, p. 86). In fact, research has shown that the so-called ‘sea-spray effect’ can impart a $^{87}\text{Sr}/^{86}\text{Sr}$ value closer to ocean water than local parent geology to terrestrial areas proximal to sea, and at distances inland from 50 metres up to several kilometres in cases where significant ocean swells cause high levels of sea-spray such as the conditions present at the island of Hawaii (Whipkey et al., 2000), known locally as the Big Island.

The tendency for ocean water to impart its own strontium isotope values to terrestrial material either saturated by it (e.g. Von Holstein et al., 2015), or simply sprayed by it (e.g. Whipkey et al., 2000) suggests that we must be cautious in the application of strontium isotope analysis to the study of waterlogged organic remains. Until a suitably reliable method for removing seawater Sr from organic remains recovered from underwater archaeological sites can be established, results from strontium isotope studies that utilise such material must be viewed with caution.

Further complicating the clear interpretation of strontium isotope values is the fact that winds currents may carry aeolian dust, and its associated Sr value, great distances. Saharan dust is clearly present in stratigraphy found in Caribbean islands, but a recent study of the Sr value for 91 trees on the Caribbean island chain of the Turks and Caicos showed that the $^{87}\text{Sr}/^{86}\text{Sr}$ for these trees did not appear to have been significantly affected by the $^{87}\text{Sr}/^{86}\text{Sr}$ value for Saharan dust, causing the authors to question the bioavailability of aeolian strontium, and what effect root depth may have on the $^{87}\text{Sr}/^{86}\text{Sr}$ values for Caribbean trees (Schultinga et al., 2018). As Mediterranean olive groves are often found near the sea and exist in geographic areas frequently covered by Saharan dust, establishing local $^{87}\text{Sr}/^{86}\text{Sr}$ values for olives stones throughout the Mediterranean may prove to be a complicated task.

Due to the ambiguity surrounding what baseline $^{87}\text{Sr}/^{86}\text{Sr}$ values could be expected for olive stone material, and the best practice for removing seawater strontium from waterlogged organic

remains, strontium isotope analysis will not be used in the isotope analysis work included in this thesis.

1.2.2 Organic residue analysis of ceramics and environmental samples

Organic residue analysis has been a well-established method for characterising the past contents of ceramic vessels since the 1970's and continues to evolve to meet new demands in archaeological science. Here, a review of the application of organic residue analysis to archaeological ceramics and environmental samples is given, with special reference to ceramics recovered from underwater contexts.

Organic residue analysis (ORA) is the extraction and characterisation of organic compounds such as lipids, waxes, esters, and resins, from material such as ceramic artefacts, organs, amorphous substances, coprolites and environmental samples. Once compounds have been recovered and isolated, the next step involves determining the original plant or animal source of compounds identified. Compounds that are relatively specific to a given plant, animal, or product have been termed 'biomarkers' (Evershed, 1993; Philp and Oung, 1988). The identification of these compounds has significantly increased what we can learn from ceramics regarding agricultural pursuits, culinary practices, and the transmission of cultural traits in the ancient world, constituting a 'biomarker revolution' (Evershed, 2008).

One of the earliest successful applications of this technique involved the chemical analysis of the so-called 'bog butters,' a white, waxy substance found during peat-cutting in Ireland and Scotland that was frequently associated with preserved wooden kegs or dishes (Thornton et al., 1970). The analysis of residues found on ceramic vessels shortly followed, with Condamin et al. (1976) publishing an influential paper on the identification of oil in ancient amphorae, in which the authors sought to determine 'whether it is possible to find scientific criteria which

allow it to be established with certainty and without any consideration *as to shape* that one given amphora contained this or that commodity' (Condamin et al., 1976, p. 195, my italics).

Similarly, Passi et al. (1981) attempted to chemically identify the contents of several Roman amphorae in order to 'test the hypothesis that individual amphora shapes were commodity-specific' (Passi et al., 1981, p. 779). Indeed, their results indicated that one of the amphorae tested may have contained sesame oil, rather than olive oil, due to higher levels of linoleic acid (C_{18:2}) and arachic acid (C_{20:0}) while the more degraded nature of oil residue recovered from a contemporary Roman oil lamp suggested that not fresh, but rancid olive oil had been contained within, which, interestingly, echoed the practices of peasants of the Apennine hills of Italy, who used rancid oil to light their homes as late as the 20th century (Passi et al., 1981, p. 784). Questions surrounding whether or not amphorae shape can be used as a determination of past vessel contents, as posed by Condamin et al. (1976) and Passi et al. (1981) are especially germane to investigations into ancient shipwreck cargoes, as we are frequently confronted with hundreds of identically-shaped amphorae, yet whether the contents of these amphorae are as homogenous as their shape may be unclear.

The types of archaeological material subjected to residue analysis quickly expanded beyond bog-butter and ceramics: bile acids from the gall bladder of a 3,200-year-old mummy were analysed (Kuksis et al., 1978), and analysis of the steroids present in a 2,000-year-old preserved human coprolite offered an early insight into palaeodiet (Lin et al., 1978). While Lin et al. (1978) were able to analyse coprolites themselves, Knights et al. (1983) utilised a different approach, when analyses of a Roman defensive ditch in Scotland yielded sterols which suggested that humans had defecated there, indicating an unexpected use for the ditch and offering evidence for the diet of the Roman soldiers in the form of partially digested wheat fragments (Knights et al., 1983, p. 139).

This early combination of geoarchaeology with residue analysis illustrates the wide-reaching potential for residue studies; not only applicable to artefacts, testing the chemical properties of the soil itself offered insight into site formation dynamics and patterns of site usage. As

geoarchaeology and soil analysis began to be featured more frequently in the scientific literature, questions arose as to what extent lipids and other biomarkers may migrate between the soil and the ancient potsherds buried within it. An influential study demonstrated that migration of lipids between the soil that constitutes the terrestrial deposition environment and porous ceramic pot sherds is negligible (Heron et al., 1991). Yet to what extent this is also true for amphorae sherds buried in seafloor sediments found on ancient shipwreck sites has not yet been determined. However, in some exceptional cases, shipwreck amphorae are found intact, and still stoppered, with extant liquid remains inside that are, ostensibly, protected from contamination.

The combination of separation through Gas Chromatography and characterisation through Mass Spectrometry became standard in the 1990's, allowing residue studies to mature as a proper discipline within archaeological science (Brown and Brown, 2011, p. 54). This period also saw important questions being asked regarding the validity of the prevalent sampling strategies used to select ceramic sherds for analysis. In studies such as the analysis of lipids in Roman amphorae and oil lamps conducted by Passi et al. (1981) discussed above, sherds from the base of the vessel were deliberately sought, as it was assumed that fragments from this area would be the most likely to yield lipids. A landmark study by Charters et al. (1993) questioned this approach. By analysing sherds from the base, body and rim of ceramic vessels, the authors were led to conclude that based on the use of the vessel, lipids could be expected to accumulate in different parts of the container (Charters et al., 1993, p. 212). Furthermore, the authors suggested that by analysing the pattern of lipid accumulation in a given vessel, it would be possible to deduce modes of use, as certain cooking processes could cause lipids to accumulate in a particular region of the vessel (Charters et al., 1993, p. 212).

This paper has been widely cited as influential in the development of residue studies (Evershed, 2008, Brown and Brown, 2011). It has proven influential for several reasons. First, it questioned the validity of the dominant sampling strategy, calling into question the accuracy of results from previous residue studies of ceramic artefacts that had exclusively tested sherds from the base of vessels. Second, it suggested that by incorporating sherds from different areas of a vessel *contents* and *use* could be distinct sets of information available to the archaeological

scientist through residue analysis. And third, it set a benchmark for all future sampling strategies in ceramic residue studies, in which a number of sherds from each vessel were tested. Unfortunately, this high standard for comprehensive sampling is difficult to attain, as the destructive nature of residue testing demands that museums and government agencies that are the custodians of the ancient material are willing to part with sherds from every part of a vessel.

In a 1993 paper entitled 'Biomolecular Archaeology and Lipids,' Richard Evershed crystallised the concept of 'archaeological biomarkers' as 'substances occurring in organic residues that provide information relating to human activity in the past' (Evershed, 2008, 1993). While this early paper focused on the diagnostic potential of lipids in residue studies, the archaeological biomarker concept itself was quite broad and encompassed many classes of molecules including ancient DNA. What united all 'biomarkers,' according to Evershed, was analysis at the molecular level. The great success archaeological scientists had in analysing lipids recovered from the walls of ceramic vessels was not found in initial attempts to characterise wine residues (Evershed, 2008, p. 904). Many of these early attempts to identify wine residue utilised a technique known as Fourier Transform Infrared Spectroscopy (FTIR).

Fourier Transform Infrared Spectroscopy (FTIR) is a technique that can be applied to the analysis of archaeological material. Put simply, 'When IR [infrared] radiation is passed through a sample, some radiation is absorbed by the sample and some passes through (is transmitted). The resulting signal at the detector is a spectrum representing a molecular "fingerprint" of the sample. The usefulness of infrared spectroscopy arises because different chemical structures (molecules) produce different spectral fingerprints' (*ThermoFisher Scientific FTIR Manual*, 2016).

Patrick McGovern, a scholar primarily concerned with the origins and history of wine, enlisted colleagues to attempt to identify tartaric acid and phenolic carboxylic acid ('biomarkers' for wine residue) in ceramic containers from Godin Tepe, Iran which date from between 3,500 and 2,900 B.C.E., through Transmission and Diffusion-reflectance Fournier Transform Infrared Spectroscopy (DRIFTS) (Michel and McGovern, 1993, p. 409). While these

attempts initially appeared successful, the authors of this study admit that ‘although the DRIFT analysis provides strong evidence for tartaric acid, it does not unambiguously identify the compound. Separation and characterization of tartaric acid by chromatography, followed by spectroscopic analysis, is one way to gain greater certainty’ (Michel and McGovern, 1993, p. 411). Shortly thereafter, another article was published which claimed to have identified the earliest wine residues yet found, from a Neolithic ceramic vessel, also from Iran, but dating from a period far earlier than the Godin Tepe jar, with a production date of around 5,000 B.C.E. (McGovern and Michel, 1996, p. 480). This study also used FTIR but, in addition, incorporated Liquid Chromatography.

Another test was added to McGovern’s wine detection routine: the Feigl spot test. A simple and cost-effective procedure, this test completed his three-part system for detecting wine: FTIR, High Performance Liquid Chromatography (HPLC), and the Feigl spot test. In 2009, McGovern et al. published their results from analysis of an Egyptian amphora from Abydos, which was dated to circa 3,150 B.C.E. In this paper, the authors claimed to have definitively confirmed the presence of tartaric and syringic acid, the principle biomarkers for *Vitis vinifera*. As discussed below, the efficacy of these methods in detecting wine residues is still under debate, as many of the results from Feigl spot tests which McGovern claimed positively identified wine residue, were not replicable by other archaeological scientists working with the same material in different laboratories (Stern et al., 2008).

Another method was recently developed that in addition to identifying wine residue may also be able to determine whether the wine was red or white. After separation through Liquid Chromatography (LC), residues were previously characterised with mass spectrometry, however, Guasch-Jané et al. (2004) illustrated that separation through LC followed by a tandem mass spectrometry approach (LC/MS/MS) could not only confidently identify tartaric acid, one of the primary biomarkers for *Vitis vinifera*, but could also identify syringic acid, which is present in red wine, but not white wine. Using this method, the researchers were able to show that based on the presence of syringic acid in only some of the containers, but tartaric acid in both, certain wine jars recovered from the tomb of Tutankhamun contained white wine, while others contained red (Guasch-Jané et al., 2006, 2004). This approach is now hailed as

the most effective for identifying wine residue in ancient ceramic containers (Evershed, 2008) and offers useful insights into ancient Egyptian alcohol consumption practices.

As discussed above, separation through gas chromatography has been utilised successfully since the 1970's and continues to be the predominant method of separation prior to characterisation through mass spectrometry. However, 'for some applications liquid chromatography (LC) has proved to be a more suitable alternative for the separation phase (Brown and Brown, 2011, p. 62).' This fine-tuning of techniques that allows for more specific characterisation of residues has also occurred in the world of lipids. Since the earliest studies on bog butters (e.g. Thornton et al., 1970), the analysis of lipids has formed a central component of ancient residue analysis.

Recent advances, however, have allowed archaeological scientists to discriminate between animal carcass fats and animal dairy fats, offering tremendous insight into the early origins of secondary products from animal husbandry. A landmark study by Evershed et al. (2008b) analysed over 2,200 pottery fragments from the Near East and South Eastern Europe and provided some of the earliest evidence for dairying yet found. This technique utilises stable isotope analysis of carbon, as the $\delta^{13}\text{C}$ value of the $\text{C}_{18:0}$ is different between adipose fats and dairy fats (Evershed et al., 2008b, p. 528). Like the detection of syringic acid discussed above, this study represents a trend towards greater specificity in residue analysis; rather than simply concluding that a vessel contained wine it is now sometimes possible to tell 'red' or 'white,' and rather than concluding that a vessel contained cow fat, it now may be possible to differentiate between the lipids derived from meat products and the lipids derived from dairy products in archaeological ceramics.

As the Mendeian amphora recovered from the Tektaš Burnu wreck that contained butchered beef bones (Carlson, 2003) has shown, bovine remains are a possible content of Classical or Hellenistic shipwreck amphorae. What is not yet known, however, is whether the analytical techniques used to differentiate between adipose lipids and dairy lipids discussed above are applicable on ceramic vessels recovered from underwater contexts. In this thesis, ceramic amphorae samples from the 4th century C.E. Babuljaš shipwreck, Croatia thought to have

contained olive oil are analysed, as are a series of Bronze Age ceramic cookware vessels recovered from beneath the Babuljaš shipwreck. This wreck lies in approximately 5 metres of seawater and is located only 15 metres from a small, uninhabited island off the Dalmatian coast. Rising sea levels since the Bronze Age, combined with processes of colluvium have caused a significant number of Bronze Age artefacts to be located on the seafloor proximal to the island. As domestic cookware is perhaps more likely to contain residues than transport containers due to greater absorption as a result of thermal cooking processes (Evershed, 2008a; Peña, 2007), these Bronze Age ceramic samples from underneath the Babuljaš could yield quite interesting results: if animal lipids are found, it may be possible to test whether this method for differentiating between adipose lipids and dairy lipids is possible on ceramics recovered from underwater contexts. Thus, the work conducted in this thesis will further contribute to our knowledge of what analysis it is possible to conduct on underwater artefacts.

1.2.2.1 Organic residue analysis of sealed shipwreck amphorae

The overwhelming majority of archaeological residues will not be pure substances, but rather mixtures of a variety of substances (Evershed, 2008a). It is therefore necessary to separate the constituent parts of a mixture into their pure forms prior to characterisation. Shipwreck contexts may add further complications: cargo items may spill out of amphorae as the wreck settles on the seafloor. As sacks or baskets decompose, their original contents can swirl, settle, and slip into broken or unstoppered amphorae, driven by ocean currents or biological activity. As will be discussed in greater detail in later chapters, the recovery of very small numbers of grape seeds, olives, or pomegranate seeds from the toes of shipwreck amphorae can cause ambiguity, as it may be unclear whether these were the original contents or entered the vessels during post-depositional mixing. Therefore, amphorae that remain intact and sealed are both rare and precious, in that the integrity of their contents as original is undisputed. Two Roman shipwrecks, the Madrague de Giens and the Albenga, have yielded several such amphorae, and bear a multitude of similarities including the period from which they date (100 – 60 B.C.E.),

the vast number of amphorae on board (6,000 – 10,000), the types of amphorae identified (Lamboglia 2 and Dressel 1, among others), and the nature of the cargo assumed to have been held in these vessels (Italian wine destined for Gaul) (Arobba et al., 2014; Formenti et al., 1978; Pomey, 1982; Tchernia, 1987).

One of the very earliest forays into the organic residue analysis of amphorae included one such artefact. An intact and sealed Lamboglia 2 type amphora was recovered from the Madrague de Giens shipwreck, France, and upon inspection, appeared to be full of its original contents (Formenti et al., 1978, p. 95). The authors used chromatography in an attempt to identify tartaric acid and phenolic acid, biomarkers of wine, but the results were inconclusive and suspicions about the vessel contents could not be confirmed (Formenti et al., 1978, p. 99). This early example of residue analysis conducted on the contents of ceramics recovered from underwater contexts perhaps strengthened the justification for a traditional approach to amphora interpretation on shipwreck sites: the Lamboglia 2 type amphorae are generally assumed to have contained Italian wine (Martinez Ferrarez et al., 2015, p. 278) and, in this case, scientific analysis was unable to disprove these suppositions. Further, this early study illustrates a phenomenon which continues to complicate the identification of wine residues: the biomarkers of wine are water-soluble and difficult to detect, even in exceptional cases where one is dealing with an intact and sealed ‘wine’ amphora.

Surpassing the Madrague de Giens in terms of size, a large merchant ship dated to between 100-90 B.C.E. was found in 42 metres of water off the west coast of Italy near the town of Albenga (ancient Albingaunum), and at 40 metres x 10 metres, has proven to be one of the larger Roman merchant ships ever found (Arobba et al., 2014, p. 226). The ship was carrying over 10,000 amphorae when it sank, the majority of which are the Dressel 1B type, with some Lamboglia type amphorae also included in the cargo (Arobba et al., 2014, p. 227). As with amphorae from the Madrague de Giens shipwreck, numerous amphorae on the Albenga remained sealed. Seizing this unique opportunity to perform analysis on the actual liquid contents of a 2,000-year-old transport container, Arobba et al. (2014) selected one of the Dressel 1B amphorae with an exceptionally well-preserved stopper of lime-pozzolana (with a

preserved pinecone adhered to the lime) and examined the contents through a combination of scientific methods.

Stable isotope analysis was performed on the dark, brownish liquid recovered from the stoppered amphorae, which, based on the normative approach to amphora interpretation, was expected to be wine. The isotope analysis was supplemented by a palynological study, pH test, and elemental analysis (Arobba et al., 2014, p. 229). The liquid recovered from the amphora was placed in a centrifuge and the resulting sediment was subjected to elemental analysis with interesting results: a far higher level of lead, aluminium and sodium were detected than had been expected (Arobba et al., 2014, p. 229). While the aluminium likely derived from the lime-pozzolana paste used to stopper the amphora, the lead was more difficult to explain, but historical evidence suggests that fruity syrups prepared in metal cauldrons were often added to wine in the ancient world in an effort to produce a sweeter taste, as described in Pliny's *Natural History* 14.11 (Eisinger, 1982).

The stable isotope analysis conducted on the Albenga sediment sample resulted in a $\delta^{13}\text{C}$ value of -27.6‰, which is consistent with C_3 plants, which includes *Vitis vinifera* and organic residue analysis detected tartaric acid, further indicating the liquid was wine (Arobba et al., 2014: 230). In reality, as the majority of plants are C_3 plants, the isotope analysis did not yield results that specifically indicated a grape-product, but the organic residue analysis results appear far more conclusive. Advances in methods since the Formenti et al. (1978) paper, which used simple chromatography, may explain why results from the Madrague de Giens sample were inconclusive, while the Arobba et al. (2014) study utilised gas chromatography / mass spectrometry (GC-MS) and liquid chromatography (LC), which resulted in a positive detection of tartaric acid in the Albenga shipwreck sample.

The palynological study of the amphorae liquid and sediment identified over 45 types of pollen, and while a high proportion were indeed *Vitis vinifera* pollen, many other types of plant pollen was represented in the sample (Arobba et al., 2014, p. 232). Table 2 details the 10 most highly represented taxa of arboreal pollen that were presented in the study's original table. Here we

gain insight into the environmental conditions proximal to the vineyard where the grapes grown, that appears to have consisted of a wide variety of trees and herbs. It is interesting to note that *Quercus* (oak) pollen was detected more frequently than grapevine pollen in what, according to all other tests conducted by the author's, appears to be a genuine sample of ancient wine. This seems to illustrate the propensity for arboreal pollen to permeate the local environment: if the grapes themselves, or the empty amphorae, or the grape press, were coated with microscopic oak pollen, these pollen grains could easily make their way inside a wine vessel, and onto an ancient ship.

Table 2. Ten highest represented taxa from the palynological study of liquid and sediment from a sealed Dressel 1B amphora, Albenga shipwreck (after Arobba et al., 2014, p. 232).

Genus	n.	%	n./cm ³
<i>Quercus pubescens</i>	57	15.0	4038
<i>Vitis</i>	48	12.7	3400
<i>Quercus ilex</i>	47	12.4	3329
<i>Pinus</i>	26	6.9	1842
<i>Olea</i>	16	4.2	1133
<i>Cupressaceae</i>	10	2.6	708
<i>Ostrya</i>	9	2.4	638
<i>Erica</i>	8	2.1	567
<i>Corylus</i>	7	1.8	496
<i>Alnus cordata</i>	6	1.6	425
<i>Pistacia</i>	4	1.1	283

The approach used to analyse the extant liquid in a Dressel 1B amphora from the Albenga shipwreck which incorporates several scientific methods to characterise a residue (Arobba et al., 2014), is one that should be emulated when performing destructive analysis on scarce archaeological materials. While the elemental, molecular and isotopic analysis indeed confirmed the author's suppositions that the liquid contained inside the amphora was ancient wine, further insights into the environmental conditions and wine production methods of 2nd to 1st century B.C.E. Italy are now possible. As this thesis takes a tandem approach of both organic residue and DNA analysis of shipwreck amphorae, it is interesting to note what the implications of the pinecone stopper and wide variety of pollen found in the Albenga sample

could be. Perhaps the custom of placing a pinecone in the bottom of a stopper may cause a positive identification of *Pinus* DNA in future aDNA studies of wine transport containers. This gives us pause to wonder whether a positive identification for *Pinus* DNA would indicate resinated wine or simply the use of a pinecone stopper. Indeed, it appears that exogenous ‘contamination’ could actually be archaeological in origin as it could occur while the grapes are growing (as illustrated by the palynological evidence), during production (one imagines pollen could have entered the wine during grape pressing), fermentation or decanting. DNA ‘contamination’ could even result from the organic material used to enclose the amphora, such as a pinecone.

1.2.2.2 Organic residue analysis of unsealed shipwreck amphorae

The majority of ceramic transport containers on ancient shipwrecks such as the Uluburun, Cape Gelidonya, Mazotos, Kyrenia, and Babuljaš are found unstoppered (Demesticha, 2011; Haldane, 1993; Katzev, 1969; McGovern and Hall, 2016) therefore some level of mixing is expected amongst the numerous organic cargo items that can be included in an ancient shipwreck assemblage.

In the case of the Uluburun shipwreck, where grape seeds were found both in the bottom of several amphorae and scattered throughout the sediment within the shipwreck, the decomposing remains of the organic cargo likely mixed together on the seafloor like a ‘bathtub filled with fruit punch’ (McGovern and Hall, 2016, p. 600, citing Pulak pers. comm. Sept 22, 2008). A total of 153 Canaanite jars were found on the Uluburun shipwreck, the majority of which had visible lumps of resin that were subsequently identified as *Pistacia*, or terebinth resin (Mills and White, 1989, p. 39), while other jars contained both lumps of resin and waterlogged grape pips. This fact caused McGovern to hypothesize that the majority of the Canaanite jars recovered from the Uluburun shipwreck carried resinated wine, while a small minority of the jars carried terebinth resin only (McGovern and Hall, 2016, p. 601), challenging the widely-published view that terebinth resin formed a substantial element of the cargo (e.g. Haldane,

1993; Mills and White, 1989; Stern et al., 2008). At the heart of this issue is the question of how the grape pips arrived inside the Canaanite jars, with McGovern contending that the grape pips accompanied the wine inside the jars, with others speculating that post-depositional mixing allowed them to enter the jars after the wrecking event.

A debate emerged between the McGovern group (based at the University of Pennsylvania Museum of Archaeology and Anthropology, commonly called 'Penn Museum') and a group of archaeological scientists from the University of Bradford. More than questioning individual findings, many archaeological scientists questioned whether or not the methods used by McGovern et al. were indeed suitable for the detection of ancient wine residues (e.g. Evershed 2008b, Stern et al., 2008). Members of the Bradford group questioned the identification of wine residues by McGovern and his colleagues through FTIR, claiming that this technique could not sufficiently distinguish subtle differences in the carboxylic acid and carbonyl absorption regions and therefore did not offer the sensitivity required to positively identify wine residue (Stern et al., 2008, p. 2198). The success rate of FTIR in the detection of tartaric acid in the Uluburun resin cargo reported by McGovern and Hall (2016, p. 602), would seem to support the contentions of the Bradford group. Of the five available samples below, all are simply shown as 'inconclusive' for the FTIR tests (Table 3).

Table 3. Results for Feigl spot test, FT-IR, and LC-MS-MS of Uluburun shipwreck resin, after McGovern and Hall, 2016, p. 602.

Uluburun resin sample (amphora no.)	Penn Museum laboratory preliminary results	Univ. Bradford laboratory results	Penn/TTB laboratories LC-MS- MS results
KW 48	Sample unavailable	Negative	Unavailable
KW 102	Inconclusive FT-IR	Negative	Borderline positive
KW 144	Borderline positive Feigl test and inconclusive FT-IR	Negative	Negative
KW 165	Inconclusive FT-IR	Negative	Negative
KW 181	Positive Feigl test and inconclusive FT-IR	Negative	Positive
KW 215	Inconclusive FT-IR	Not run	Positive

McGovern and Hall (2016) replied by asserting that the subtle differences were indeed distinguishable, and that members of the Bradford group had failed to identify these minute differences in their own research (referring to Stern et al., 2008, Figs. 2 – 4), which had attempted to characterise syringic acid, terebinth resin, and L-tartaric acid from vessels recovered from the Uluburun shipwreck cargo. McGovern’s earlier admission in his 1993 article that this technique could not unambiguously identify wine residue does not appear to help his case, while his staunch reliance on FTIR to detect wine residue despite his own admissions regarding its insufficiency is puzzling. Due to concerns regarding the efficacy of this method in characterising wine residues, FTIR will not be used as a residue analysis method in this thesis.

McGovern seems so adamant that the Uluburun carried a large cargo of wine that he appears to argue himself into a corner: while responding to the challenge from Stern et al. (2008) that these vessels did not originally carry wine McGovern writes ‘Stern et al. do not mention that all the Uluburun amphoras, as found, lacked intact stoppers or other closure. They do not discuss the extensive mixing of contents within the hull of the ship. Wine or another grape product – fresh grapes, raisins, vinegar, etc. – could have spilled from one amphora into another when the ship went down and during some 3,400 years thereafter. More recent materials might also have contaminated the amphoras’ contents’ (McGovern and Hall, 2016, p. 605). While

contamination through the permeation of wine residues over the wreck is certainly a possibility, McGovern's argument completely undermines his own findings and conclusions that the Canaanite jars contained wine. The statement that 'more recent materials might also have contaminated the amphoras' contents' could easily have been confirmed or denied by analysing the seafloor sediment surrounding the shipwreck. Without such a control, this statement appears to question the validity of every residue analysis study of shipwreck cargo items ever conducted, indeed, including his own work. Fortunately, the data presented in this thesis will include results from analysis of the seafloor sediments which surrounded the ceramic vessels being analysed, and ideally this will help to clarify the extent to which intrusive DNA, residues or macro-remains could affect the outcome of analysis of vessel contents.

Determining whether loose grape pips found their way into Canaanite jars carrying terebinth resin or are remnants of poorly filtered wine is a complex endeavour and may be an example of an instance in which aDNA and residue analysis may be unable to provide more information than analysis of macro-remains. The source of biomarkers identified in an unstoppered ceramic container may derive from elements of the cargo, but do not necessarily represent the original contents of the jar itself. To better understand the 'bathtub filled with fruit-punch' phenomenon of ancient shipwreck diagenesis, aDNA and residue analysis of the shipwreck sediment itself is necessary. This key element of the present study can hopefully shed light on site formation dynamics of deep Mediterranean shipwrecks, by allowing us to understand whether or not residues and DNA derived from the cargo, or extraneous sources, have permeated the site. By comparing results from the seafloor samples with the aDNA and residues extracted from ceramics, and comparing results from soil samples with the aDNA and residues extracted from the ceramic samples from the terrestrial site of Yeronisos, it may be possible to determine what the source of the aDNA or residue is, and to what extent these elements are present in the surrounding deposition environment.

1.2.3 Background of ancient DNA research

This section offers a review of the application of ancient DNA research to materials recovered from coastal and maritime archaeological sites, the pitfalls encountered in previously published studies, the promise DNA analysis may hold for the field of underwater archaeology, and what future directions are likely to help answer long-standing questions about the human past.

Deoxyribonucleic acid (DNA) is the molecule that contains genetic information for a given organism and is present in every cell. Humans, animals, plants, bacteria, and fungus all contain DNA, so more than offering information solely on the genetic history of humans, DNA research can offer insights into all classes of organisms (Brown et al., 2015; Brown and Brown, 2011; Gutaker and Burbano, 2017; Shapiro and Hofreiter, 2014). As such, successful extraction and identification of DNA molecules could offer species-specific (or at least genus-specific) information to the archaeological scientist. However, such research can be difficult, technical, and expensive, as only minute amounts of ancient DNA (aDNA) are likely to be preserved, and the threat of contamination from exogenous sources is ever present (Cooper and Poinar, 2000, p. 1139).

Early aDNA research conducted on archaeological materials involved the cloning and analysis of DNA extracted from the preserved soft tissues of animals (Higuchi et al., 1984), humans (Pääbo, 1985), and to a lesser extent, plants (Goloubinoff et al., 1993; Poinar et al., 1993), primarily from museum specimens that had originally been excavated from terrestrial sites. With the development of extraction and amplification techniques that successfully recovered DNA from archaeological bone (Hagelberg et al., 1989; Hagelberg and Clegg, 1991), a new class of material was available for archaeological scientists to investigate.

However, as ancient DNA laboratories sought ever-older specimens to study, concerns were soon raised regarding the authenticity of the DNA recovered. An early study sought to isolate and amplify DNA from the leaf of an extinct tree species, found locked in amber recovered from amber mines of the Dominican Republic. The authors claimed that as the amber itself was 35 to 40 million years old, this study ‘essentially doubles the age of DNA that can be recovered and sequenced from extinct plants’ (Poinar et al., 1993, p. 677). These and similar

claims of successful multimillion-year-old DNA retrieval were challenged (Austin et al., 1997; Bada et al., 1994; Hebsgaard et al., 2005; Palmer et al., 2011: 147; Walden and Robertson, 1997), and it is interesting to note that the primary author of the Dominican amber study, Hendrik N. Poinar, soon after published an article calling for more rigorous standards for aDNA research (Cooper and Poinar, 2001), perhaps to help others avoid such mistakes in the future.

In the decade that followed this well-timed call for more stringent controls in aDNA research, the prevalent method of Polymerase Chain Reaction (PCR) amplification of aDNA began to be replaced. The PCR approach had several limitations: a different PCR was required for each sequence being studied which used up a considerable amount of the aDNA available, primers could only be designed for the DNA of organisms that had already been studied, and it was not a suitable method for exceptionally short base pair fragments (Brown, 1999; Brown et al., 2015). The replacement of PCR with the so-called ‘next-generation sequencing’ technology rapidly began to have a considerable impact on the field of aDNA studies (Mardis, 2008; Orlando et al., 2015). Next Generation Sequencing (NGS) allows all of the aDNA present in a sample to be amplified, without the need for specially designed primers. Areas of investigation that were previously difficult or impossible with the PCR approach are becoming considerably more realistic with the advent of NGS (Brown et al., 2015, p. 207), while the streamlined nature of sample preparation offered by NGS can significantly decrease the time and costs necessary to conduct DNA studies on archaeological materials (Hofreiter et al., 2015; Knapp and Hofreiter, 2010; Mardis, 2008). DNA ‘capture’ methods, in which a specific type of DNA is fished out of metagenomic DNA pools with molecular ‘bait’ (e.g. Avila-Arcos et al., 2011) now offer a way to target DNA of interest among the thousands of species amplified in these large datasets.

These methodological developments, from cloning DNA fragments inside vectors of *Escherichia coli* bacteria, to PCR, to NGS, increased the practicality of ancient DNA analysis while simultaneously lowering the cost. This created the potential for genetic studies of archaeological material to answer long-standing questions about human evolution and migration events (Green et al., 2009; Ko, 2016; Krause et al., 2010; Matisoo-Smith, 2015, p.;

Meltzer, 2015; Pääbo et al., 2004; Reich, 2018; Schroeder et al., 2019) animal domestication (Eriksson et al., 2008; Larson et al., 2010, 2007; Pires et al., 2019; Rubin et al., 2010; vonHoldt et al., 2010), and plant domestication and the accompanying the spread of agriculture (Allaby et al., 2015; Brown et al., 2015; Jones and Brown, 2000; Nistelberger et al., 2016; Palmer et al., 2011; Wales, 2012; Wales et al., 2016).

The majority of ancient DNA studies cited above have analysed archaeological material recovered from terrestrial sites. However, on closer inspection, a number of important ancient DNA studies have utilised archaeological material recovered from submerged sites, a trend that is growing as more inundated sites are investigated. Now, with the advent of NGS and shotgun metagenomics, more research has begun to focus on ancient and environmental DNA recovered from lacustrine, coastal and maritime archaeological sites. As with artefacts and environmental samples from terrestrial sites, ancient DNA analysis of inundated sites may provide insight into human evolution, disease, migration events, animal domestication, plant domestication, and climate change in the past.

1.2.3.1 Potential and limitations: faunal and human skeletal remains

While the potential for aDNA studies of material recovered from submerged sites to reveal previously unknown aspects of the human past is clear, it is necessary to explore limitations and pitfalls that may be encountered in this work, as well as the potential this field holds.

DNA analysis of skeletal remains from shipwrecks has a long pedigree: the study that reported the earliest successful extraction and amplification of DNA from bone involved a pig bone from the *Mary Rose* shipwreck (Hagelberg and Clegg, 1991), in addition to several human bones from terrestrial sites. This illustrates the long history of aDNA studies conducted on material from underwater archaeological sites. Further faunal remains from the *Mary Rose* have been analysed in recent years, including a dog bone that was shown to bear genetic similarities

to modern-day terriers and the Jack Russell breed (Zouganelis et al., 2014). This allows us insight into aspects of breeding and pet ownership in the Tudor period. The exceptional preservation of food stores on the *Mary Rose* allowed excavators to sample the pig bone discussed above, as well as cod remains from the dried cod that so often featured in sailors' rations from this period. Both DNA and stable isotope analysis of these fish bones were undertaken and offered insight into the relatively global nature of food procurement and seafaring in the Tudor period (Hutchinson et al., 2015). While shipwrecks hold great promise for the study of faunal remains, submerged settlement sites hold similar potential for the study of human remains.

Settlement sites inundated due to sea-level rise offer several unique benefits to archaeologists. No subsequent habitation by modern communities intrude upon their preserved stratigraphy, and no currently inhabited modern buildings preclude their excavation. DNA analysis of faunal or human skeletal remains from submerged settlement sites such as Doggerland (Cossins, 2015; Gaffney et al., 2009, 2007) could shed light on important transitions in the Mesolithic, while inundated Neolithic sites off the Levantine coast may offer insights into early farming communities (Galili et al., 1997, 1993).

One such site appeared to offer early evidence for a disease which has long afflicted human populations. HersHKovitz et al. (2008) claimed to have isolated and amplified tuberculosis DNA from two 9,000-year-old skeletons recovered from the underwater Neolithic settlement site of Atlit Yam, Israel (HersHKovitz et al., 2008). While the authors concede that 'No demonstrable amplicons of human nuclear microsatellite DNA were obtained from the bone samples' (HersHKovitz et al., 2008, p. 4) they remained confident that the conditions on the underwater site are conducive to ancient DNA preservation.

Unlike a shipwreck, which may quickly sink into an anoxic layer of seafloor sediment, the site formation dynamics of Atlit Yam appear to have been quite different. Gradually rising sea levels at the end of the Neolithic would seem to suggest that hydrolytic processes resulting from the inundation of sea water could have accelerated the degradation of the DNA in the skeletons

recovered from this site. The authors state that pathologies observed on the skeletal remains are consistent with tuberculosis (Hershkovitz et al., 2008, p. 3) and that these lesions offer further confirmation for the presence of tuberculosis at this site. In addition, a connection is drawn between the presence of domesticated cattle bones at Atlit Yam and the discovery of tuberculosis, adhering to the theory that early cases of tuberculosis (TB) were contracted as a result of animal husbandry.

A response from Wilbur et al. (2009) questioned these findings and provided several salient points as to why destructive analysis in biomolecular archaeology should only proceed when a relevant scientific question remains unanswered. Attempts to identify ever older cases of TB in humans may not have great scientific value without additional information about the strains involved, while the myriad taphonomic processes which may degrade the DNA of various TB strains on archaeological sites means that a negative result would not definitely preclude the presence of TB at that site (Wilbur et al., 2009).

Donoghue et al. (2009) offered a response to these criticisms, defending the use of destructive analysis of human remains in the interest of investigating the health of an early farming community, rather than attempting to detect the earliest known case of TB in humans (Donoghue et al., 2009). The authors did not address the fact that it is now well-established tuberculosis in humans did not derive from proximity to cattle, as they argue that it is still necessary to examine early cattle herding communities to better understand the relationship between proximity to bovines and TB in humans (Donoghue et al., 2009, p. 2799).

A prescient point offered by Wilbur et al. (2009) is that *Mycobacterium* includes dozens of species that commonly exist in soil and water and any skeletal remains that have been in contact with soil and water for millennia would likely contain environmental mycobacterium (Wilbur et al., 2009, p. 1993). This argument has far-reaching implications for NGS and metagenomic datasets. Characterising, understanding, and analysing the DNA that is present in the archaeological deposition environment remains a fundamental way to exclude environmental contamination as a factor in ancient DNA studies of both terrestrial and underwater sites.

Failure to collect soil samples from the submerged settlement, and adjacent inundated areas where no trace of human habitation could be identified, thus constituting a ‘natural’ area of seafloor, would have offered a baseline for what DNA is naturally present in this environment, and thus would not relate to anthropogenic activities.

If DNA from environmental mycobacteria can survive in soil, perhaps any TB recovered from skeletal remains was a simple case of contamination from exogenous sources in the soil. As no human DNA amplicons survived within the skeletal remains (Hershkovitz et al., 2008) it is curious that DNA derived from pathogens present in the bones would be preserved. A test of the DNA present in the soil from the area of inhumation and an area outside the inhumation would have provided more salient arguments regarding the source of the DNA.

Despite challenges in the Hershkovitz et al. (2008) study, there is great potential for the use of aDNA analysis on human remains from underwater archaeological sites. Recently, a human skeleton was discovered on the Classical era Antikythera shipwreck, Greece (Merchant 2016). DNA analysis of this individual is ongoing (Schroeder, in preparation), and, if successful, may help illuminate aspects of mobility, seafaring, and sailors in the Classical Mediterranean. News reports suggested that this would be ‘a feat never attempted before - DNA analysis on someone who has been under the sea for 2,000 years’ (Marchant, 2016, p. 462). This statement is, however, not strictly accurate as DNA analysis was attempted on the human skeletal remains recovered from the submerged Neolithic burial site at Atlit Yam (Hershkovitz et al., 2008), and while no human DNA amplicons were successfully detected for analysis in this study, it was certainly attempted.

Some of the most important discoveries of the past two decades involve the growing and branching of the hominin evolutionary tree. Denisovans, Neanderthals, and *Homo floresiensis* (Aiello, 2015; Green et al., 2006; Krause et al., 2010; Meyer et al., 2012; Reich, 2018) walked the earth at the same time as anatomically modern humans and appear to have interbred. Prehistoric cave sites that were near ancient coastlines will inevitably now be submerged due to rising sea levels. If soil samples from cave sites in terrestrial contexts have yielded ancient

hominin DNA (Slon et al., 2017), it remains a distinct possibility that submerged cave sites may yield both artefactual evidence for hominin occupation and hominin DNA.

In addition to cave sites, submerged landscapes such as Beringia may also hold clues to as to processes that resulted in the peopling of the Americas. Perhaps in the ongoing search for ancient caves and landscapes that hold material evidence of early humans, we should now begin to search in earnest under the sea.

1.2.3.2 SedaDNA from submerged sites

As NGS supplants PCR as the predominant method for characterising the complex assemblages of DNA present in environmental samples and microbial communities in human remains (Key et al., 2017), a greater awareness of the potential for the preservation of DNA within environmental samples has been realised. Metagenomics is the characterisation of the numerous genomes present within a sample (Zepeda Mendoza et al., 2015, p. 745), and recent applications to archaeological material have focused on environmental DNA (eDNA) recovered from submerged landscape sites and inland lake environments (Niemeyer et al., 2017; Sjögren et al., 2017; Smith et al., 2015). For example a study which used NGS to characterise plant species present in a Greenland lake core found that macro-fossils, pollen, and DNA could be used together to present a more accurate picture of the total biodiversity present in both the aquatic lacustrine environment and the terrestrial environment nearby, as neither the DNA, macro-fossils or pollen represented the total range of species present in the sediment (Pedersen et al., 2013, p. 165). Alsos et al. (2018) have additionally shown that eDNA analysis of lake sediments collected from 11 lakes in Norway detected up to 49% of the vascular plant species recording within 2 metre of the lakeshore, with the remaining 51% not detected (Alsos et al., 2018). This further supports Pedersen's argument that the examination of environmental DNA recovered from archaeological sites should be used as a complementary approach to the study

of pollen, macrofossils, micromorphology, and other existing methods for characterising past environments (Pedersen et al., 2015, p. 1).

Investigations into Mesolithic paleosols at the submerged site of Bouldner Cliff near the Isle of Wight, UK, yielded both palynological and DNA information, offering insight into Mesolithic environmental conditions for this region (Smith et al., 2015). Paleosols represent the old land surface, and, when identified, can provide a tremendous amount of information for the geoarchaeologist, palaeobotanist, and prehistorian (French, 2003). This investigation sought to illuminate aspects of the shift from the Mesolithic to the Neolithic (Smith et al., 2015, p. 999) as agriculture spread from the Mediterranean littoral to northern Europe. This old land surface was dated to between 8,030 and 7,980 years B.P. and represents a period in the British Isles for which there is little terrestrial archaeological evidence and is little understood (Smith et al., 2015, p. 999).

While archaeological artefacts like corded fibre, burnt hazelnut shells and worked flint already provided the excavators with early examples of such technologies (Smith et al., 2015, p. 999), it was the identification of ancient wheat DNA that allowed this investigation to challenge the current understanding of the movement of domesticated staple crops in prehistory. Prior to this discovery, the earliest evidence for wheat on the British mainland came from sites dated to around 6,000 B.P., meaning that the Bouldner Cliff discovery pushed back the earliest example of wheat in this region by 2,000 years, and appeared to be 400 years earlier than the oldest example of wheat on the adjacent European mainland (Smith et al., 2015, p. 998).

Of primary importance to this study was whether or not DNA can move vertically through layers of sediment, and if so, had this occurred at Bouldner Cliff? It appears that in this case, the paleosol was well-sealed since it was covered by a peatbog prior to inundation by the ocean, which the authors suggest would have inhibited the vertical movement of DNA (Smith et al., 2015, p. 999). While several other members of the *Poaceae* (true grasses) family were represented both in the DNA and pollen evidence, it is interesting that the DNA from *Triticum* (wheat) became more and more prevalent in the upper levels of the sediment core (Smith et

al., 2015, p. 1000). The palynological evidence did not yield the large pollen type associated with proximal cereal production, and the authors conclude that the wheat was not grown onsite (Smith et al., 2015, p. 1000) but may have been transported as milled flour for consumption at the Bouldner Cliff site. A companion piece in the same volume explored how wheat may have come to Britain far earlier than previously expected, and how agricultural products can appear in the archaeological record before the earliest evidence for their local production (Larson, 2015).

Concerns were raised by Weiß et al. (2015) that the number of reads assigned to wheat in the Smith et al. (2015) study was too low to authenticate as ancient in origin, and the authors proposed new methods to authenticate DNA as ancient in cases involving a very low number of reads (Weiß et al., 2015). As authentication of DNA reads is absolutely essential in aDNA studies, the results from the Smith et al. (2015) study may need to be revisited in order to securely establish the presence of wheat in the British isles 2,000 years earlier than previously suspected.

As metagenomics data generated by NGS ostensibly amplifies and sequences all recovered DNA, it has been hoped that work in this area will make it possible to reconstruct the full complement of ancient environments (Rawlence et al., 2014), which would be of great benefit to archaeological investigations into the past (Larson, 2015). Yet, the eDNA evidence from lake cores suggest it is unlikely we would detect the ‘full complement’ using current methods, as distance from the deposit and taphonomical constraints appear to limit the full representation of nearby plant species (Alsos et al., 2018). These tandem studies that assess how well NGS data can detect nearby plant species which have been independently confirmed by palynological or modern survey data (e.g. Pederson et al., 2015; Alsos et al., 2018) are exceptionally useful in understanding the limitations of our research, and ways to supplement it.

DNA research conducted on environmental samples from lakes and ocean cores can be very useful in reconstructing ancient environments (Alsos et al., 2018; Larson, 2015; Pedersen et

al., 2015; Rawlence et al., 2014; Smith et al., 2015; Willerslev, 2003). As the necessity to understand climate change grows, it behoves us to fully investigate past extinction events, the expansion and contraction of woodlands, and how early humans responded to these events. DNA analysis of ocean cores and lake deposits are predicted to be an increasingly important line of evidence in our understanding of climate change in the past.

1.2.3.3 Plant remains

As discussed in section '2.1 Isotope analysis of macro-remains', the study of archaeobotanical remains can offer insights into both ecological and cultural aspects of the human past (Bogaard, 2013). However, the majority of research has relied on terrestrial charred plant remains and phytoliths (e.g. Hillman, 1981; Jones, 1984; Jones, 1996; Harvey and Fuller, 2005; van der Veen, 2007; Lightfoot and Stevens, 2012; Fiorentino et al., 2012; Styring et al., 2017). Waterlogged archaeobotanical remains from submerged settlement sites, shipwrecks, and ancient harbours could present a new line of evidence for both isotope analysis, and aDNA research.

One study compared the grape DNA detectable in charred grape pips from a terrestrial archaeological site to waterlogged grape pips from inundated sites of similar antiquity and found that the waterlogged specimens yielded longer base pair fragments than the charred (Manen et al., 2003, p. 728). One specimen, *V2*, was recovered from sediment layers dating to the 5th century port at the Greek colony of Marseille. Microsatellite analysis showed a greater commonality with grape varieties from France or Austria/Germany, rather than Greece (Manen et al., 2003). This discovery challenges the model of clockwise trade patterns, which sees local demand for wine in Gaul as fuelling the trade of wine for Gallic slaves in Marseille (Tchernia, 1980; Unwin, 1991). With this evidence for local viniculture as early as the 5th century B.C.E., perhaps the plethora of Dressel 1 amphorae recovered from sites in France from this period are better interpreted as evidence for wine connoisseurship and increased consumption, rather than a desperation for wine.

Oak timbers from the *Mary Rose* shipwreck were investigated through DNA analysis in an attempt to establish a provenance for the wood, as oak has strong phylogeographic identifiers. Initial results indicated that plant chloroplast DNA was difficult to recover as PCR inhibitors in the waterlogged wood presented challenges, so the authors tested new pre-treatments before extraction and successfully managed to obtain oak DNA from one of the 10 archaeological samples (Speirs et al., 2009). This echoes the findings of Fors (2008) which showed that sulphur contamination in waterlogged wood can inhibit conservation efforts, making waterlogged wood, with its highly porous nature, likely to exhibit signs of contamination from the underwater environment, and present difficulty in analysis (Fors, 2008).

Recent work that utilised high-throughput methods rather than PCR has examined the decay of DNA within waterlogged plant material and found that maternally inherited chloroplast haplotypes in 21 wood samples analysed showed genetic similarities to locally grown trees of the same species (Wagner et al., 2018) indicating further promise for studies of waterlogged wood to provide insights into artefact provenience.

More recent studies utilising NGS have demonstrated that waterlogged grape pips at sites spanning the Iron Age (circa 5th century B.C.E.) to the Medieval period (circa 1,200 C.E.) can yield sufficient amounts of endogenous DNA to determine their genetic relationships to modern cultivars (Ramos-Madrigal et al., 2019; Wales et al., 2016), which can offer important information pertaining to the inception and spread of viniculture throughout western Europe. Close genetic relationships among samples from several Roman sites were detected, suggesting clonal propagation of these specific examples of grape vines in antiquity (Ramos-Madrigal et al., 2019).

Like grapevines, the olive tree occupies a special place in Mediterranean history, and underwater archaeological sites have yielded some of the most significant discoveries of olive remains. The Levantine coast off modern-day Israel features a series of submerged prehistoric sites, in addition to the submerged Neolithic inhumations at Atlit Yam discussed above, a site called Kfar Samir has yielded early evidence for olive cultivation and processing in the Neolithic

(Galili et al., 1997). The largest individual deposit of Bronze Age olives was found inside a Canaanite jar on the Uluburun shipwreck, Turkey, with over 2,000 olive stones preserved in one vessel (Haldane, 1993). As more submerged sites are explored, the potential increases.

Olive stone samples were collected from Kfar Samir submerged site and analysed with PCR along with additional desiccated olive stones of a similar antiquity discovered at the Qumram cave (Elbaum et al., 2006). After comparing DNA from both desiccated and waterlogged olive pits, it appears that desiccated organic remains yield the most viable example of ancient DNA from preserved olive stones (Elbaum et al., 2006, p. 86).

In addition to the mode of taphonomic preservation, Elbaum et al. (2006) point out that one must consider how these agricultural products were treated prior to deposition: do olives that have been pickled in brine yield different results than fresh olives? The notion that quick desiccation contributes to the survival of ancient DNA in organic remains echoes the findings of Pääbo et al. (1985) which suggested that superficial mummy tissue that had dried out quickly yielded more ancient DNA than internal mummy tissues. A further issue raised by Elbaum et al. (2006) revolves around post-excavation practices: the authors note that the degradation of aDNA in the waterlogged olive pits may have occurred rapidly after excavation, as the pits were removed from their anaerobic environment and exposed to oxygen and sunlight (Elbaum et al., 2006, p. 86). This highlights an important concern for aDNA studies; the treatment of finds immediately after excavation can determine what scientific analysis is feasible in the future. With the growing importance of aDNA research, it is vital that excavators take care to carefully handle and properly store at least a selection of recovered organic remains.

If results from the Manen et al. (2003) study comparing DNA recovery from charred and waterlogged grape pips can be amalgamated with the work of Elbaum et al. (2006) that compared DNA recovery from waterlogged and desiccated olive stones, it suggests that among charred, waterlogged and desiccated archaeobotanical remains DNA preservation is least likely in charred remains, moderate in waterlogged remains, and potentially well-preserved in desiccated remains.

Ancient DNA studies have generally sought to extract DNA molecules for previously living organisms including humans, animals and plants. However, in 2008 and 2012 a series of studies attempted a novel form of ancient DNA extraction; rather than extracting DNA from a preserved organic material, the authors attempted to extract DNA from the ceramic matrix of shipwreck amphorae that had lain on the seafloor for well over 2,000 years. Inorganic mineral matrices such as the inorganic phase of bone apatite can act as substrates to which DNA can bind and be protected from degradation (Grunenwald et al., 2014), while the fired clay fabric of ceramic vessels can similarly protect organic residues (Evershed, 2008a, p. 910). It is therefore theoretically possible that DNA from past vessel contents can bind to the interior walls of amphorae.

In 2008, Hansson and Foley explored a new avenue for ancient DNA research by analysing the interior walls of two amphorae recovered from a shipwreck off of Chios in an effort to recover DNA derived from the past contents of the vessel (Hansson and Foley, 2008). One amphora was produced on the nearby island of Chios, and one amphora was of an unknown type. While the results from the unknown amphora type were limited, the authors identified olive, thyme and oregano DNA in the ceramic scrapings from the Chian amphora (Hansson and Foley 2008, 1174). These findings challenged the belief that the shipwreck had been transporting a cargo of fine Chian wine, due to the closely held association between Chian style amphora and the luxury wine that was produced on Chios in antiquity. The authors caution against 'further assumptions that unlined fourth century B.C. amphoras contained wine,' adding that 'additional investigations into the diversity of the islands agricultural production and trade should be considered' (Hansson and Foley, 2008, p. 1174).

This argument is indeed borne out by historical evidence from ancient authors such as Pliny, who state that the best mastic resin came from Chios (Pliny, *NH* 12.72). The discovery of several Chian amphorae containing hundreds of whole olives on the Mazotos shipwreck (Demesticha, 2011; Margaritis et al., in preparation) further strengthens the assertion that Chian amphora did not exclusively contain wine. While evidence from ancient authors and the Mazotos shipwreck, which is contemporary with the sites from which these analysed amphorae

came, may align with the assertion that Chian amphorae could contain a variety of products, questions remain about the validity of the results from the DNA analysis. It is worth noting that scrapings were not taken from the exterior surface of the amphorae, which has been standard practice in residue analysis studies of archaeological ceramics for some time (e.g. Charters et al., 1993), as a way to discriminate between endogenous and exogenous sources of residue.

Hansson and Foley assert that the conditions on the shipwreck could contribute to the preservation of ancient DNA, as protection from UV light and a lack of contact with modern terrestrial plant species would both limit the degradation of the DNA and protect the ceramic matrix from exogenous sources of plant DNA (Foley et al., 2012, p. 391; Hansson and Foley, 2008, p. 1171). Cognizant that DNA could be present in the underwater deposition environment, primers were designed to prevent the amplification of algal DNA, and either target regions of high conservation, which would allow for the identification of more than one species or designed to amplify one specific species.

While the 2008 study had tested transport amphorae recently recovered from the Oinousses shipwreck, a follow up study by Foley et al. (2012) applied this method to a series of amphorae from underwater contexts that have been stored and archived by the Ministry of Cultural Tourism Ephorate of Underwater Antiquities in Athens, Greece. These amphorae had been dry and stored for, in some cases, up to 15 years (Foley et al., 2012, p. 391). To amplify the DNA obtained, primers were designed to amplify species 'expected to be part of the early Greek diet and trade...' (Foley et al., 2012, p. 393). As will be discussed further below, a confirmation bias is at play in designing an experiment in this manner.

The 2008 study had taken scrapings from the interior of the amphorae, a destructive method, while the 2012 study attempted a non-destructive method of swabbing the interior of the amphorae with a swab saturated with lysis buffer (Foley et al., 2012, p. 391). Indeed, as discussed above, the high standards set for exhaustive testing of all areas of ceramic containers as promoted by Charters et al. (1993) for organic residue analysis studies is difficult to replicate

as obtaining permission to destroy many areas of a vessel is less likely to be granted. For this reason, the implications of the work of Foley et al. (2012) for future non-destructive testing of ceramics to determine past vessel contents appeared to offer an undeniable benefit to archaeologists, ceramic specialists, and conservators.

Through non-destructive analysis by swabbing with a lysis buffer, the authors claim to have had '43 DNA hits from 27 swabbed samples' and '16 DNA hits from 18 scraped samples' (Foley et al., 2012, p. 394). The authors stated that ancient DNA from pine, olive, ginger, juniper, and oregano, among other species of plants, had been successfully detected through non-destructive analysis. It appears that no swabs were taken from the exterior of the vessels. The stated success rate in detecting what the authors claim to be genuinely ancient DNA is unusually high. To provide some context, in a 2013 study of the ancient origins of domestic pigs in the Levant, 177 samples taken from actual pig bones only yielded 34 viable ancient DNA samples (Meiri et al., 2013), or a 19.2% success rate. The low rate of endogenous DNA recovery in the Meiri et al. (2013) study is far more in line with typical results for palaeogenomic research.

The HersHKovitz et al. (2008) study, the Hansson and Foley (2008) and Foley et al. (2012) studies share three important commonalities. First, PCR was used in these studies to amplify preselected DNA; tuberculosis DNA from human remains, and terrestrial plant DNA from shipwreck amphorae. As the presence of either type of DNA in this archaeological material was purely speculative a confirmation bias may have been at play in both these studies. The authors had decided before analysis what DNA they expected to find. Second, methods for authenticating aDNA have been greatly enhanced (Briggs et al., 2009, 2007; Jónsson et al., 2013), and it is now standard practice to assess damage patterns of cytosine deamination and nucleotide misincorporation in order to confidently assign an archaeological origin to recovered DNA fragments. Third, no environmental samples were collected, extracted, and analysed alongside the archaeological material.

This latter point is particularly important as research moves towards NGS and metagenomic datasets that amplify all DNA present in a sample. Demonstrating that the DNA recovered from the archaeological material was not present in environmental samples taken from the artefact's surroundings would greatly enhance the credibility of the results presented. For future studies of ancient DNA from coastal and maritime sites, it is vital to characterise what DNA is naturally present in the underwater deposition environment, before conclusions are drawn based on the detection of these species in archaeological samples from submerged contexts.

If the non-destructive swabbing of shipwreck amphorae described by Foley et al. (2012) can genuinely recover ancient DNA derived from past vessel contents, this would prove to be revolutionary. Non-destructive methods are always preferable when dealing with a resource as scarce as archaeological material, and one could imagine access being granted to conduct this swabbing on ceramic vessels otherwise beyond the reach of archaeological scientists due to their rarity, intact nature, or inherent beauty. The sampling strategy issues raised by Charters (1993) would cease to matter, as presumably curators would allow all areas of a vessel to be swabbed non-destructively. Yet, perhaps it is too early to celebrate the impending halcyon days of non-destructive analysis. Just as it is necessary to understand what DNA is present on underwater sites, it is also necessary to understand what DNA is present where the analysed artefacts are stored. The shipwreck amphorae swabbed by Foley et al. (2012) had been kept in museum storage conditions for up to 15 years. It is therefore possible that the terrestrial plant DNA detected may have derived from the storage environment, rather than the past contents of the amphorae.

The research presented here aims to characterise the DNA recovered from ceramic transport containers, archaeological resin, and environmental samples from five Mediterranean shipwrecks and one terrestrial site. It is hoped that by analysing environmental samples of seafloor sediments taken from both inside and outside of the various shipwreck sites, as well as samples of the water column above these wrecks, we can better understand what DNA is present on these sites. Destructive analysis is undertaken on ceramics, resin, and environmental samples. The non-destructive swabbing method described by Foley et al. (2012) is retested on

ceramic vessels from two Late Bronze Age shipwrecks, and critically, swabs have been taken of the storage environments in which these artefacts are housed.

By establishing a baseline for what species can be detected in seafloor sediment, seawater, and the museum storage areas where these shipwreck artefacts are housed, a more accurate interpretation of the DNA recovered from archaeological material may be possible. In addition, recovered DNA fragments have been scrutinised for the characteristic damage patterns associated with ancient DNA. By establishing what recovered DNA is ancient or modern or modern in origin, we can avoid assigning archaeological meaning to what may simply be environmental contamination.

1.3 The sites and their settings

The artefacts analysed in this thesis were recovered from five ancient shipwrecks that represent a broad range of Mediterranean history: from the Late Bronze Age (the Uluburun and the Cape Gelidonya shipwrecks of Turkey), the Classical (the Mazotos and Kyrenia shipwrecks of Cyprus), to the twilight of the Hellenistic (1st century B.C.E. terrestrial site of Yeronisos, Cyprus), and the late Roman (the 4th century C.E. Babuljaš shipwreck, Croatia). The majority of these sites are located in the Eastern Mediterranean, with the exception of the Babuljaš shipwreck, on the Dalmatian coast of Croatia (Figure 5).

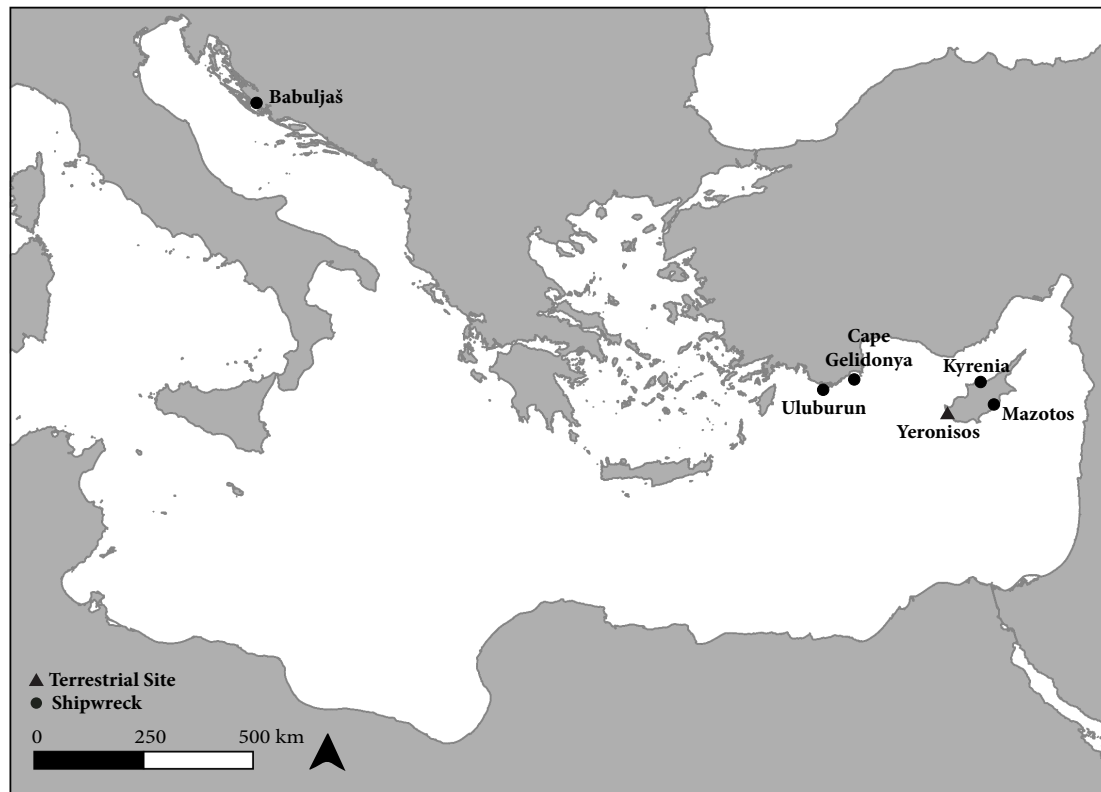


Figure 5. Map of shipwreck locations.

While sometimes disparate both temporally and spatially, these five ancient shipwrecks, and one terrestrial site, share a fundamental similarity: an assumption has been made about the nature of the cargo or commodities contained within the ceramic vessels found there. An additional similarity is that the amphorae on the ships were not manufactured nearby the site of the wrecking event. The ceramic transport containers recovered from the five shipwrecks included in this study are thought to have been manufactured at various sites throughout the Mediterranean and had been transported some distance before the catastrophic sinking of the ship (Figure 6).

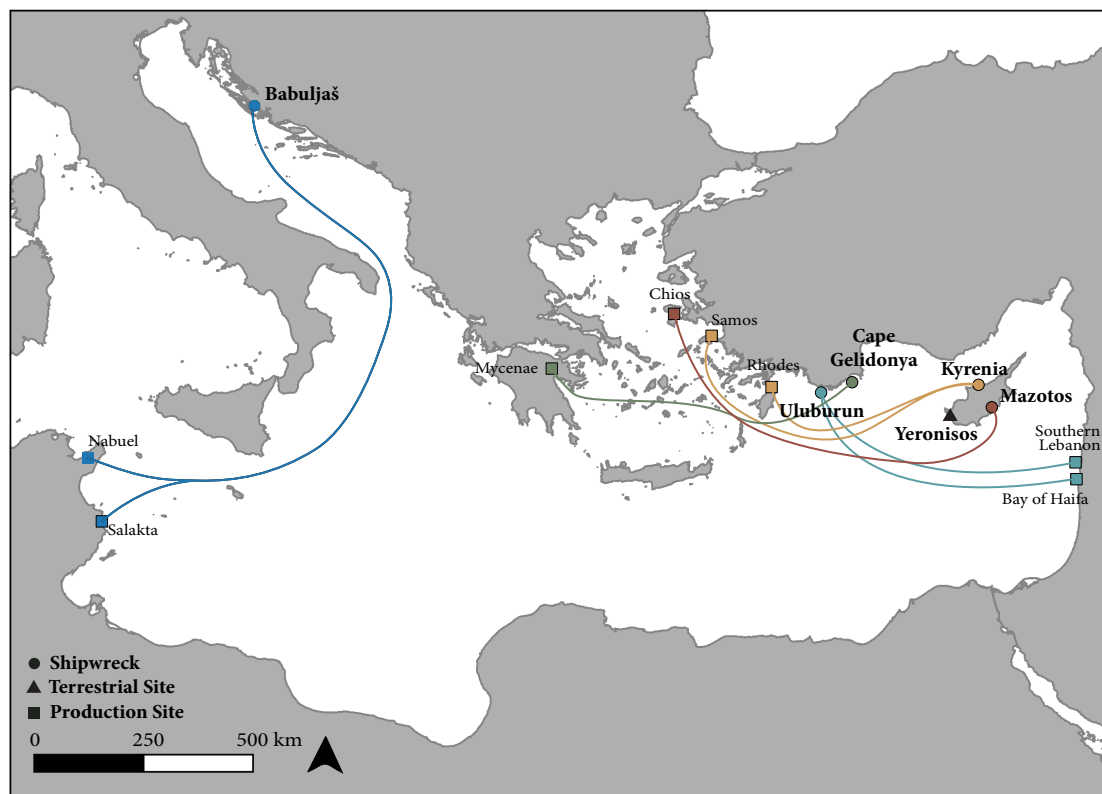


Figure 6. Proposed production locations for transport jars analysed in this study, with connections to shipwreck locations.

Petrographic analysis of the Canaanite jars from the Uluburun shipwreck has shown at least two distinct fabric types. One fabric type is predominant among the 153 recovered jars, and likely originating near the Bay of Haifa (designated fabric HM2), while another fabric type is also present (designated fabric RM6) and appears to come from southern Lebanon (Pulak, in preparation). Two vessels of each fabric have been analysed through non-destructive swabs.

The stirrup jar from the Cape Gelidonya shipwreck analysed here is thought to have been produced in the Aegean, as this vessel shape is closely associated with Mycenaean exports.

The Rhodian, Chian, and Samian amphorae analysed in this study are named for the Greek islands on which it is thought they are produced: Rhodes, Chios and Samos. The shapes of these Greek amphorae and their typology has been much discussed in the literature (e.g. Grace 1934, 1946, 1961; Whitbread, 1995, Lawall, 2010), and their identification was provided by the Principal Investigator of each shipwreck site (e.g. Demesticha, 2011; Swiny and Katzev, 1973). The Africana III / Keay 25 amphorae from the Babuljaš shipwreck, Croatia, are thought

to have been manufactured in North Africa, with amphorae of the same type produced in Sullectheum/Salakta in Byzacena (Bonifay and Leffly, 2002), and Neapolis/Nabuel on Cape Bon (Bonifay et al., 2009).

1.3.1 The Mazotos shipwreck, Cyprus

Early surveys and subsequent excavations of this wreck located off the south coast of Cyprus found that the majority of the cargo consisted of amphorae produced at some point in the late 5th or early 4th century B.C.E. on the Greek island of Chios, indicating that this island is the most likely place of origin for the ship's cargo (Demesticha, 2011).



Figure 7. Chian amphorae from the Mazotos shipwreck.

Prior to the end of the 5th century B.C.E. Chian amphorae featured a distinctive bulbous neck, which was replaced by a straight neck by the end of the century (Whitbread, 1995, p. 136). The amphorae on the Mazotos shipwreck are of the latter type, which aids in the dating of this wreck (Figure 7).

The island of Chios was well known in antiquity for producing some of the finest wine in the region (Athenaeus, *Deipnosophistae*, Ath. 1.26b, 1.29e, and esp. 1.32f;) and it is this agricultural product that the distinctive amphorae of Chios are almost exclusively associated (Boardman, 1967; Sarikakis, 1986). Silver coins minted on Chios depict their distinctive amphorae, with a bunch of grapes pictured above the vessel, further strengthening our association between Chian amphorae and wine. Wine from Chios appears to have been a benchmark for comparison: a papyrus from Ptolemaic Egypt dating to 257 B.C.E. describes the ancient Bethanath estate in Judea that boasted 80,000 vines, which claimed to produce wine that was indistinguishable from the celebrated wines of Chios (Koh and Cline, 2014; Skeat, 1974). However, whole olive macro remains recovered from at least three Chian wine amphorae on the Mazotos wreck have shown empirically that whole olives were also transported in these so-called ‘wine’ amphorae.



Figure 8. Southern Aegean type jar designated 'mushroom-rim-knob-toe' from the Mazotos shipwreck found to contain whole olives.

A much smaller number of an additional type of amphorae are also present on the wreck: this type is more globular in shape, is thought to come from the southern Aegean, and has been designated ‘mushroom-rim-knob-toe’ amphorae by the Principal Investigator of the site. Whole olive stones have been found in one such jar (Figure 8). Notably, both amphorae from

Chios and southern Aegean amphorae found on the Mazotos shipwreck contained whole olives.

Here we come to the crux of the matter: how valid is the assumption that the Chian 'wine' amphorae found on the Mazotos shipwreck truly contained wine? An image from the Mazotos shipwreck may offer insight into the utility of traditional approach to amphorae interpretation, and why it has found such favour with excavators of ancient shipwrecks. In this image (Figure 9) we can see dozens of the hundreds of nearly identical Chian amphorae, with one southern Aegean type amphorae also in the frame. Once the Chian amphorae had been properly identified, it is very tempting to make a reasonable assumption as to what they contained, and in turn, the nature of the cargo on board this ship. The alternative, investigating the contents of every amphorae, is costly and time consuming. Yet, of the comparatively small number that have been investigated, several revealed an unexpected content of whole olives.



Figure 9. Mazotos shipwreck site, dominated by Chian amphorae, thought to have contained wine.

In this thesis, investigation of the macro-remains of olive stones recovered from these ‘wine’ amphorae by stable isotope analysis may be able to answer questions about the geographic origin of these remains, and the environmental conditions in which they were grown. No assumptions have been made as to what the southern Aegean amphorae type contained in the past, but the discovery of whole olive stones in one such vessels demonstrates that they at least occasionally contained olives.

Both organic residue analysis and shotgun metagenomic DNA analysis are performed on several of these ‘wine’ amphorae from the Mazotos shipwreck in order to investigate what these vessels may have contained and assess how effective these techniques are in the recovery of molecular evidence for their past contents.

1.3.2 The Kyrenia shipwreck, Cyprus

Discovered off the northern coast of Cyprus near the village of Kyrenia, this Hellenistic shipwreck is located at a depth of 27.4 metres, and initial surveys in the late 1960’s revealed a cargo primarily composed of Rhodian amphora, with an additional nine amphora types present (Katzev, 1969). Further excavation revealed that in addition to approximately 400 amphorae, the cargo contained stone mill blocks of a dark volcanic stone thought to originate in the Cycladic islands (Katzev, 1969), as well as wooden utensils, a large copper cauldron, fishing weights, coins, and domestic cookware, both fine and coarse (Steffy, 1985, p. 72). Whole almonds discovered in 12 amphorae led the excavators to deduce that these nuts constituted an element of the cargo (Katzev, 1969). Lead-filled anchors were also recovered from the wreck (Van Duivenvoorde, 2012). Amphorae recovered from the Kyrenia shipwreck and thought to have been produced in Rhodes, Samos, and Kos are analysed here.

As with the amphorae from the Mazotos shipwreck discussed above, the primary question posed is whether or not the ceramic transport containers recovered from the Kyrenia shipwreck truly did contain the wine they are assumed to have held. In order to determine what these

amphorae may have held, both organic residue analysis and shotgun metagenomic DNA analysis has been performed on sherds from the Kyrenia shipwreck. While it is known that almonds formed part of the cargo, it remains to be seen whether evidence for additional types of unexpected contents may be found in these ancient ceramic sherds excavated decades ago.

As the majority of the cargo consisted of amphorae from the island of Rhodes, which was the centre of a prolific wine trade, it has been assumed that the primary cargo the Kyrenia shipwreck was wine (Katzev, Swiney, personal communication). Amphorae produced on the island of Rhodes had a wide distribution around the Mediterranean littoral, as evidenced by the discovery of Rhodian amphorae on the El Sec shipwreck, found off the coast of Majorca, Spain (Arribas, 1987; Parker, 1992b, p. 392). Nearly contemporary with the Kyrenia shipwreck, the El Sec shipwreck dates from between 390 and 340 B.C.E., and the recovery of Rhodian amphorae from this site serves to demonstrate the great distance that Rhodian amphorae were transported aboard ships in this time period. The widespread presence of Rhodian amphorae in the archaeological record outside of Rhodes provides evidence for the robust export economy the island enjoyed in the Classical and Hellenistic periods. While exports of wine undoubtedly formed a significant part of Rhodes' economy throughout the Classical and Hellenistic periods (Lund, 2011, p. 280), it remains a possibility that some Rhodian amphorae contained other products.



Figure 10. Rhodian amphora from the Kyrenia shipwreck.

The shape of Rhodian transport amphora evolved during their long period of production between the 4th century B.C.E. and the 2nd century C.E (Whitbread, 1995, p. 53). The Rhodian amphorae recovered from the Kyrenia shipwreck and analysed here are of the earlier type (Figure 10), that lack what would later become the distinctive angled handles by which Rhodian amphorae are so easily distinguished in the archaeological record.

Widespread references to Rhodian wine by ancient Greek authors such as Athenaeus, and a corpus of stamped amphorae studied by classicists has led to a close association between Rhodian amphorae and a content of wine (Whitbread, 1995, p. 54). A smaller version of the Rhodian amphora is also present amongst the cargo of the Kyrenia shipwreck, this type has been designated 'Rhodian fractional amphora' (Figure 11). While a direct evidence for what these smaller jars contained has not been found, it is assumed they also contained wine.



Figure 11. Rhodian fractional amphora recovered from the Kyrenia shipwreck.

While Rhodian amphorae dominate the cargo assemblage of the Kyrenia shipwreck, amphorae produced on the island of Samos were also found amongst the cargo. Amphorae of the ‘Samian’ type are difficult to define accurately given the possibility that similar shapes were produced elsewhere such as Miletus and in Asia Minor, where numerous examples of ‘pseudo-Samian’ (Whitbread, 1995, p. 129) dilute what may already be a flimsy designation. The Samian amphorae found on the Kyrenia shipwreck were clustered in the bow and stern section of the ship (Whitbread, 1995, p. 127) and are assumed to have contained wine. There is additional evidence from terrestrial sites that wine was carried in vessels of this type (Shipley, 1987, p. 16). However, a papyrus document which dates from 295 B.C.E. mentions a shipment of oil destined for Alexandria, Egypt contained within Samian jars (Grace, 1971; Whitbread, 1995, p. 122), and an olive branch was used as a device on silver coins minted in Samos during this period (Whitbread, 1995, p. 123). This perhaps suggests a content of olive oil, rather than wine, in the Samian amphorae recovered from the Kyrenia shipwreck.

A sherd from a Koan amphora from the Kyrenia wreck is also analysed here. Generally assumed to have contained wine, both Pliny (N.H. XVI, x, 78 -79) and Athenaeus (I, 32 – 33) describe Koan wine as having been mixed with large quantities of seawater (Whitbread, 1995, p. 82).

1.3.3 The Babuljaš shipwreck, Croatia

The currently uninhabited islet of Babuljaš is located on the Dalmatian coast of Croatia, adjacent to the modern town of Pakoštane (Figure 12). A heavily looted shipwreck dated to the 4th century C.E. has been observed on the submerged northern slope of Babuljaš at depths between 5 and 7 metres. Excavations have revealed a cargo predominantly composed of Africana III/ Keay 25 amphorae (Figure 13 -14), with a secondary cargo of African Redslip Ware (ARS). Based on these cargo items, an origin of North Africa has been proposed for the ship's last voyage.



Figure 12. Babuljaš islet, Croatia.



Figure 13. Amphorae sherds during excavation, Babuljaš shipwreck.

Africana III/Keay 25 amphorae are most often assumed to have contained olive oil (Bonifay et al., 2009; Bonifay and Leffly, 2002), as the trade in vast quantities of olive oil from the Roman provinces of Africa Proconsularis and Tripolitania is well attested by ancient authors.



Figure 14. Africana III/Keay 25 amphorae sherds from the Babuljaš shipwreck.

However, a recent study of the organic residues contained in 32 amphorae of this type revealed evidence for *Pinaceae* products and tartaric acid, indicating a content of wine rather than olive oil (Woodworth et al., 2015). Such results are significant, as they challenge long-held assumptions about the contents of certain types of amphorae. For example, Africana II amphorae dating from circa 200 C.E. were recovered from the Plemmirio wreck, Sicily, and are assumed to have contained olive oil (Gibbins, 2001). Based on this assumption, investigators suggested that these amphorae represent physical evidence for the shift from dependence on Spanish olive oil to a reliance on North African oil as the main source of long-distance olive oil importation in the Roman Empire (Gibbins, 2001, p. 311). If important theories on ancient trade are to be built on assumptions surrounding past vessel contents, the necessity to accurately determine what these amphorae actually contained becomes clear.

In addition to amphorae, a secondary cargo of African Red Slip Ware (ARS) has also been recovered from the Babuljaš shipwreck (Figure 15). This type of pottery was produced in North Africa and shipped overseas to be traded throughout the Roman empire (Hayes, 1972, p. 13). This wreck shares some similar features to the Dramont E shipwreck, which also carried some Africana III/ Keay 25 jars and a secondary cargo of ARS (Santamaria, 1995).



Figure 14. African Redslip Ware (ARS) bowl found on the Babuljaš shipwreck.

1.3.4 The Uluburun shipwreck, Turkey

Located off the south coast of Turkey near the city of Kas, in the province of Antalya, this wreck was discovered on a steep slope at between 44 and 52 metres depth (Bass, 1987; Cleary and Meister, 1999; Pulak, 1998). Named for a nearby promontory on the Turkish coast, the *Uluburun*, or 'Grand Cape,' this shipwreck dates from the Late Bronze Age and is thought to have sank around 1,350 B.C.E. (Pulak, 1997, p. 233).

Survey and excavation of this site revealed a cargo composed primarily of copper ox-hide ingots, over 100 cobalt and turquoise-blue coloured glass ingots, around 153 Canaanite jars containing approximately one tonne of amber-coloured resin, in addition to sundry items including 'logs of Egyptian ebony, an elephant tusk and part of another, hippopotamus teeth, ostrich eggshells,

tortoise shells, the doors of murex shells, an amphora of orpiment (a pigment, yellow trisulphide arsenic), and a variety of fruits and spices, including figs, pomegranates, grapes, olives, safflower, and coriander' (Bass, 1995, p. 1428). The high value of the cargo, the age of the ship, and the thorough nature of the excavation have ensured that the Uluburun remains one of the most well-known and extensively researched ancient shipwrecks ever found (Bass, 1995, 1987; Cleary and Meister, 1999; Haldane, 1993; Monroe, 2010; Pulak, 1998, 1988). Even the remains of a stowaway mouse were recovered from amongst the wreckage of the ship and analysed by morphometrics to see if the origins of this small mammal could provide clues as to ports visited along the last journey of the ship (Cucchi, 2008).



Figure 15. Canaanite jar KW 1553 from the Uluburun shipwreck.

This research focuses on the contents of the Canaanite jars recovered from the Uluburun shipwreck (Figure 15), the majority of which are vessels of known contents. First produced on the Levantine coast of what is modern-day Lebanon and Syria, 'Canaanite jars' were first defined by Virginia Grace in 1956 (Öniz, 2016, p. 5). Following their production in, and export from, the Levant circa 1,600 – 1,500 B.C.E., production of vessels of the same shape began in Egypt in the 15th century B.C.E. and images of these vessels are depicted in Theban tomb paintings that show scenes of winemaking (Öniz, 2016, p. 5). It is perhaps for this reason that Canaanite jars are often assumed to have contained wine. What is empirically known from terrestrial excavations is that significant numbers of Canaanite jars have been found in Late Bronze Age layers in the Aegean, specifically on Crete and in Southern Greece including

Tyrinis and Mycenae (Rutter, 2013, p. 56), which has led scholars to perceive an increase in Eastern Mediterranean trade in this period (Öniz, 2016, p. 5).

A small number of Canaanite jars on the Uluburun were found to include contents other than resin. Included in this research are three jars known to have contained resin, one found to be full of olive stones, and one found to contain several pomegranate seeds (Figure 15), but also bits of wood and additional detritus from the decomposing ship.

The contents of the 153 Canaanite jars were first established by the macro-remains of amber-coloured resin, then characterisation by GC-MS (Mills and White, 1989). Still, a debate emerged as to the true nature of their past contents. Bass had stated that the total weight of resin amounted to approximately one tonne (Bass, 1995, p. 1428); such an amount appeared to indicate that the resin itself was a significant element of the cargo. Yet others disagreed; a theory was put forth by McGovern and Michel (1996) that the Canaanite jars aboard the Uluburun actually carried resinated wine, and that the remnant resin left at the toe of the jars would have been sold on or reused after the wine was sold or consumed (McGovern and Michel, 1996). The evidence cited by McGovern and Michel (1996) to support these claims included the macro-remains of grape pips found in the toes of certain jars, and the results from a 'Fiegl spot-test' which indicated the presence of tartaric acid, a biomarker of wine. Stern et al. (2008) countered these claims, and tested several of the same resin samples analysed by McGovern and Michel, but with a larger range of techniques including repeating the 'Fiegl spot-test,' and adding analysis by Fourier Transform Infrared Spectroscopy (FTIR), and High Performance Liquid Chromatography Tandem Mass Spectrometry (HPLC-MS-MS). Their results indicated that there was no molecular evidence to support a past contents of resinated wine.

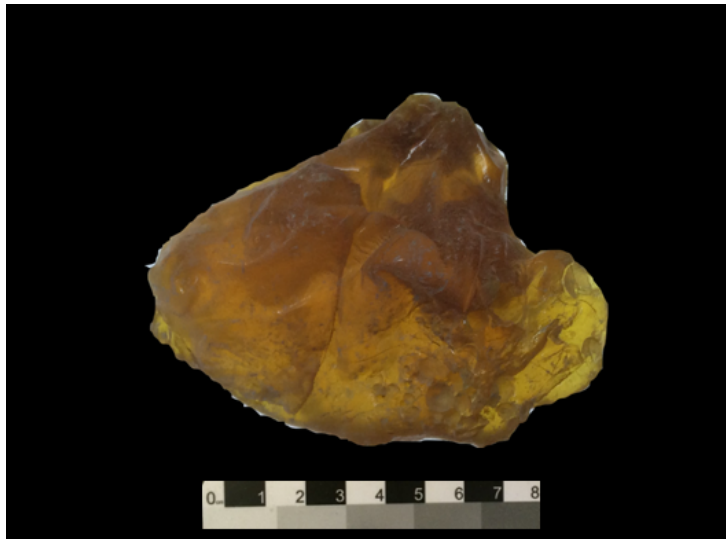


Figure 16. Resin recovered from Canaanite jar KW 605, Uluburun shipwreck.

Here, Canaanite jars known to have contained amber-coloured resin at their toe have been swabbed non-destructively to determine whether there is any DNA evidence for wine, or DNA derived from the resin itself (Figure 16). In addition, samples of resin have also been analysed by both DNA analysis and organic residue analysis, to determine if the new developments in shotgun metagenomics can provide new insights into this debate surrounding the true contents of these Canaanite jars. If evidence for resinated wine is revealed, it would vindicate the claims of McGovern and Michel (2016). If only evidence for the resin itself is revealed, it would further confirm the critiques offered by Stern et al. (2008).



Figure 17. Small pilgrim flask from the Uluburun shipwreck.

In addition to Canaanite jars, dozens of ‘pilgrim flasks’ were found on the Uluburun shipwreck site (Figure 17), in at least three sizes. None of these vessels retained a visible lining on their interior surface. This type of vessel is first noted in the Levant in the Late Bronze Age, appearing in archaeological assemblages that date between 1,550 B.C.E. and 1,400 B.C.E. (Dov Ber Markon and Gibson, 2007, p. 423). A globular vertical flask shape that is likely a precursor to locally produced pilgrim flasks was exported from Mycenaean communities into the Eastern Mediterranean regions of Egypt, Cyprus and the Levant. Here, this shape appears to have been more popular as an export than it was in contemporary Mycenaean communities (Gallorini, 2015). These Eastern Mediterranean communities soon began to produce local versions of this closed-vessel type, and Egypt imported pilgrim flasks manufactured in Syro-Palestine.

At terrestrial archaeological sites in Egypt that are contemporary to the Uluburun shipwreck, pilgrim flasks are often found associated with Canaanite jars, leading some scholars to postulate that pilgrim flasks contained a commodity that would be added to the wine in Canaanite jars to flavour it (Bourriau and Phillips, 2016; Gallorini, 2015). The discovery of dozens of pilgrim flasks on the Uluburun shipwreck, potentially associated with the 153 Canaanite jars, seems to

support this theory. Yet the question remains as to what kind of liquid would be transported in a closed-shape vessel, ready for suspension, that came in at least three clear sizes. This research tests whether the non-destructive swabbing of shipwreck pilgrim flasks can reveal molecular evidence for the contents held within these vessels in the past.

1.3.5 The Cape Gelidonya shipwreck, Turkey

Dating from slightly later than the Uluburun shipwreck, the Cape Gelidonya ship most likely sank around 1,200 B.C.E., and carried a cargo of copper ingots, scrap metal, and coloured beads (Bass, 1967, p. 163). The relatively modest nature of the cargo has led some researchers to conclude that this ship was likely manned by private entrepreneurs, rather than representing a state-controlled royal consignment (Muhly et al., 1977, p. 353).

Like the Uluburun shipwreck, Canaanite jars were also conveyed on this ship, however their state of preservation was considerably lower than the Canaanite jars from the Uluburun shipwreck, many of which remain virtually intact. One Canaanite jar from the Cape Gelidonya shipwreck was subjected to non-destructive swabbing (jar P2).

While the Canaanite jars found on the Uluburun shipwreck retained macro-remains indicating their past contents, including resin, olive stones, and pomegranate seeds, the Canaanite jars from the Cape Gelidonya shipwreck did not retain any macro-remains which demonstrate what they contained in the past. The original excavators referred to the few Canaanite jars recovered as ‘water jars’ (original excavation diaries, personal observation 2017), but there does not appear to be any corroborating evidence to support this designation.

In addition, one of the several stirrup jars recovered from the Cape Gelidonya shipwreck was also swabbed for analysis (stirrup jar 1.2.95). Widely produced on Crete, stirrup jars are considered to be the preferred bulk transport containers for liquid commodities in the Late Bronze Age Aegean (Pratt 2016, 27). Evidence from contemporary records written in Linear B suggest that oil and wine were variously contained in these vessels (Pratt 2016, 53). The

stirrup jars found on the Cape Gelidonya shipwreck are assumed to have contained olive oil, and in some cases the seafloor proximal to their in-situ location appeared to be stained with an oily substance (Pulak, personal communication).

1.3.6 Yeronisos Island, Cyprus

This small, rocky island located off the southwest coast of Cyprus, was first inhabited in the Chalcolithic period, followed by around 3,500 years during which it appears no human habitation took place. A new period of occupation began on Yeronisos island in the Hellenistic era (Connelly, 2005, p. 169). Finely carved stone architectural remains and sherds of fine-ware pottery suggest an elite presence on the island, where a large circular platform has been interpreted as an element of a temple of Apollo (Connelly, 2009, p. 302).

The ceramic artefacts found at Yeronisos are somewhat perplexing, however, as a high proportion of fine vessels and several elegant unguentaria (small, piriform jars thought to have contained unguents) would appear to indicate high status inhabitants (Figure 18 - 19). Contrarily, widespread evidence for reuse, re-fashioning, and mending of pottery suggests that the inhabitants were slow to replace the pots and jugs they possessed (Connelly, 2005, p. 176).



Figure 18. Unguentarium from Yeronisos island, thought to have contained precious liquids.

Several vessels preserve so many holes that have been mended with lead, it begs the question whether the cost of the lead would have exceeded the cost of replacing the pot (Connelly, 2005, p. 176). Widespread reuse of pottery was common in the ancient world (Peña, 2007). However, it is the combination of luxury wares with clear evidence for refashioning and mending that makes the ceramic assemblage of Yeronisos unique. Archaeological evidence for reuse has implications for the scientific analysis of these artefacts, as it is possible that palimpsests of residue and perhaps the associated DNA, could accumulate inside the ceramic matrix of such vessels after years of continued use.



Figure 19. Unguentarium from Yeronisos.

Another interesting facet of the Yeronisos pottery assemblage is the decided dearth of transport amphorae, while 'wine' pitchers and 'wine' vessels abound: lagynoi and oinochoe, pitcher-like vessels thought to have contained wine, are common finds on the island (Figure 20). This begs the question as to how wine arrived at Yeronisos, if not in transport amphorae, and if the many *oinochoe* and *lagynoi* discovered did in fact contain wine.



Figure 20. Oinochoe, a 'wine' jug, from Yeronisos island.

1.4 Conclusion

As methods in archaeological science advance, it is possible to apply new techniques to both recently excavated, and previously excavated, shipwreck material. Ancient shipwrecks make ideal case-study sites for examining the efficacy of newly developed methods. Often containing hundreds of 'identical' amphorae, we may be able to see patterns, or anomalies, emerge amongst what is assumed to be a homogenous cargo. By incorporating data from the occasional preservation of macro-remains inside maritime transport containers, we are allowed a control of sorts, in that as we test these scientific techniques, in some instances the past vessel contents are already known. This allows us immediate insight into how effective a method is by establishing whether this method reveals molecular evidence for the known contents.

Comparing what new information can be gleaned from either ancient DNA shotgun metagenomic analysis, or the more traditional residue analysis of the same artefact can provide great insight into which of these techniques is most effective for determining past vessel contents. Past vessel contents are not always as straightforward as previously thought, as demonstrated by the macro-remains of unexpected cargo items on numerous ancient Mediterranean shipwrecks. With this in mind, the research presented here will explore these unexpected contents by subjecting waterlogged organic remains to stable isotope analysis and testing both destructive and non-destructive methods of organic residue and ancient DNA analysis on ceramics transport containers from five ancient Mediterranean shipwreck sites, and one terrestrial site.

Fundamentally, this research seeks to illuminate the diverse content types and uses of amphorae aboard ancient shipwrecks. The archaeological evidence from ancient shipwrecks calls into question our tidy assumptions surrounding the trade, transportation, and packaging of commodities in the past. In light of the fact that ancient shipwrecks can offer such vivid and direct information on trade in the ancient world, understanding what they were trading is

fundamental to our interpretations of the ancient economy. Detailed work on the uses of amphorae found on terrestrial sites from the Classical period (Lawall, 2016) and the Roman world (Peña, 2007) has successfully shown that amphorae re-use appears to have been far more common than previously thought. As Lawall puts it: 'While some scholars remain sceptical of the extent to which such re-use occurred and hence the scale of this problem it seems to me that if the occurrence is clear in even a few archaeological cases, the ancient reality must have been even more significant' (Lawall, 2016, p. 264).

Whether an amphora carries its original contents, or has been re-used, may not be immediately apparent. By seeking molecular evidence for past vessel contents and characterising aspects of the underwater deposition environment, this research attempts to provide greater insight into what shipwreck amphorae contained, and how best to detect it. Stable isotope analysis may be able to answer questions regarding the origins and purpose of waterlogged botanical remains found on shipwreck sites. Organic residue analysis can help to confirm or deny assumptions made about the contents of mass-produced amphorae carried on ships in the ancient Mediterranean. DNA analysis of ancient shipwreck material may hold great promise, but it is necessary to revisit work that used methods now surpassed by Next Generation Sequencing, metagenomics, and bioinformatics.

Patterns of exchange, commercial interests, agricultural and social transformations are all tied up with our understanding of what was being produced, packaged, and traded, where these items were being sent, and by whom. As the transport container par excellence of the ancient world, understanding what the amphorae found on shipwrecks contained is vital for reconstructing ancient economic phenomena and developments. Here, we explore scientific methods that will allow us to do exactly that.

Chapter 2 – Stable Isotope Analysis

TITLE:

Stable Isotope analysis of waterlogged olive stones from the Mazotos shipwreck

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Abstract

The use of stable isotope analysis on waterlogged archaeological plant material remains largely unexplored. Here we present the results from a small, preliminary isotopic study of olive stones recovered from the 4th century B.C.E. Mazotos shipwreck, Cyprus. Analyses of the stable isotopes of carbon and nitrogen are conducted on 20 ancient, waterlogged olive stones from the Mazotos wreck and 10 modern olive stones from Chios, Greece. The extent of lipid retention, and preservation of original isotopic composition is explored. The possibility of determining the geographic origin of the Mazotos olive stones by isotope analysis is assessed.

2.1 Introduction

Underwater archaeology has provided unique insights into olive (*Olea europea*) cultivation, transportation, and consumption in the ancient Mediterranean. From the submerged prehistoric settlements off the Carmel Coast which yielded olive pits, pollen and wood, as well as evidence for olive oil processing (Galili et al., 1993; 1997), to the largest single deposit of olives from the Late Bronze Age found in a Canaanite jar on the Uluburun shipwreck (Haldane, 1993), and the olive-filled transport amphorae found on Archaic and Classical wrecks (Bound and Vallintine, 1983; Demesticha, 2011; Frey, 1982, p. 5; Haldane, 1993; Lanza, 2011; Santamaria, 1995), submerged archaeological sites can provide a wealth of information about the transportation and use of the fruits from this emblematic plant.

In 2006, an ancient shipwreck was discovered off the southern coast of Cyprus near the village of Mazotos. The majority of ship's cargo consisted of amphorae produced on the Greek island of Chios in the 4th century B.C.E., therefore Chios was most likely the harbour whence the ship started its last voyage (Demesticha, 2011). Chios was well known in antiquity as a producer of fine wine, and it is this agricultural product with which the island and its distinctive amphorae are most often associated (Athenaeus, *Deipnosophistae*, Ath. 1.26b, 1.29e, and esp. 1.32f; Boardman, 1967; Sarikakis, 1986).

However, since the discovery of this shipwreck and the subsequent excavation seasons, at least 25 Chian amphorae have been found to contain diverse quantities of olive pits. While this remains a relatively small proportion of the 500+ amphorae observed at the site, evidence is mounting that the Mazotos ship carried a heterogeneous cargo that included more than just wine. Only three of these amphorae had significant numbers of pits (over 1,000) that indicate without any doubt that they were originally shipped full of olives; groups of 5-100 olive stones were found in 22 amphorae, which could either be the remnants of larger quantities or the

result of post-depositional site formation processes. Thus far, more than 10,000 olive stones have been recovered from excavated amphorae, as well as numerous stones collected through sieving the excavated seafloor sediment within the shipwreck area. While the majority of the cargo on this ship was most likely the famed wine from Chios, evidence is mounting that a significant number of whole olives were carried on board. In order to investigate the preservation of isotopic composition, ancient growing conditions, possible geographic origins and the purpose of the Mazotos olives stones, stable isotope analysis has been conducted on 20 stones.

While the effects of charring on the isotopic composition of archaeobotanical material has been widely explored (Fraser et al., 2013; Poole et al., 2002; Styring et al., 2013), and desiccated remains to a lesser extent (Evershed et al., 1997; Metcalfe and Mead, 2019), little research has been done on what effect water logging may have on the stable isotope ratios of organic remains, or whether or not it is possible to analyse ancient waterlogged material using the same methods of isotopic analysis that have been used so effectively on charred plant remains. This paper explores the use of stable isotope analysis as a method to determine the environmental and cultivation conditions under which the waterlogged olive stones from the Mazotos shipwreck were originally grown, and whether such information can allow us insight into their geographic origin, or purpose aboard the vessel.

2.2 Context

2.2.1 Stable isotope analysis: Environmental conditions

Analysis of the stable isotopes ^{12}C and ^{13}C can offer insights into the environmental and atmospheric conditions under which a plant grew (Malainey, 2010). The ratio of carbon stable isotopes is given as a “delta” (δ) value and is expressed by the following formula:

$$\delta = \left[\frac{R_{\text{Sample}}}{R_{\text{Standard}}} \right] \times 1000 \text{ permil}$$

In this formula, R represents the $^{13}\text{C}/^{12}\text{C}$ ratio for $\delta^{13}\text{C}$ values (Marino and Deniro, 1987). There are several factors that can lead to variation of stable isotope values in plant populations. These include photosynthetic pathway, species, latitude/altitude, and water availability (Bird and Ascough, 2010, p. 1530).

2.2.2 Geographic origin

Results from the stable isotope analysis of carbon and nitrogen have been used to argue for either single or multiple origins for archaeological examples of agricultural products from Bronze Age Greece (Heaton et al., 2009), Bronze Age Syria (Fiorentino et al., 2012), and Iron Age England (Lightfoot and Stevens, 2012). Broadly, it is the range of isotopic values that can indicate either a single origin or multiple origins for plant remains. A large range can be indicative of diverse growing conditions, suggesting that plants were grown in spatially distinct areas, and experienced different levels of rainfall, sunlight or temperature. Fiorentino et al. (2012) used such data to argue that the Bronze Age site of Ebla was an important centre for the redistribution of agricultural products: the charred barley found there shows a wide range of isotopic values, which the authors interpreted as an indication that the barley had been grown in many different fields and was subsequently transported to Ebla. In contrast, a limited range of isotopic values can indicate a single geographic origin for agricultural products. Charred wheat remains from the Bronze Age site of Assiros Toumba, Greece showed a small range of isotopic values, which mirrored the tight range of values found for inter-annual single field harvests of modern durum wheat grown nearby the archaeological site: these results were used to argue for a single (and local) geographic origin for the charred ancient wheat stores of Assiros Toumba (Heaton et al., 2009).

As with previous isotopic studies of archaeological wheat (Heaton et al., 2009), barley (Fiorentino et al., 2012), and Pistacia resin (Stern et al., 2008), it is necessary to first establish what variability in isotopic values for carbon and nitrogen one can expect to find in modern

examples of the same plant species. While olive stone endocarp has not been previously been studied, stable isotope analysis has been used to combat adulteration and counterfeiting in the modern olive oil industry. Several studies have utilised isotope analysis to show that the isotopic values found in olive oil can reflect the region in which the olives were grown (Angerosa et al., 1999; Gumus et al., 2017; Portarena et al., 2014).

Here, comparison with a control sample set of modern olives grown on the Greek island of Chios (the most likely port where the ship's cargo was loaded) allows us to determine a baseline $\delta^{13}\text{C}$ value for olive stone endocarp. Once established, the range of isotopic values from the Mazotos shipwreck olive stones are compared with the range of values from the set of single origin olive stones from Chios in order better understand if post-depositional alterations to the isotopic composition has taken place, if lipids are retained within the ancient olive stone endocarp, and to assess the possibility of determining geographic origin based on the stable isotopes of carbon and nitrogen.

2.3 Materials and Methods

2.3.1 Recovery and Selection

Excavation at both the bow and the stern end of the shipwreck yielded olive remains (Demesticha, 2011). Twenty olive stones were systematically selected in order to represent both the bow and stern portions of the shipwreck, as well as three different manners of recovery: from inside an excavated amphora, directly from a trench, and sieve-collected (Figure 21).

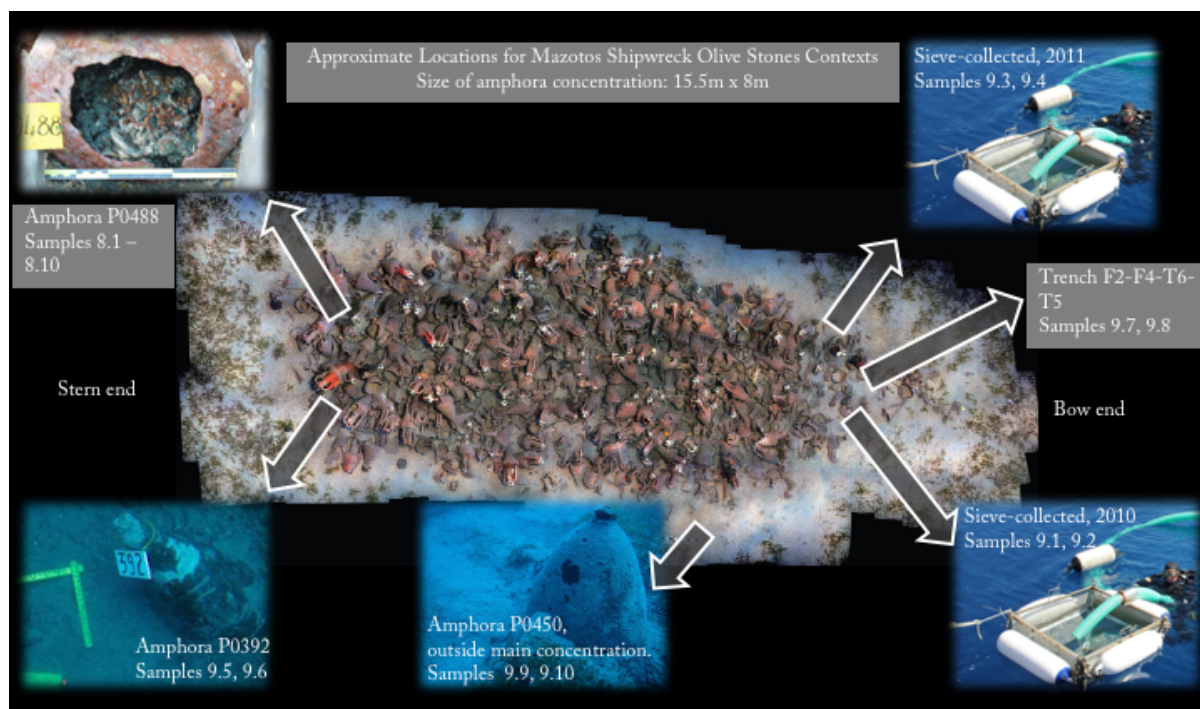


Figure 21. Mazotos olive stone recovery locations.

With a distance of 15.5 metres between bow and stern and located at a depth of 44 metres (Demesticha, 2011) where the current is negligible, it seems unlikely that post-depositional processes would have moved olive stones from the stern to the bow, suggesting that the many olive stones found in sediments at the bow end of the ship were originally stowed somewhere nearby.

Ten stones were selected from a Chian amphorae found at the stern of the wreck (amphora P0488, Figure 22), with two additional olive stones selected from another Chian amphora also located at the stern (amphora P0392, Figure 23).

Two more olive stones were selected from a non-Chian amphora, which seems to belong to a large group of south-eastern Aegean containers, called 'mushroom-rim' (Demesticha, 2011) for their heavy, over-hanging rims (amphora P0450, Figure 24, Figure 25).

A total of six olive stones recovered from the seafloor sediment were selected for analysis: two were recovered directly from a trench at the bow end of the wreck (Trench F2-F4-T6-T5) and four were sieve-collected on the surface.



Figure 22. Chian amphora P0488 full of olive stones.

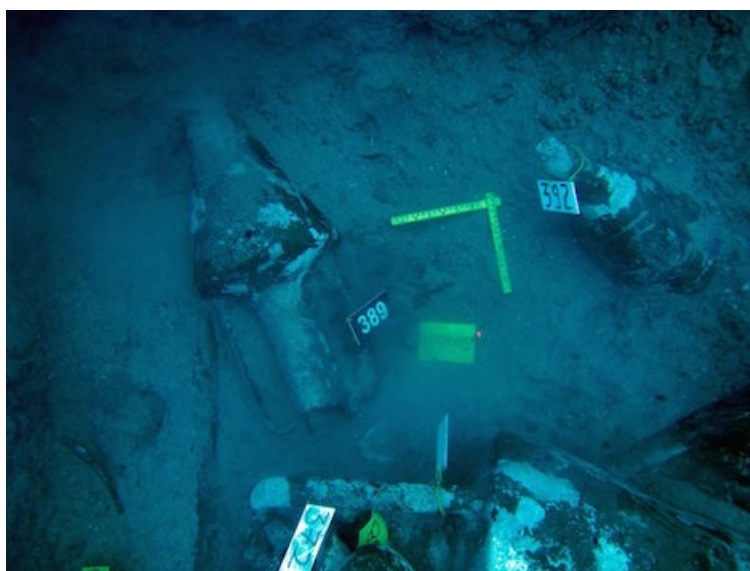


Figure 23. Chian amphora P0392, in situ.



Figure 24. Amphora P0450, southern Aegean-type amphora.



Figure 25. Amphora P0450, in situ.

2.3.2 Olive anatomy

Whole olives consist of a thin skin (exocarp), which surrounds a fleshy interior (mesocarp), and at the centre is a hard stone (endocarp) that encases a soft, lipid-rich kernel, which is the embryonic olive tree.

2.3.3 Initial Examination

All olive stones recovered from the Mazotos shipwreck consist of olive endocarp, as the soft, fleshy mesocarp has either decayed after deposition or has been consumed by marine organisms. During examination of the extant olive endocarp, it was noted that nearly all of the 20 Mazotos shipwreck olive stones showed signs of consumption by marine organisms, which left marks on the exterior surface of the endocarp (Figure 26).



Figure 26. Sample 8.1, with signs of consumption by marine organisms.



Figure 27. Sample 9.1 showing aperture through which marine organisms may have entered to access kernel.

By gaining access to the interior of the olive stone through the aperture where the stem enters the endocarp it appears that these organisms were able to consume the soft, lipid-rich kernel. When the endocarp was opened, what at first appeared to be an extant kernel was in fact an empty seed coat. Consequently, only the endocarp is preserved in this waterlogged collection.



Figure 28. Sample 8.3 with empty seed coat.

All archaeological olive stones were measured, photographed and examined both before and after freeze-drying. It was noted that the gross anatomical morphology of the olive endocarp, and signs of consumption by marine organisms, was easier to observe after freeze-drying (Figure 29, Figure 30).



Figure 29. Sample 9.1 prior to freeze-drying.



Figure 30. Sample 9.1 after freeze-drying.

Included as a control sample set are 10 modern olive stones purchased from a market on the island of Chios, Greece (Figure 31). These were organically grown on one orchard on the island. As olive stone endocarp has not previously been analysed through stable isotope analysis, these modern samples are included in order to determine what range of $\delta^{13}\text{C}$ values can be expected for modern olive stone material grown in one geographic region in the Eastern Mediterranean. These results will be compared with the range of $\delta^{13}\text{C}$ values found for the ancient waterlogged olive stone material. While 10 modern samples will not give definitive indication of the possible range of $\delta^{13}\text{C}$ values for olive stone endocarp grown throughout the entire island of Chios, the data from the contemporary samples can assist in indicating whether or not the isotopic composition of the ancient samples has drastically altered post-deposition.



Figure 31. Chios market olive stone after de-fleshing.

2.3.4 Sample Preparation

All samples were initially prepared for analysis at the Dorothy Garrod Laboratory for Isotopic Analysis, McDonald Institute for Archaeological Research, University of Cambridge, where similar work has been conducted on archaeobotanical material (Lightfoot and Stevens, 2012). The Mazotos shipwreck samples were rinsed with double distilled H₂O, and one olive stone was submerged in pH3 solution in order to determine whether or not seafloor sediment or marine organism remains had contaminated the samples, the most common contaminants for terrestrial samples being carbonate precipitates and humic/fulvic acids (Fiorentino et al., 2015). No reaction from carbonate precipitates was observed in the pH3 so it was deemed unnecessary to proceed with a full pH3 rinse.

All samples were measured for length and width, examined using a Leica M205C microscope, and photographed with a Leica DFC 425 microscope camera. The waterlogged Mazotos samples were examined and photographed both in their waterlogged state and after freeze-drying. Samples were first placed in a Revco -20° freezer for 3 hours then moved to a Revco -70° freezer for 12 hours. Next the samples were dried in a ThermoFisher ModulyOD 230 Freeze-drying Digital Unit at -41° for 12 hours. Once dried, the samples were examined and photographed for a second time. In their dry state, it was far easier to observe features on the exterior surface of the endocarp and assess their level of consumption by marine organisms. Once these features had been noted and recorded, the samples were pulverized with an agate mortar and pestle.

The modern samples from Chios presented greater difficulty in preparation. The lignin and cellulose-rich composition of fresh olive endocarp is extremely durable, and after attempts to pulverise this material with a mortar and pestle, the fragments were too large for the desired weight of 1mg. After extensive experimentation, the most effective method of pulverization proved to be grinding the samples into coarse fragments with a steel percussive mortar and pestle, then agitating the coarse fragments in an Eppendorf tube with four sterilised steel balls in a Qiagen Tissue Lyser for 120 seconds at 20 agitations per second. The Tissue Lyser significantly aided in pulverizing the samples to a homogenous powder. As the modern Chios samples had not come into contact with archaeological sediments it was not necessary to

perform the pH3 wash process. In order to remove any remaining H₂O, the modern samples from Chios were freeze-dried as above.

2.3.5 Lipid extraction

A test was conducted to determine if lipids were retained in the archaeological material and what effect this may have on the isotopic data. One ancient olive stone from Chian amphora P0488 (Sample 8.1) and one modern Chios market olive (Sample 5.6) were divided into two equal parts. Half of the pulverised olive stone material was subjected to a solvent extraction (5 mL 2:1 CHCl₃/MeOH x 3) with sonication at 40° C for 20 minutes.

A small difference in isotopic values was observed in the results from the extracted and non-extracted half of each olive stone. As expected, the modern Chios market olive stone showed a greater difference in absolute values from the extracted and non-extracted sides, with an average from the triplicate runs for the non-extracted side resulting in a $\delta^{13}\text{C}$ value of -23.9‰ and the extracted side -23.6‰, a difference of 0.3‰. The archaeological olive stone appears to have retained fewer lipids, with a $\delta^{13}\text{C}$ value of -24.6‰ for the non-extracted side and -24.5‰ for the extracted side, a difference of 0.1‰. While this test has shown that a significant amount of remnant oil is unlikely to be preserved in shipwreck olive stones, the fact remains that any remnant oils could affect the isotopic results, as olive oil is far more depleted in ¹³C than the cellulose-rich olive endocarp, with an average $\delta^{13}\text{C}$ of around -31.0‰ to -27.5‰ (Portarena et al., 2014). As a precautionary measure, all archaeological and modern olive stones in the study were subjected to lipid extraction. Once dried, 1mg amounts of pulverised olive endocarp material ($\pm$ 0.10mg) were placed in a tin capsule in duplicate.

2.3.6 Mass Spectrometer

Initial analyses were conducted at the Godwin Laboratory, Department of Earth Sciences, at the University of Cambridge. The lipid extraction protocol and post lipid-extraction analyses

were conducted at the Research Laboratory for Archaeology and the History of Art, University of Oxford. Analyses were performed using an automated elemental analyser coupled in continuous-flow mode to an isotope-ratio-monitoring mass-spectrometer (Costech elemental analyser coupled to a Finnigan Delta V mass spectrometer). Stable isotope concentrations are measured as the ratio of the heavier isotope to the lighter isotope relative to an internationally defined scale, VPDB for carbon and AIR for nitrogen (Hoefs, 1997). Isotopic results are reported as δ values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) in parts per 1000 or ‘permil’ (‰) values, where $\delta^{15}\text{N}_{\text{AIR}} = [({}^{15}/{}^{14}\text{N}_{\text{sample}} / {}^{15}/{}^{14}\text{N}_{\text{AIR}}) - 1] \times 1000$. Based on replicate analyses of international and laboratory standards, measurement errors are less than $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. The isotopic standards used are IAEA standard of caffeine for carbon and nitrogen; in-house laboratory standards of nylon, alanine and bovine liver standard for carbon, nitrogen and atomic C/N ratios.

In order to compare carbon isotope values between modern and ancient samples it is necessary to correct for the ‘Suess Effect.’ Atmospheric CO_2 has increased since the Industrial Revolution and as more CO_2 enters the atmosphere, there is proportionately less ^{13}C in the air as ^{12}C is the more prevalent carbon isotope (Keeling, 1979). The National Oceanic and Atmospheric Administration calculates that the $\delta^{13}\text{C}$ levels have dropped from their pre-Industrial Revolution value of -6.5‰ to -8‰ at present (Shoemaker et al., 2010). As the modern samples were formed after the Industrial Revolution and the ancient samples were formed before, it was necessary to correct for the discrepancy in atmospheric CO_2 by raising the $\delta^{13}\text{C}$ values of the modern samples by $+1.5\text{‰}$ in order to make an accurate comparison.

2.4 Results

2.4.1 Nitrogen isotope results

The amplitude peaks for the $\delta^{15}\text{N}$ results were insufficient for obtaining reliable nitrogen isotope results (an amplitude peak height of between 3,000 – 13,000mV is desirable and these

runs showed amplitude peak heights of <1,000mV). This could be due to the 1mg weight of the sample being an insufficient amount of matter to produce the desired amplitude peaks for nitrogen during combustion in the initial stages of the mass spectrometer. As ancient olive material has rarely (if ever) been subjected to stable isotope analysis, this process of trial and error is informative for future studies. The amount of human bone collagen material required for isotope analysis, for example, is well established, while the great variety of ancient botanical material available to archaeological scientists has yet to be tested as thoroughly as human remains and thus requires further experimentation to establish weight standards and acceptable standard deviations for the wide range of botanical material types (Lightfoot and Stevens, 2012). After replicate runs before and after lipid extraction, no additional ancient olive stone material remained to repeat the analysis with a greater sample weight. We suggest at least 2mg of pulverised olive stone material for future studies to obtain sufficient amplitude peaks for reliable $\delta^{15}\text{N}$ data. However, the 1mg weight used here was sufficient to produce reliable results for the $\delta^{13}\text{C}$ values.

2.4.2 Carbon isotope results

A summary of the carbon stable isotope results is given in the following table (Table 4).

Table 4. Summary of results.

Origin	Max. $\delta^{13}\text{C}$ ‰	Min. $\delta^{13}\text{C}$ ‰	Mean $\delta^{13}\text{C}$ ‰	Range	SD	Replicates
Modern Chios (n=10)	-21.5	-24.2	-22.9	2.7‰	0.9‰	2
P0488 Mazotos (n=10)	-22.6	-26.5	-24.1	3.9‰	1.1‰	2
Various Mazotos (n=10)	-21.4	-24.8	-23.3	3.4‰	1.1‰	2

The modern olive stones from Chios have a mean $\delta^{13}\text{C}$ value of 22.9‰, a standard deviation of 0.9‰, and a range of $\delta^{13}\text{C}$ values from -21.5‰ to -24.2‰. This establishes that modern olive stone endocarp is less depleted in ^{13}C than modern olive oil, which has a range around -

31‰ to -27.5‰ (Portarena et al., 2014, p. 13). This further suggests that the amount of lipids preserved within olive stone endocarp will inevitably contribute to the $\delta^{13}\text{C}$ values found for such material, as greater amounts of remnant oil will lower the value. The Mazotos olive stones recovered from Chian amphora P0488 show $\delta^{13}\text{C}$ values ranging from -22.6‰ and -26.5‰ with a mean of -24.1‰, and a standard deviation of 1.1‰. Here we see a larger range of $\delta^{13}\text{C}$ values and higher standard deviation than the modern sample set, a range of 3.9‰. In addition to the 10 olive stones recovered from Chian amphora P0488, a further 10 olive stones recovered from various areas of the wreck have been analysed. Samples 9.1 and 9.2 (sieve recovered from the bow) have a relatively large range in $\delta^{13}\text{C}$ values from -21.4‰ to -24.7‰, a range of 3.3‰. Samples 9.3 – 9.10 (collected from a trench at the bow, Chian amphora P0392 and southern Aegean amphora P0450) share close absolute values and similar ranges of values. Results for all 30 olive stones analysed are shown in a box plot chart (Figure 32).

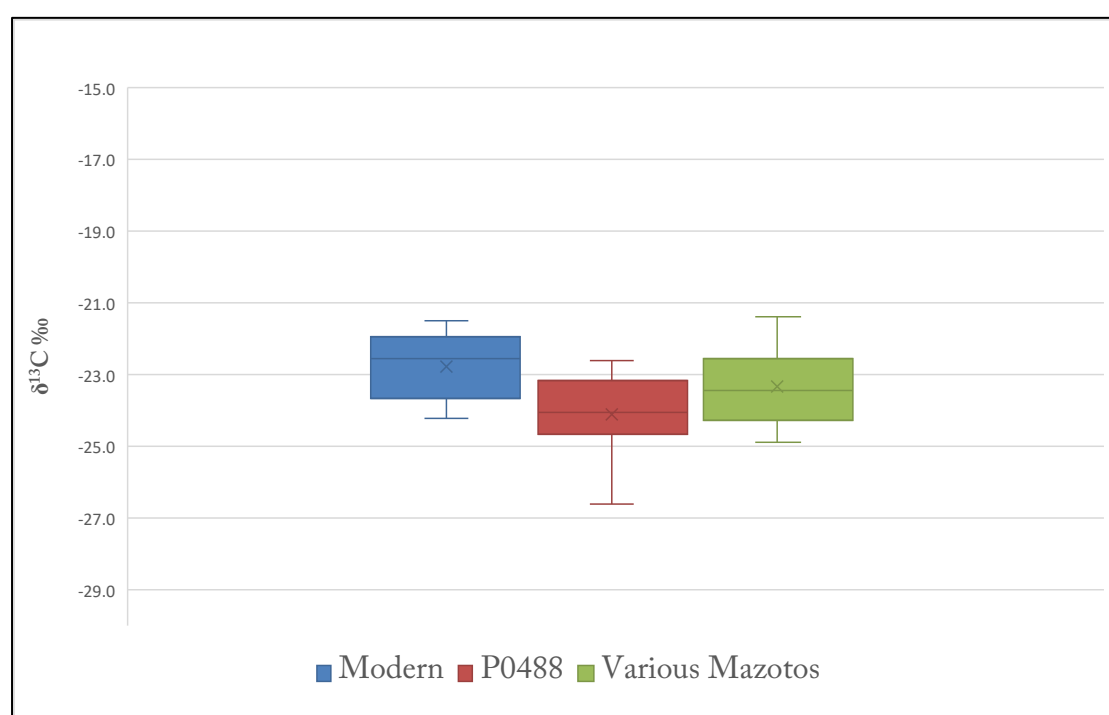


Figure 32. Box plot showing carbon isotope values for all samples. Blue is modern olive stones, red is olive stones from Chian amphora P0488, and Green is olive stones collected from various locations on the Mazotos shipwreck.

The chart below details $\delta^{13}\text{C}$ as well as specific recovery context to allow for a more detailed visual inspection of the data (Figure 33). The total range of values for the Mazotos shipwreck olive stones extends from -21.4‰ to -26.5‰, with a mean of -23.7‰ and a standard deviation

of 1.1‰. This means that there is a range in values of 5.1‰, a far higher range than that found for the 10 modern Chios olive stones including in this study, which have a range of 2.7‰.

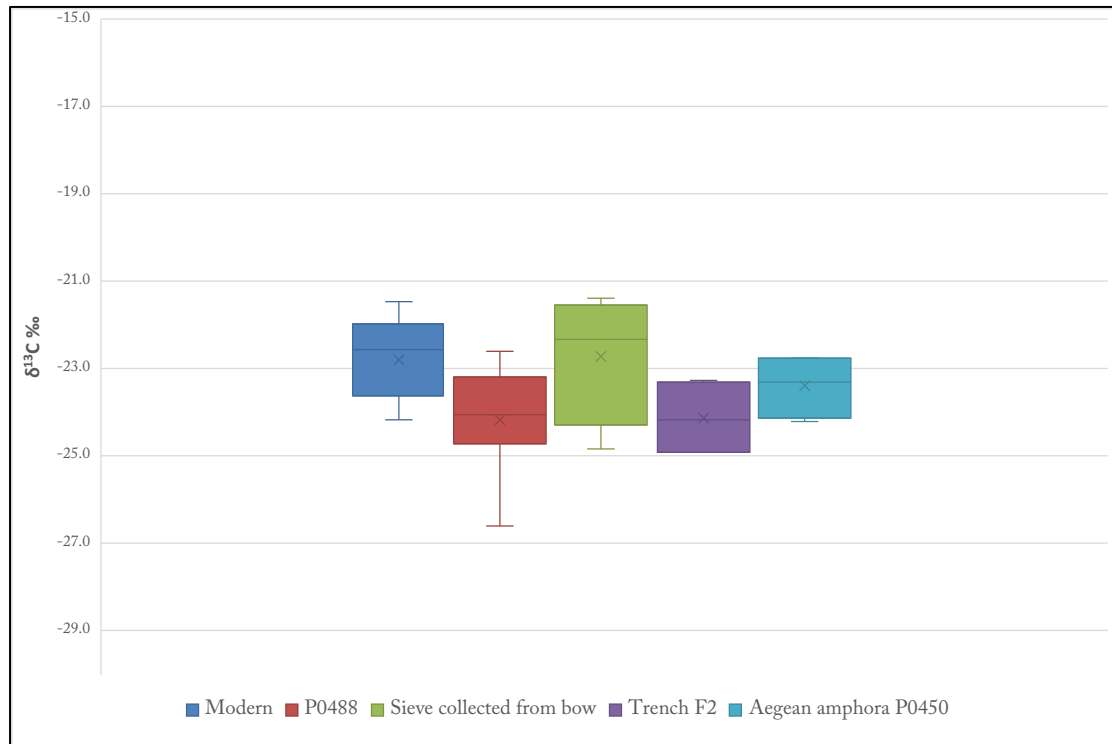


Figure 33. Carbon isotope results for olives stones by recovery context.

As shown in Figure 33, there is considerable overlap in values for the Mazotos olive stones and the modern olives from Chios, yet the stones recovered from Chian amphorae P0488 demonstrate the greatest variability in values, perhaps suggesting that the olives contained within this amphora were grown in a variety of locations. In order to better understand whether this wide range of isotopic values is statistically significant, several statistical tests were performed on the data using Microsoft Excel 16:28. First, it was necessary to determine if the values found for these olive stones show a normal distribution. In order to determine this, a Shapiro-Wilk test for normality was conducted (Shapiro and Wilk, 1965).

The data for $\delta^{13}\text{C}$ values for the olive stones analysed here show a normal distribution according to the Shapiro-Wilk test. These tests were undertaken using the online version of the Shapiro-Wilk test (Dittami, 2009).

In Table 5, results from an F-test comparing the isotopic values found for the ancient and modern olive stones are shown.

Table 5. Results from F-test comparing ancient olive stones (Variable 1) and modern olive stones (Variable 2) sample sets.

F-Test Two-Sample for Variances		
	<i>Variable 1</i>	<i>Variable 2</i>
Mean	-23.75	-22.78
Variance	1.41842105	0.87066667
Observations	20	10
df	19	9
F	1.62912066	
P(F<=f) one-tail	0.22921615	
F Critical one-tail	2.94765205	

The alpha value for this test was set to 0.05, as is standard in statistical tests in Biology and Archaeology (Bailey, 1992). In Table 5 we see that the P value is greater than the alpha value 0.05, with a value of 0.22. This means we accept the null hypothesis that there is not a statistically significant variance between Variable 1 (ancient olive stones) and Variable 2 (modern olive stones).

A major caveat for these tests is the fact there are not an equal number of ancient and modern olive stones represented in this set of samples. With double the number of ancient stones (20 ancient stones compared to 10 modern) it remains a distinct possibility that the large range of values found for the ancient olive stones is due to the greater number of this type of sample included in the study. Future research would benefit from analysing an equal number of ancient and modern samples, in order to simplify comparison of the values obtained.

2.5 Discussion

2.5.1 Range of $\delta^{13}\text{C}$ values for archaeobotanical remains

Results from the carbon isotope analysis of the modern olives stones from Chios indicate that a range in $\delta^{13}\text{C}$ values for randomly selected olive stones grown in the same season on one Eastern Mediterranean island can be at least 2.7‰ for olive endocarp. The range in values for the Mazotos shipwreck olive stones is 5.1‰. However, the range of $\delta^{13}\text{C}$ values can be easily influenced by a single outlier, as is shown in the Mazotos olive stones recovered from Chian amphora P0488 where a single stone (8.2) yielded a $\delta^{13}\text{C}$ value of -26.6‰, deviating from the average of the other stones recovered from this amphora by 2.7‰. If this outlier is included, the average for these 10 olive stones is -24.2‰, but if this outlier is excluded, we arrive at an average of -23.9‰. Several important studies of archaeobotanical remains have arrived at conclusions regarding the origin of their samples based on the range of $\delta^{13}\text{C}$ values, however, as discussed above, this may be problematic given the susceptibility of range to be altered by a single outlier. A selection of the previous studies that utilised stable isotope analysis as an indication of geographic origin or inter-annual harvests for archaeobotanical material are discussed below, in order to provide context for the results from this preliminary study of waterlogged botanical remains.

A range of 3.2‰ found for inter-annual single field harvests of modern durum wheat grown in Greece was used to argue for a single (and local) geographic origin for the ancient wheat samples recovered from the Bronze Age site of Assiros Toumba (Heaton et al., 2009). While durum wheat is a different type of material than olive endocarp, it is interesting that results from the Heaton et al. study (2009) show a range in $\delta^{13}\text{C}$ values of 3.2‰ for durum wheat grown in the Eastern Mediterranean which is similar to the range of 2.7‰ found for the olive stones from Chios, also grown in the Eastern Mediterranean. Conversely, according to the Fiorentino et al. (2012) study that compared modern barley to archaeological barley from Elba, the range found for modern barley samples in Syria was a mere 1.5‰ – 2.0‰. This suggests that the range in $\delta^{13}\text{C}$ values for unprocessed C_3 plant material grown in one geographical area of the Eastern Mediterranean depends on the type of plant material in question. While the far larger range in $\delta^{13}\text{C}$ values of 5.1‰ for the Mazotos shipwreck olive stones clearly exceeds the ranges found in other studies for plant material from one geographic origin, this is mainly due to the one outlier olive stone from Chian amphora P0488. This indicates that statistical analysis of variance may offer greater insight than a simple calculation of the range in $\delta^{13}\text{C}$ values for unprocessed C_3 plant material. Another interesting possibility is that a large range in values

may indicate remains from inter-annual harvests; Portarena et al. (2014) have shown that for modern olive oil annual variability may have a greater effect on isotopic values than geographic origin (Portarena et al., 2014).

In another study, isotope analysis conducted on archaeological resins recovered from transport containers was compared with isotope values of tree resins with known geographic provenance in order to identify areas of probable geographic origin for the ancient material (Stern et al., 2008). Some of the tree resins analysed originated in Chios and Cyprus, providing further comparative data of typical ranges in isotope values from two geographical locations relevant to this study. The values for *Pistacia lentiscus* resin from Chios are -25.3‰ and -24.7‰, a range of 1.4‰. The samples from Cyprus are of resin from two separate species, *Pistacia lentiscus* and *Pistacia terebinthus* however these also have similar values of -24.1‰ and -26.2‰, a range of 2.1‰. The large range of 5.1‰ for the Mazotos olive stone samples is larger than the ranges observed for durum wheat, barley, and tree resin samples of known provenance from one geographic location in the Mediterranean region.

The range in $\delta^{13}\text{C}$ values for the waterlogged archaeological material is almost double that of the modern set of olive stones from Chios, and exceeds the range found for durum wheat, barley and tree resin from a known provenance. Adhering to the conventional confidence interval utilised in biology of 95%, which requires a P value of 0.05 or lower to indicate statistical significance (Bailey, 1992), the results indicate that while there is indeed a large range for the Mazotos olive stones, there is not a statistically significant difference between the ancient and modern olive stones.

What this does indicate is that the difference in isotopic composition between the two is not statistically significant, suggesting that the marine deposition environment may not, in fact, significantly alter the original isotopic composition of olive stone endocarp. This suggests that future isotopic studies of plant material recovered from the marine environment may be beneficial to our understanding of submerged archaeological sites.

2.5.2 Range of $\delta^{13}\text{C}$ for modern olive oil

How does this range of values compare with those found in other isotopic studies conducted on modern olive products? In an early study, Angerosa et al. (1999) investigated isotopic variance in modern olive oil as a means of determining geographic origin. Olive oil from six Mediterranean countries was analysed in an attempt to determine what $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values could be expected from oil produced in these geographic areas (Table 6). This study concluded that oil from six Mediterranean countries generally showed $\delta^{13}\text{C}$ values in a relatively tight range: five countries had a range of 1.5‰ or less. The samples from Italy were a noted exception, showing by far the greatest range in $\delta^{13}\text{C}$ values from -31.3‰ to -27.0‰, a range of 4.3‰ (Table 6).

Table 6. Carbon and oxygen stable isotope values for modern olive oil from Mediterranean countries, after Angerosa et al., 1999.

	Greece		Italy		Morocco		Spain		Tunisia		Turkey	
	$\delta^{13}\text{C}$	$\delta^{18}\text{O}$	$\delta^{13}\text{C}$	$\delta^{18}\text{O}$	$\delta^{13}\text{C}$	$\delta^{18}\text{O}$	$\delta^{13}\text{C}$	$\delta^{18}\text{O}$	$\delta^{13}\text{C}$	$\delta^{18}\text{O}$	$\delta^{13}\text{C}$	$\delta^{18}\text{O}$
N	10	10	41	41	10	10	6	6	11	11	5	5
mean	-28.3	23.5	-29.5	20.7	-28.8	28.5	-28.9	25.4	-28.6	26.2	-28.2	24.6
SD	0.45	0.99	1.08	1.47	0.31	1.21	0.28	0.65	0.45	0.79	0.58	0.44
min	-29.1	22.3	-31.3	18.1	-29.2	27.1	-29.2	24.3	-29.6	25.3	-29.1	24.0
max	-27.6	24.9	-27.0	23.9	-28.4	30.7	-28.5	26.0	-28.2	27.9	-27.7	25.2
CI	±0.3	±0.7	±0.3	±0.5	±0.2	±0.9	±0.3	±0.7	±0.3	±0.6	±0.8	±0.6

The authors suggest the large range of values demonstrated by the Italian olive oil is due to the many varieties of olive cultivars and particular orography of the Italian peninsula (Angerosa et al., 1999). However, a closer inspection of the data published in this study reveals that a very small number of samples were represented from certain countries, perhaps too small a sample set to make an accurate assessment of geographic variability found among each nation, with only five samples from Turkey, and six from Spain (Angerosa et al., 1999). Rather predictably, of the six Mediterranean countries included in the Angerosa et al. study (1999), Italy showed the greatest variability in $\delta^{13}\text{C}$ and had the largest number of samples included, 41. Rather than being due to the particular orography of Italy or the numerous cultivars found there, it seems perhaps more likely that the inclusion of over eight times the number of samples from Italy than those from Turkey, a geographic region far larger than the Italian peninsula, may account for the variability found from the $\delta^{13}\text{C}$ values of Italian olive oil.

What this study claims to be a relatively homogenous isotopic signature for Turkish olive oil is more likely a result of the small sample size. A more recent study of the isotopic variation of

modern Turkish olive oil, for example, included 49 samples from the Aegean and Marmara regions and found values between -27.7‰ and -31.0‰ for these samples, a range of 3.3‰ (Gumus et al., 2017). As the absolute values found for oil from specific regions in Turkey overlapped with absolute values found for olive oil from Tunisia, Morocco, and Greece, the authors additionally incorporated principal component analysis of trace elements of Fe, Zn, Ca and Cu. A combination of trace element analysis and isotopic data was found to be more efficacious in distinguishing geographic origin than $\delta^{13}\text{C}$ values alone (Gumus et al., 2017, p. 1724).

An even larger study examined 387 olive oil samples produced in Italy over three successive years, 2009, 2010, and 2011 (Potarena et al., 2014). Even for this large sample set, the range in variation of $\delta^{13}\text{C}$ was 3.5‰, a smaller range than the 5.1‰ found for Mazotos olive stones, however it is not clear if outlier values were included or excluded from these studies. By examining the variation among inter-annual harvests from the three years included, the authors concluded that annual isotopic variation might be greater than the regional variation found for Italy (Potarena et al., 2014, p. 13). That is, clusters of isotopic values may be more likely to indicate what year olive oil was harvested in, rather than pinpointing a geographic region.

2.5.3 Potential insights into growing conditions

Studies have shown that water availability may be the most significant contributing factor to $\delta^{13}\text{C}$ values in C_3 plants (Hartman and Danin, 2010; Wallace et al., 2013). The inclusion of the modern sample set from Chios allows for a comparison between the $\delta^{13}\text{C}$ for these samples and the known levels of precipitation and temperature during their formation. Average annual rainfall in Chios is 656 mm, however the majority of precipitation occurs during the winter. The average rainfall in August (the period of endocarp and kernel formation) is a measly 1 mm (*weather-and-climate-information.com*, 2015). With average July temperatures of 27.5° (Ibid), it is reasonable to infer that the olive stones from Chios included in the study were formed under hot and dry climatic conditions.

The similarity in mean $\delta^{13}\text{C}$ for the Chios and Mazotos samples suggests that the Mazotos olives were grown under climatic conditions that were similar to the modern climatic conditions of Chios. However, the mean $\delta^{13}\text{C}$ does not reflect the large range of values found for the Mazotos olive stones. This relatively large range (5.1‰) could indicate that during sclerification of the olive stone endocarp, the olive trees that produced the ancient shipwreck olive stones experienced varying levels of access to water.

Predictably, the mean $\delta^{13}\text{C}$ value observed for all olive stone samples included in this study (ancient and modern) is -23.4‰, significantly higher than the mean $\delta^{13}\text{C}$ value of -27‰ for all C_3 plants (Bird and Ascough, 2012), which is consistent with the findings of Hartman and Danin (2010) that show plants grown during the hot, dry summer season in the Eastern Mediterranean tend to have higher $\delta^{13}\text{C}$ values than those grown in the same location during the cool, wet winter. However, it is necessary to account for the Suess effect when making any comparison between ancient and modern plant material, yet even if the mean for all C_3 plants is raised by +1.5‰ to -25.5‰, the mean value for olive stone endocarp remains higher, and appears to indicate arid growing conditions.

As seasonal variation in atmospheric CO_2 is anti-correlated to the trend in the $\delta^{13}\text{C}$ value of the atmosphere (Shoemaker et al., 2010), it is necessary to investigate the possibility that regional flowering time is related to $\delta^{13}\text{C}$ value, as endocarp sclerification occurs eight weeks after flowering time (Connor, 2005). Research on variability in flowering time has shown that temperature is the most significant factor for the flowering time of *Olea europaea* L. (Orlandi et al., 2010). However, the endocarp and kernel of the olive stone form in the summer during a time of consistent atmospheric $\delta^{13}\text{C}$ value. It is the fleshy mesocarp of the olive that continues to form in the autumn (Sanz-Cortés et al., 2002) as $\delta^{13}\text{C}$ values shift, and thus the $\delta^{13}\text{C}$ values for this fruity material type are far more likely to be affected by seasonality than the endocarp and kernel.

What remains unclear, for both the ancient and modern sample sets, is to what extent position within an olive grove may affect the $\delta^{13}\text{C}$ of olive stone endocarp. Preferential access to sunlight and water can vary within one grove based on a tree's position on a slope, which cardinal direction the tree faces, and the density of the surrounding canopy. These variables would

consequently cause a variation in the $\delta^{13}\text{C}$ of the fruits these trees produced. Bonafini et al. (2013) found a strong variation in the $\delta^{13}\text{C}$ between grasses grown in shaded woodland and those grown out in the open, with a variation of up to 5‰. Their tentative conclusion was that access to sunlight could be a greater factor in raising or lowering $\delta^{13}\text{C}$ than the uptake of respired CO_2 from the forest floor, but the effect of this depleted CO_2 could not be ruled out as a contributing factor (Bonafini et al., 2013, p. 3934). However, it should be noted that different plant material can show different levels of $\delta^{13}\text{C}$ within the same plant, as has been shown for *Setaria italica* (foxtail millet), where leaves on the same plant showed a range of $\delta^{13}\text{C}$ of up to 2.9‰, while the greatest range for the panicles of one foxtail millet plant was 1.6‰ (Lightfoot et al., 2016). It seems possible, then, that there is greater isotopic variation in the leaves and blades of grass than in sclerified plant material such as olive stone endocarp.

To better understand what variation in $\delta^{13}\text{C}$ can be found within the fruits from trees in one olive grove, island, or region, additional research involving a larger sample set of modern olives with well-established provenance is necessary. Such an experiment would ideally include olive samples grown over several years from multiple groves on Chios, facing different cardinal directions, and at different positions on the slope. The subtle microclimates created by slope, aspect, rainfall, and shade could well account for some of the variability found in the isotopic values of the olive stones analysed here. While it is vital to establish this data for modern olive stone sample sets in order to have a basis for comparison with ancient samples, the findings of Portarena et al. (2014) which indicate that inter-annual variability based on differences in temperature and precipitation may be an even greater factor than specific geographic origin means that any such study would also have to account for inter-annual variability in harvests.

2.5.4 Taphonomic factors in culinary preparation and the marine environment

Preservation type must be also considered as a factor in $\delta^{13}\text{C}$ values for the samples included in this study. If water logging by seawater were shown to have a significantly deleterious effect on the isotopic composition of organic material, this would greatly reduce the potential for isotope analysis of organic remains from underwater archaeological sites to yield useful results. In addition, research is needed on the ancient culinary practices used to make whole olives edible,

and what affect this process may have on the stable isotope composition of whole olives. A bitter compound called oleuropein in fresh olives makes uncured whole olives virtually inedible. Exclusion of oleuropein by preserving in brine has been used to produce edible olive fruits for centuries (Yada et al., 2007), but the potential effects on the isotopic signature of olive stone endocarp created by the preservation process, and to what extent the ancient method of whole olive curing differed from that used today, as some industrial olive curing uses lye (Yada et al., 2007), must be examined further. Both the marine environment and preservation through exposure to brine will significantly raise the amount of NaCl in olive fruits, but what effect this has on the isotopic values of olive endocarp remains unclear.

Issues surrounding contamination by salts and the marine environment may be avoided if different protocols are tested. Procedures for isolating the carbohydrate element of waterlogged wood are well established for ^{14}C dating, where acid-base-acid and bleaching treatments are used to isolate the cellulose from lignin and other compounds found in waterlogged wood (Brock et al., 2010). The inconsistency of olive stone endocarp, composed of cellulose, lignin, and as we have seen from this work, remnant lipids, suggests that variations found in the $\delta^{13}\text{C}$ may result from unintended incorporation of these heterogeneous constituents. Accidental inclusion of a simply oilier part of olive stone endocarp could lower the $\delta^{13}\text{C}$, thus creating what appears to be greater variation in isotopic composition than actually exists for one material type. If only the cellulose of olive stone endocarp can be isolated, by protocols used in ^{14}C dating (e.g. Brock et al., 2010), more consistent results that reflect the true isotopic composition of the material may be achieved. These protocols have been shown to successfully mitigate potential contamination by the marine environment on waterlogged wood from shipwreck sites (Lorentzen et al., 2014), as such, the potential incorporation of these protocols into shipwreck olive stone material seems to be a promising way to avoid interference by marine contaminants in this material. In addition, experimental analysis could be employed to test what effect water logging by seawater has on the isotope values of botanical remains, including olive stone endocarp.

2.6 Conclusion

2.6.1 Geographic origins

Stable carbon isotope analysis has been conducted on olive stones from the Mazotos shipwreck and modern olive stone samples from the Greek island of Chios. Results show that the $\delta^{13}\text{C}$ values for the olive stone samples from Mazotos do not have a statistically significant difference from modern olive stones from Chios used for comparative analysis in this study. While the range of $\delta^{13}\text{C}$ values found for the ancient olive stones exceeds those found for botanical remains grown in one geographical location examined in previously published studies that investigate geographic origins through isotope analysis, range alone is not a robust way to determine provenance as it is susceptible to be greatly altered by a single outlier. While no published data on the $\delta^{13}\text{C}$ of olive stones could be found, comparisons have been drawn between the ranges in isotopic values found for modern olive oil (Angerosa et al., 1990; Portarena et al., 2014; Gumus et al., 2017), and charred archaeobotanical remains including durum wheat (Heaton et al. 2009), barley (Fiorentino et al., 2012), and tree resin (Stern et al., 2008) grown in the Eastern Mediterranean.

While a statistically insignificant difference between the ancient and modern sample set may not provide definite answers regarding geographic origin, it may provide several insights into the archaeological material: first, it appears to indicate that the marine deposition environment has not had a seriously deleterious effect on the isotopic composition of these ancient shipwreck olive stones. Otherwise, we would expect to see drastically different values for the ancient and modern samples sets. Second, no statistically significant difference between the ancient and modern sets may indicate that the various olive stones recovered from different areas of the Mazotos shipwreck including the bow and stern, as well as inside Chian and southern Aegean amphorae types, may share a common geographic origin. It is an interesting possibility that a southern Aegean style amphora (P0450) and an amphora from Chios (P0392) may have contained whole olives from the same geographic location, or, at the very least, contained olives stones grown under very similar climatic and environmental conditions. This could have

implications for the use and re-use of amphorae in the ancient world, cargo compositions, and ship provisioning strategies.

2.6.2 Lipid retention

Based on results from the lipid extraction performed here, both ancient and modern olive stone material appears to retain some lipids within the stony endocarp. While the difference in $\delta^{13}\text{C}$ values found in the pre and post lipid extraction for the modern olive stone material was greater than that found for the ancient olive stone material (0.3‰ for the modern Chios olive stone and 0.1‰ for the Chian amphora P0488 olive stone), results consistently showed that non-extracted olive stone material had a more negative $\delta^{13}\text{C}$ value, indicating a smaller amount of ^{13}C over all. While only single samples of ancient and modern olive stones were subjected to this initial lipid extraction test, it is worth noting that over the triplicate runs of the olive stone material the direction of the difference was the same. This appears to be consistent with the fact that the $\delta^{13}\text{C}$ values of modern olive oil are lower than that of olive stone endocarp, with modern olive oil having $\delta^{13}\text{C}$ of around -31.0‰ to -27.5‰ (Portarena et al., 2014), while the 30 olive stones tested here had a mean $\delta^{13}\text{C}$ of -23.4‰. Therefore, the fact that olive stone material which has not been subjected to a lipid extraction procedure show lower $\delta^{13}\text{C}$ values than the olive stone material which has undergone lipid extraction appears to be an indication of lipid retention in the modern olive stones. For the ancient stones, however, a difference of 0.1‰ is not greater than the margin of error for mass spectrometry, indicating either a very small amount of remnant oil remains inside the endocarp, or none at all.

If even a small amount of remnant oil could be extracted from the ancient stones, however, this could have interesting future implications for pinpointing the geographic origin of ancient olive stone remains. If lipids extracted from ancient shipwreck olive stones could be collected, it may be possible to analyse the lipids themselves in order to establish where ancient shipwreck olives were grown. As the internal standard of ^{18}O isotope analysis is the Standard Mean of Ocean Water (SMOW), attempts to analyse artefacts waterlogged in the marine environment by oxygen isotope analysis would prove difficult as the artefacts would likely be contaminated by what is essentially the internal standard used to calibrate the Mass Spectrometer. If, however,

the lipids from olive stone endocarp could be extracted and isolated, it may be possible to perform oxygen isotope analysis on this material rather than olive stone endocarp.

Oxygen isotope analysis has been shown to be more effective in pinpointing geographic origin than carbon isotope analysis alone, and perhaps the dual-isotopes conceptual model of Scheidegger et al. (2000), a model that is also frequently used to derive climatic information from tree rings (Mccarroll and Loader, 2004), could be applied to these lipids by using both oxygen and carbon isotopes to investigate geographic origin. In a study that assessed 387 samples of extra virgin olive oil from Italy, the authors found at least four distinctive oxygen ‘isozones’ on the Italian peninsula alone (Chiocchini et al., 2016), indicating that if lipids can be harvested from ancient olive stone material then oxygen isotope analysis may be more capable of accurately pinpointing geographic origin.

Assessment of the various constituents of olive stone endocarp will also be helpful in understanding to what extent oxygen isotope analysis can be used on ancient olive stones; a study comparing the signals for $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in *Pinus halepensis* compared extracted wood, lignin and holocellulose and found that only the holocellulose of the analysed pine trees showed consistent relationships between the $\delta^{18}\text{O}$ and known climatic data (Ferrio and Voltas, 2005). This could theoretically also be true for olive stones, and it would be fruitful for future studies to compare isotopic signals derived from the lignin and cellulose of the same olive stone to establish what variability can be expected from different tissue types.

2.6.3 Amphorae use and re-use

The fact that two amphorae from different parts of the Aegean (P0392 and P0450) contained olive stones, rather than the wine with which these amphorae shapes are associated, is interesting, as it seems to suggest that at least one of them was reused. It is unlikely that both a southern Aegean and a Chian amphora were transported empty, after being freshly fired, to a third place to be filled with olives. This may also be indicative of the complex relationship between transport amphorae and particular contents in antiquity (Lawall, 2011, p. 32).

Perhaps wine was the prime-use content of the Chian amphorae on the Mazotos shipwreck, but it is abundantly clear that at least three of the Chian amphorae on board carried olives, most probably as an irregular content (for the types of content see Peña, 2007, p. 64). If we assume that the last port of departure where a significant amount of cargo was loaded onto the ship was Chios, then at least the south-Aegean amphora (P0450) must have been reused. Nothing on the Chian olive containers suggests that they were older than the rest - and thus, reused - but this possibility cannot be excluded. Even if the three Chian amphorae known to have contained olives were as new as the rest of the cargo amphorae, they could still be shipped with an irregular content. For the other 22 amphorae shown to contain a low number of olive stones it is possible that these stones may have entered the amphorae after the wrecking event.

2.6.4 Purpose of the Mazotos olive stones

It is difficult to determine evidence for diverse growing conditions, and therefore geographic origins, that would indicate a trade function for the Mazotos olives. Mariners made frequent stops for supplies on the way to their ultimate destination during this period, in which case diverse geographic origins for the olive stones might suggest that they were acquired from multiple locations during the journey, to provide food for the sailors on board. However, the fact that three of the four olive containers were Chian-style amphorae does not fit this narrative. In addition, the presence of olive remains at both the bow and the stern of the Mazotos shipwreck does not categorically indicate that whole olives formed part of the cargo intended for trade, as mariners would often store provisions for the crew at both the bow and the stern. Conversely, the sheer number of olives transported on board could be evidence that these olives were an element of the cargo and were intended for trade, as over 10,000 olive stones have already been found. The midship area of the wreck where the middle of the hold was located is currently under excavation, which may shed new light on the relative abundance of olive remains on this shipwreck.

After examination of the largest single deposit of Late Bronze Age olive remains ever found, Haldane (1993) argued that the large average size of the Uluburun shipwreck olives indicated

a trade function. Moreover, in the Zenon papyri we read reports of the olive trade from Chios, Knidos and other places in the Aegean to Hellenistic Egypt (Demesticha, 2011, p. 290; Kruit and Worp, 2000, p. 81). In this respect, it is also possible that whole olives were a dynamic element of the cargo, providing food during the journey with the potential to sell, barter or consume them at the ship's ultimate destination.

Perhaps seeking one simple function for the thousands of olive stones recovered from the Mazotos shipwreck is overly simplistic and does not account for their multifarious uses. Mounting evidence for the trade in whole olives gleaned from shipwreck evidence undermines arguments made by Runnels and Hanson (1986) and Hamilakis (1996) that oil processing equipment on terrestrial archaeological sites is necessary to infer widespread use and consumption of olives by a large societal demographic. Whole olives were far easier to produce and acquire than olive oil in the ancient world due to their lower cost of production (Foxhall, 2007). Increasing evidence from ancient shipwreck excavation shows that whole olives, not just olive oil, were traded and distributed widely.

Additional study of whole olive remains from ancient shipwreck sites is necessary to better understand their use place and purpose in sea-borne trade, and to establish whether or not whole olives formed an important part of the export economy of ancient Mediterranean states. Subjecting additional olive remains from a variety of underwater sites to isotope analysis could enhance our knowledge of the cultivation, trade and consumption of this important species.

2.6.5 Future directions

The inclusion of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ data would allow for greater accuracy in determining growth conditions and therefore geographic origin of the remains. Future research on ancient olives would benefit from the creation of a comprehensive data set of isotopic values for olive stones of known provenance. By establishing what level of variability in isotopic values can be expected from one olive tree, one orchard, and one entire geographic area, further inferences regarding

the geographic origin of the numerous olive stones recovered from ancient shipwrecks may be possible.

Utilising protocols developed for the ^{14}C dating of waterlogged wood, which isolate the cellulose of plant material, may provide more consistent results, by excluding the remaining heterogeneous constituents of olive stone endocarp including lignin and remnant lipids. Experimental water logging by seawater of modern olive stones samples may increase our understanding of the effect the marine environment may have on isotopic composition.

Ancient shipwreck excavations have yielded preserved olive remains in the tens of thousands, offering a unique opportunity to study an agricultural product that is often overlooked in the academic literature. Through further investigations by stable isotope analysis, the extraction of remnant lipids, and additional methods such as ancient DNA research, a wealth of information regarding geographic origin, genetic make-up, and ancient cultivation strategies such as manuring and irrigation may be attained. Olives occupy a special place in the history of the Mediterranean, and by further research perhaps we can learn more about the role the fruits from this emblematic plant played in ancient trade, transport, and diet in the past.

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Chapter 3 – Organic Residue Analysis

TITLE

Biomarkers in shipwreck ceramics and sediments: Using residue analysis to explore cargo compositions and the environment of the Uluburun, Mazotos, Kyrenia and Babuljaš shipwrecks

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Abstract

Ancient shipwrecks are often identified by their cargo of amphorae. We assume cargo composition based on amphorae shape. Frequently, shipwreck amphorae contain visible remains of unexpected contents, challenging our assumptions. In cases where amphorae appear empty, we must rely on scientific analysis to characterise shipwreck cargoes. Here, solvent extraction and alkaline fusion is used to capture biomarkers of past vessel contents from shipwreck amphorae, assumptions regarding vessel contents are assessed, and environmental samples are analysed to determine what molecules may be naturally present on the seafloor around ancient Mediterranean shipwreck sites and may migrate into amphorae sherds. The

amphorae show evidence for a variety of past contents including plant oils, and remnants of a *Pinaceae* lining, while environmental samples of seafloor sediment yielded several compounds traditionally thought to be wine biomarkers.

3.1 Amphorae and ancient shipwrecks

Ceramic transport amphorae form the bulk of archaeological remains by which ancient shipwrecks sites are discovered (Bass, 1970; Frost, 1963; Muckelroy, 1978; Parker, 1992; Throckmorton, 1965) and studied to illuminate aspects of ancient trade (Horden and Purcell, 2000; Wilson, 2011). Durable amphorae settle into mounds on the seafloor as the wooden hull structure of the ship rots away, allowing for discovery by SCUBA divers (Wilson, 2011, p. 37), and insights into the nature of the cargo the ship carried. Evidence from ancient shipwrecks is of crucial importance to our understanding of the development and modalities of long-distance trade in antiquity (Robinson and Wilson, 2011), and is vital to produce an accurate reconstruction of ancient economies. The nature of shipwreck cargoes is essential to these reconstructions.

Early studies of amphorae recovered from terrestrial archaeological sites often assigned a specific cargo type to an amphorae of a given shape and geographic origin, aided substantially by the study of stamped amphorae handles or *tituli pinti* which provided insight into the origin of these vessels, the past vessel contents and their ancient producers (Dressel, 1899; Grace, 1961, 1946, 1934). Long after the early work of Heinrich Dressel and Virginia Grace, the idea that amphorae shape can be safely used to establish function and past vessel contents has endured (Koehler, 1995; Lawall and Lund, 2011). This can lead to false assumptions regarding the goods amphorae contained, and the volume of trade in these goods (Dallongeville et al., 2011, p. 3053).

3.1.1 Unexpected cargoes

Ancient vessel contents can be investigated through the identification of preserved organic macro-remains, in particular in arid environments where organic remains can be well preserved through desiccation, and submerged archaeological sites, where organic remains are preserved by the anoxic environment. Amphorae recovered from several ancient shipwrecks have been shown to contain preserved macro-remains that demonstrate the transportation of unexpected contents: so-called wine amphorae have contained butchered beef bones (Carlson, 2003), almonds (Swiny and Katzev, 1973), whole olives (Demesticha, 2011), orpiment (Bass, 1995), and pitch (Carlson, 2003).

The discovery of amphorae containing unexpected contents on several Mediterranean shipwreck sites demonstrates why it is problematic to assume what amphorae contained based on their shape and geographical origin. Such preservation, however, is rare in the archaeological record: amphorae and other ceramic vessels recovered from terrestrial sites are unlikely to retain the macro-remains associated with their original contents. Furthermore, while macro-remains can be preserved in an underwater environment, the opposite could be said for past amphorae contents that are in a liquid or soluble state. Stoppered amphorae like those recovered from the Madrague de Giens shipwreck (Formenti et al., 1978, p. 95) are exceptional; the norm is to find unstoppered amphorae with no visible remains inside.

Wine and olive oil are perhaps the ancient Mediterranean products most often associated with amphorae (Pecci et al., 2013), and it is these liquid commodities that don't leave macro-remains that are frequently postulated to be the primary cargo of amphorae-laden shipwrecks. In the instances where shipwreck amphorae appear empty, our analysis seeks molecular evidence for their past contents, and once identified, addresses whether this molecular evidence supports the long-held assumption that amphorae shape and geographic origin indicate what specific commodity was contained within. This work also explores what unique challenges the underwater deposition environment may present in our attempts to recover molecular evidence for past vessel contents.

3.1.2 Scientific approaches to characterising shipwreck cargoes

Analysis of ceramics from a shipwreck context was one of the very earliest forays into the chemical characterisation of past vessel contents by organic residue analysis (e.g. Formenti et al. 1978). An intact and sealed Lamboglia 2 type amphora was recovered from the Madrague de Giens shipwreck, France, and upon inspection, appeared to be retain some of its original contents (Formenti et al., 1978, p. 95). The authors used chromatography in an attempt to identify tartaric acid, a biomarker of wine, but the results were inconclusive and suspicions about the vessel contents could not be confirmed (Formenti et al., 1978, p. 99). This early example of residue analysis conducted on the contents of ceramics recovered from underwater contexts perhaps strengthened the justification for the implementation of the normative approach to amphora interpretation on shipwreck sites: the Lamboglia 2 type amphorae are generally assumed to have contained Italian wine (Martinez Ferrarez et al., 2015, p. 278) and in this case scientific analysis was unable to disprove these suppositions.

Yet, the scientific validity of the traditional approach to amphorae interpretation has long been scrutinised. The authors of an early study on residues in amphorae sought to establish ‘whether it is possible to find scientific criteria which allow it to be established with certainty and without any consideration *as to shape* that one given amphora contained this or that commodity’ (Condamin et al., 1976, p. 195). Similarly, Passi et al. (1981) sought to ‘test the hypothesis that individual amphora shapes were commodity-specific’ (Passi et al., 1981, p. 779). After analysing amphorae associated with the transportation of olive oil, their results indicated that one of the amphorae tested may have contained sesame oil, rather than olive oil, due to higher levels of linoleic acid (C_{18:2}) and arachic acid (C_{20:0}) (Passi et al. 1981, p. 784). Despite these early challenges to established assumptions, the idea that the past contents of ceramic vessels can be postulated based on shape and geographic origin, here termed the ‘traditional approach’ to amphorae interpretation, has remained popular among excavators and academics.

Olive oil and wine, the main liquid products that are assumed to have been traded on ancient ships, have very different chemical composition, and as such, are supposed to leave very different biomarkers. Lipids, such as those found in olive and sesame oil discussed above, appear to have great potential for preservation in shipwreck ceramics. Lipids are the main molecular constituents of plant oils, and their inherent hydrophobicity and resistance to decay has long been recognised in archaeological science (Evershed, 1993, p. 77), and may aid in their successful recovery from inundated sites. Interpretation of lipids in archaeological samples is not always straightforward, however, in particular in samples from underwater sites, as the underwater environment may cause plant oils to exhibit molecular profiles similar to those found in animal fats, due to hydrolysis and differential solubility (Steele et al., 2010).

Tartaric acid and syringic acid (released from malvidin-3-glucoside, the main pigment in grape skins) are the two acids that have been used in the literature to reveal the presence of wine in ceramics (Guasch-Jané et al., 2004), but no clear consensus exists on the best extraction and detection methods. Analytical methods used in the literature include the use of High Performance Liquid Chromatography / Tandem Mass Spectrometry (HPLC/MS/MS) (Guasch-Jané et al., 2006, 2004), thermally assisted hydrolysis and methylation/gas chromatography/mass spectrometry (THM/GC/MS) (Garnier, 2007; Garnier et al., 2003) and gas chromatography/mass spectrometry (Pecci et al., 2013).

Furthermore, tartaric and syringic acid are extremely water-soluble and prone to leaching (Barnard et al., 2011; Pecci et al., 2013; Romanus et al., 2009), which may mean that in underwater deposition environments, even biomarkers for wine that had previously penetrated into the ceramic matrix could be lost into natural solvent of the water column. As Romanus et al. (2008) have noted, the most convincing profile for tartaric and syringic acid extracted from archaeological material came from vessels of ancient Egyptian provenance (Guasch-Jané et al., 2006, 2004) where the arid environment may have helped to halt the hydrolytic processes that leach them in wetter environments.

Further complicating the detection of wine is the fact that tartaric acid, malvidin and syringic acid are known to be present in other plants, making them less reliable biomarkers for grape products alone. For example, while tartaric acid, the principal biomarker of wine, is more prevalent in grapes than in other fruits, it does also occur in pomegranates, which incidentally formed part of the cargo of the Uluburun shipwreck (Haldane, 1993), one of the shipwrecks under investigation in the present study.

In this study, vessels of known shape which are strongly associated with a specific commodity are analysed by GC-MS to examine the survival of residues in ceramics recovered from ancient shipwreck sites. These results could help assess the usefulness of the traditional approach to amphorae interpretation by establishing if molecules associated with assumed vessel contents can be found in ceramics recovered from underwater deposition environments. Our extraction protocol was designed to allow the characterisation of lipids from oily products, the so-called biomarkers of wine (tartaric acid and syringic acid), and resinous products that were used to line amphorae. In order to investigate contamination issues that are specific to the underwater environment, samples have been also taken of the seafloor sediment from both the shipwreck area, and the natural seafloor outside the wreck area. While it has been established that lipids are unlikely to migrate between soil and potsherds on terrestrial sites (Heron et al., 1991), the extent to which molecules and compounds used as biomarkers can migrate between seafloor sediment and ceramics has not yet been sufficiently investigated.

3.2 Materials and Methods

3.2.1 Sample descriptions

A total of 33 ceramic samples have been analysed from three ancient Mediterranean shipwreck sites and one terrestrial site (Table 7).

Table 7. Ceramic samples analysed in this study.

Site	Artefact	Code	Contents	Assumed contents
<i>Mazotos shipwreck</i>				
<i>Chian amphora</i>		426	Olives	Wine
		P0824	-	Wine
		P0784	-	Wine
		P0863	-	Wine
		P0841	-	Wine
		P0643	-	Wine
		P0746	-	Wine
		P074	-	Wine
		P0293	-	Wine
		P0394	-	Wine
<i>Kyrenia shipwreck</i>				
<i>Rhodian fractional amphora</i>		069	-	Wine
		414	-	Wine
<i>Rhodian mushroom-rim amphora</i>		760	-	Wine
		468	-	Wine
		507	-	Wine
<i>Samian amphora</i>		551 P46	-	Wine
		A97	-	Wine
<i>Koan amphora</i>		534	-	Wine
<i>Torpedo jar</i>		P174	-	-
<i>Babuljaš shipwreck</i>				
<i>Africana III/Keay 25 amphora</i>		A114	-	Olive oil
		A115	-	Olive oil
<i>Bronze Age cooking pot</i>		A118	-	-
<i>Yeronisos island</i>				
<i>Lagynos</i>		P.15.17	-	Wine
		P.05.03	-	Wine
<i>Oinochoe</i>		P.13.12	-	Wine
		P.94.49	-	Wine
		P.94.23	-	Wine
<i>Spouted jug</i>		P.05.07	-	-
		P.94.18	-	-
<i>Unguentarium</i>		P.04.20	-	Unguent
		P.14.02	-	Unguent
		P.08.01	-	Unguent
<i>Chamber pot</i>		P.93.12	-	Feces

Ceramic samples from the Mazotos shipwreck consist of sherds of Chian amphorae found loose on the seafloor (Figure 34), while the majority of the sherds from the Kyrenia shipwreck are small fragments of relatively intact vessels (Figure 35).



Figure 34. Chian amphora sherd P0239 from the Mazotos shipwreck.



Figure 35. Rhodian amphora sherd 468 from the Kyrenia shipwreck.

Six samples of the resin cargo from the Uluburun shipwreck, as well as six environmental samples are analysed here to determine the compounds present (Table 8). Environmental samples were collected from the Mazotos and Babuljaš shipwreck, each set consisting of one sample of seafloor sediment collected from the shipwreck area, and one sample collected from a sufficient distance away from the wreck area to constitute natural seafloor. At the Mazotos and Babuljaš shipwreck sites, the samples of natural seafloor sediment were collected as far away as possible from the wreck sites, according to diver safety constraints. The Babuljaš site

is very shallow, and sediment was taken from an area of seafloor 50 metres away from the wreck site. At 42 metres depth, diving on the Mazotos shipwreck site requires decompression so in this case a sediment sample was taken from top levels of seafloor adjacent to the wreck. The fifth seafloor sediment sample was collected from the inside of Canaanite jar 1553 on the Uluburun shipwreck. A soil sample was also collected from the terrestrial site of Yeronisos at a depth of 1.8 metres and proximal to several ceramic containers including a vessel thought to be a chamber pot (artefact P93.12).

Table 8. Samples of shipwreck cargo (resin) and environmental samples analysed.

Site	Sample type	Code	Sampled from -
<i>Uluburun shipwreck</i>			
	<i>Resin</i>	A36	KW 39
		A34	KW 49
		A31	KW 144
		A32	KW 605
		A35	KW 215
		A33	KW 591
	<i>Seafloor sediment</i>	L3561	KW 1553
<i>Mazotos shipwreck</i>			
	<i>Seafloor sediment</i>	A120	Outside shipwreck area
		A121	Inside shipwreck area
<i>Babuljaš shipwreck</i>			
	<i>Seafloor sediment</i>	A117	Outside shipwreck area
		A116	Inside shipwreck area
<i>Yeronisos island</i>			
	<i>Soil sample</i>	A25	P93.12

The six resin samples from the Uluburun shipwreck, including the one pictured in Figure 36, have been analysed here in order to assess what biomarkers might be obtained from this material, and to further inform the debate surrounding whether or not the many Canaanite jars on the Uluburun shipwreck contained resin or resinated wine (McGovern and Hall, 2016; McGovern, 2003; McGovern and Michel, 1996; B. Stern et al., 2008).



Figure 36. Resin sample found in Canaanite jar KW 605 from the Uluburun shipwreck.

3.2.2 Lipid extraction

The exterior surface of all sherds was cleaned with an aluminium oxide air wick, and a sample was obtained by drilling to a depth of 2 – 5 mm with a clean drill bit. Amphora sherds and environmental samples were solvent extracted to recover lipids from all sherd powders and sediment samples (1 g) by sonication in a DCM:MeOH (2/1 v/v 3 x 5 mL) together with an internal standard (10 mg *n*-Tetratriacontane) to quantify the amount of lipids in the sample. The resulting Total Lipid Extracts (TLEs) were combined and dried under a gentle stream of N₂. These were silylated with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane at 70° C for one hour and dried again under N₂. Ten mg of internal standard (*n*-hexatriacontane) was added to each sample after dilution into 100 µg of hexane prior to immediate analysis by GC-MS to assess lipid recovery. 'Blank' samples were also prepared alongside each batch of archaeological materials and subjected to identical protocols.

After solvent extraction, the remaining ceramic sherd powder and sediment samples were subjected to a secondary extraction protocol by alkaline fusion to extract tartaric and syringic acid (Guasch-Jané et al., 2004; Pecci et al., 2013). The 1 g of remaining ceramic sherd powder was treated with 6 mL of KOH (1 M concentration) at 70° C for 90 minutes, acidified with HCL (2 M concentration), then 2 mL of ethyl acetate were added to the acidified solution and the two phases were allowed to separate. The ethyl acetate supernatant was collected. The extraction with ethyl acetate was repeated twice and the resulting extracts were combined and dried under a gentle stream of N₂. These were silylated with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane at 70° C for one hour and dried again under N₂. Ten mg of internal standard (*n*-hexatriacontane) was added to each sample that have been diluted in 100 µL of hexane prior to immediate analysis by GC-MS for quantitation.

In addition to the established destructive analysis method above, a method of non-destructive residue analysis was tested on three vessels from the Kyrenia shipwreck. This method has been used previously on ceramics of a comparable age (Gerhardt et al., 1990) and was conducted by Vic Garner and Guy Lord in 2008. Methanol was placed in three intact amphorae, removed after a period of hours with a glass pipette, and stored for future analysis. These solvents were silylated, dried, and diluted as above.

3.2.3 Gas Chromatography – Mass Spectrometry (GC-MS)

GC-MS was carried out on all samples using an Agilent 7890 A Series chromatograph attached to an Agilent 5977 B mass-selective detector with a quadrupole mass analyser (Agilent technologies, Cheadle, Cheshire, UK). All samples were initially screened using a splitless injector maintained at 340 °C. The carrier gas used was helium, and inlet column head-pressure was constant. The column (DB-5 ms) was coated with 5% phenyl-methylpolysiloxane (30 m × 0.25 mm x 0.25 µm; J&W Scientific, Folsom, CA, USA). Oven temperature was set at 50 °C for 2 min, then raised by 10 °C min⁻¹ until 325 °C was reached, where it was held for 15 min until the end of the run. The ionization energy of the mass spectrometer was 70 eV and

spectra were obtained in a scanning mode between m/z 50 and 1,000. Hexane ‘blanks’ were injected regularly throughout the sequence to monitor for any carryover between analytical runs.

3.3 Results

3.3.1 General description of GC-MS results

After solvent extraction, samples yielding total lipid concentrations of $> 5 \mu\text{g}$ were considered to be interpretable. The majority of the samples analysed met this criterion. Diterpenes are the most common molecules extracted from the ceramic samples (Table 9).

Table 9. Summarised results of solvent extractions and alkaline treatments of pottery samples. See above for determination of biomarkers. Res.=resin, Pit.=pitch, TA=tartaric acid, SY=syringic acid.

Site		Lipid yield		Oil biomarkers	Conifer biomarkers			Pistcia biomarkers	Beeswax	“Wine biomarkers”	
		Min $\mu\text{g/g}$	Max $\mu\text{g/g}$		Res.	Pit.	Tar			TA	SY
Kyrenia shipwreck	Amphorae - destructive analysis	5	671	2		1	3				
	Amphorae - Non destructive analysis	94	18,648				3				
	Domestic pottery	0	20		1						
Mazotos shipwreck	Amphorae	0	614			2	3				
Babuljaš shipwreck	Amphorae	2	134	1							
	Bronze Age cooking pot	0	11		1						
Uluburun shipwreck	Resin	n.d.	n.d.					6			
Yeronisos island	Domestic pottery	13	181	1					1	1	2

The detection of abietic acid, pimaric acid, dehydroabietic acid (DHA), didehydroabietic acid (di DHA), 7-oxo dehydroabietic acid (7-oxo-DHA) indicate the presence of conifer exudates, more or less degraded, while retene indicates that the products have been heated, probably to make pitch (Colombini et al., 2003; Pollard and Heron, 1996). The identification of methyl-dehydroabietic acid (Me-DHA) and methyl-7-oxo-dehydroabietic acid (Me-7-oxo-DHA), as shown in Figure 37b, indicate that the product was heated in the presence of wood, to produce conifer tar (Pollard and Heron, 1996, p. 240). Some solvent extracts yielded lipid profiles mainly constituted of fatty acids and including a high quantity of unsaturated fatty acids ($C_{18:1}$, $C_{20:1}$; Figure 37a). In most of those samples, alkaline fusion revealed degradation products of unsaturated fat, including the *threo* and *erythro* isomers of the 9,10-dihydroxyoctadecanoic acid and diacids ($C_{5:0}$ - $C_{16:0}$). Furthermore, some samples yielded linear long-chain alcohols (C_{22} to C_{28}) and phytosterols (β -sitosterol, stigmastanol, etc) in the solvent extracts, suggesting a plant origin (Figure 37a). Sulphur was also identified in numerous samples.

In addition to fatty acids and polar diterpenes (DHA, di-DHA, abietic and pimaric acid, 7-oxo-DHA), alkaline treatment revealed a large diversity of small organic compounds (Figure 37). Among them, however, tartaric and syringic acids were not detected in any of the ceramic vessels included in this study (Table 9), despite their designation as 'wine' amphorae according to the traditional approach to amphorae interpretation. Syringic acid, however, was detected in nearly all of the environmental samples of seafloor sediment.

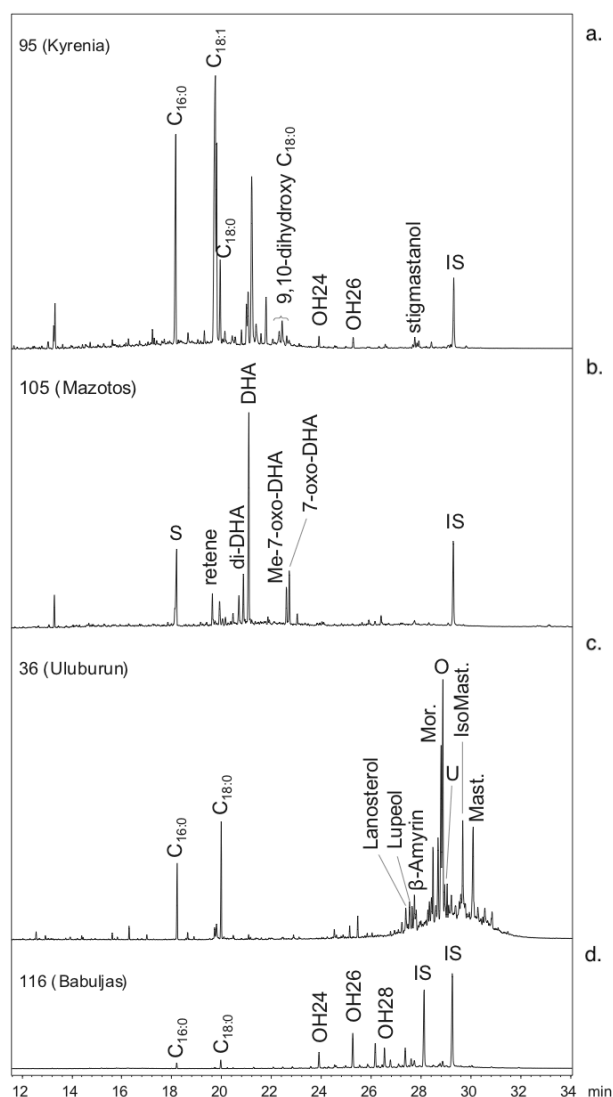


Figure 37. Partial gas chromatograms following solvent extraction of amphorae sherds, showing evidence of plant oil (a); conifer tar (b), Pistacia resin (c) and partial gas chromatograms of the lipid extracts from a sediment sample (d). Cxx:x: fatty acids; DHA: dehydroabietic acid; OHxx: linear alcohol; S: sulphur; Mor.: moronic acid; O: oleanonic acid; Mast: masticadienoic acid; IS: internal standard.

3.3.2 Mazotos shipwreck ceramics

Two types of content have been identified in amphorae from the Mazotos shipwreck. Of the 10 sherds analysed here, five yielded very low quantities of lipids (P836, P643, P74, 426 and 394), while five revealed a high quantity of diterpenes (Figure 37b). Amphorae P293, 784 and 746 seem to have been coated with conifer tar (due to the presence of retene, Me-DHA and Me-7-oxo-DHA), while the others (P841 and P824) were more likely sealed with conifer pitch.

3.3.3 Kyrenia shipwreck ceramics

The ceramic sherds from the Kyrenia shipwreck yielded compounds that are indicative of a variety of past contents, with various quantity of preserved lipids (from 0 to 18 mg/g). The highest quantity of lipid extracted (between 0.1 and 18 mg/g of lipids) are found in the samples prepared using the non-destructive extraction method, probably because the surface layer, where a *Pinaceae* lining is most concentrated, but also the most likely to be contaminated, has not been removed prior to extraction. Nearly all of the Kyrenia samples showed evidence for plastic contamination. As the Kyrenia shipwreck amphorae and their associated sherds have not been stored in plastic, the source of the plastic contamination found in the analysis of these artefacts remains unclear. Plastic contamination in the sea has been suggested as a source for these molecules, but it is worth recalling that single use plastic items were not commonly used and disposed of in the sea in the early 1970's when these artefacts were recovered.

Three types of amphorae content seem to arise from this analysis. Six amphorae revealed high quantity of diterpenes and their natural degradation products (DHA, di-DHA, 7-oxo-DHA, abietic acid). The presence in most of the samples of degradation products of diterpenes, formed when conifer exudates are heated (retene), for some, in the presence of wood (Me-DHA and Me-7-oxo-DHA) suggests that conifer tar was used in five amphorae (414, 534, 136, 468, P174), and conifer pitch in one Rhodian amphora (760). Small amounts of fatty acids were detected in all these samples, but their small quantity prevents a clear interpretation.

Plant oil seems to have been contained in both of the two tested amphorae from the Greek island of Samos, because of a large quantity of C_{18:1} and its degradation products that were detected (Figure 37a). Furthermore, sample 551 P46 yielded linear long-chain alcohols (C₂₄ to C₂₈) and plant sterols (β -sitosterol and stigmastanol). Finally, one Rhodian amphora (69) yielded only trace amounts of heated conifer product and fatty acids, suggesting either that the content was entirely degraded, or that the main content of this amphora was some kind of non-

fatty substance. That both heated and unheated conifer products were utilised on the Kyrenia ship confirms earlier work that compared a tar-like substance on a wooden strake from the ship's hull to the resinous amphorae lining (Beck and Borromeo, 1990).

3.3.4 Babuljaš shipwreck

A sherd from one of the two Roman amphorae yielded lipids in sufficient quantities to make an interpretation. Amphora sherd 114 contained fatty acids ($C_{12:0}$ - $C_{28:0}$), with high quantity of unsaturated fatty acids ($C_{18:1}$, $C_{20:1}$), together with long chain linear alcohols (C_{22} - C_{28}), and plant sterols (sitosterol, stigmastanol, sitostenone) that suggest the presence of plant oil. The presence of squalene is more difficult to interpret, as it occurs naturally in olive oil, but can also be absorbed into the ceramic matrix during post-excavation handling of the pots, together with cholesterol. However, the absence of cholesterol, a compound more stable than squalene, in this sample could then be further evidence of a past oily content, rather than be indicative of contamination through post-excavation handling.

3.3.5 Uluburun resin

Six samples of the amber-coloured resin identified as *Pistacia* resin (Mills and White 1989) were analysed to explore the debate surrounding whether the 140 Canaanite jars recovered from the Uluburun shipwreck contained only resin (Stern et al., 2008) or resinated wine, suggested by the presence of grape pips recovered from the bottom of several of these jars (McGovern and Hall, 2016; McGovern, 2003). Of the six resin samples analysed here, which were recovered from Canaanite jars number KW 144, KW 605, KW 591, KW 49, KW 215 and KW 39, all yielded typical biomarkers of *Pistacia* resin (Figure 37c), already identified in previous studies, including moronic acid, oleanonic acid, ursolic acid, masticadienonic acid, isomasticadienonic acid and their dihydro derivatives (Brettell et al., 2015; B. Stern et al., 2008), but none tested positive for biomarkers of wine, suggested that these vessels contained

resin, and not resinated wine, as postulated previously by Stern et al. (2008). However, as is discussed below, the solubility of wine biomarkers may impede their recovery in artefacts from submerged contexts.

3.3.6 Yeronisos island ceramics

The samples from this site are characterised by large scale contamination by modern products. Together with compounds originating from plastic (phthalates and benzoic acid derivatives), cosmetic additives have been identified. Octocrylene, octinoxate and homosalate are common UV filters found in sunscreen. N, N-Diethyl-meta-toluamide (DEET) are used in insect repellent, while tocopherol acetate and cetyl 2-ethylhexanoate are compounds frequently used in skin creams. The presence of very high quantity of sterols (up to 40 µg/g of sample), together with unusually high quantity of C_{18:2} and C_{18:1} (up to 15 µg/g of sample) also suggest modern contamination. The presence of such large amounts of modern contaminants makes the accurate interpretation of the rest of the molecular compounds extracted from these samples very difficult. Indeed, fatty acids, linear alkanes, small triacylglycerols are largely used in cosmetics and could originate from the content of the archaeological ceramics or from modern contamination.

A wine jug, or *lagynos*, P.15.17, expected to have contained wine, was found to contain degraded beeswax (wax esters W40-50, max W48; linear alkanes C27-C33, max C27 including unsaturated C33 and small quantity of linear long chain alcohol). In addition, small quantity of fatty acids and retene and Me-7-oxo-DHA) occur in the sample. However, the presence of modern contaminant in the sample (including plasticizers and UV filters) suggest that the beeswax could be part of a sunscreen composition.

The solvent extraction results of a second *lagynos* from Yeronisos (vessel P.05.03) yielded what appear to be contamination products from the modern cosmetics industry, while the alkaline fusion treatment yielded traces of syringic acid, a biomarker of wine, as well as traces of conifer exudate.

Interestingly, one extract (vessel P.14.02, *unguentarium*) recovered after alkaline fusion revealed tartaric acid, malonic acid, fumaric acid, tartronic acid and 4-coumaric acid, succinic acid and malic acid. Although a lot of these compounds are ubiquitous, they are generally found together in wine (Garnier and Valamoti, 2016). Of all the ceramic vessel samples included in this study, here we see the most convincing profile for wine biomarkers. It is interesting to note that this is one of the few vessels included in this study that was not assumed to have contained wine, but rather, based on the vessel's diminutive size and piriform shape, was assumed to contain perfume or unguents. The fatty acids present may also indicate plant oil as a previous content of this vessel but is difficult to securely interpret due to the contaminants from modern cosmetics.

3.3.7 Environmental samples

Comparison between solvent extracts from the environmental samples and ceramic samples from the Mazotos and Babuljaš shipwrecks reveal clear differences in the molecular distribution, suggesting the absence of contamination from the surrounding environment penetrating the ceramic sherds included in this study. Fatty acids ($C_{16:0}$ - $C_{18:0}$) are present in very low amount in all those environmental samples. Long chain alcohols (C_{24} - C_{28}) are the main constituent from Babuljaš sediments (Figure 37d). Plant sterols (stigmasterol, sitosterol, stigmastanol) are present in sediments from Babuljaš and Mazotos. Although the same molecular compounds are also detected in archaeological samples, their distribution is clearly different.

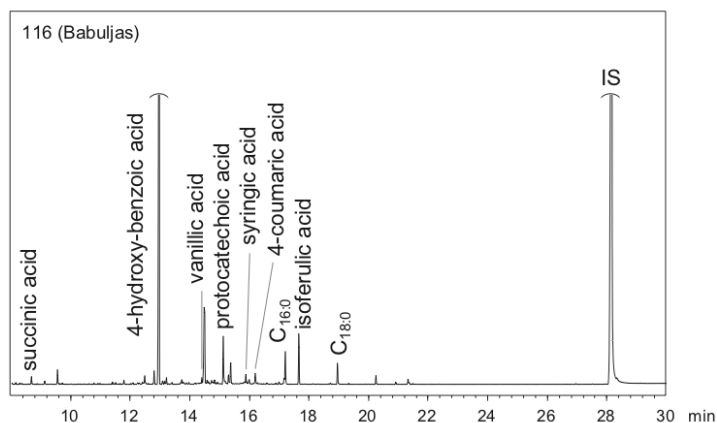


Figure 38. Partial gas chromatograms following alkaline treatment of a sediment sample. Cxxx: fatty acids; IS: internal standard.

One sample of what appeared to consist mainly of sand, shells and other constituents of marine sediment, was collected from the inside of Canaanite jar KW 1553 from the Uluburun shipwreck. Fatty acids (mainly C_{16:0} and C_{18:0}, C_{19:0}, C_{24:0}), as well as compounds associated with the *Pistacia* resin, primarily triterpenes (lanosterol, amyirin, oleanonic acid, masticadienoic, isomasticadienoic acid, and several other compounds as yet unidentified), also found in numerous other jars, indicate that molecules from the vessel contents have permeated the sediment that was collected inside this jar.

The alkaline treatment of the environmental samples revealed a large diversity of small organic compounds that have been identified in the pottery samples (Figure 38): succinic acid, vanillin, acetovanillone, syringic acid, syringaldehyde, vanillic acid 3-and 4-hydroxybenzoic acid, ferulic acid, 4-coumaric acid, protocatechoic acid, boric acid, lactic acid, glycolytic acid, arsenous acid, and phloretic acid.

3.4 Discussion

3.4.1 Assumed cargoes versus molecular results

Of the 33 ceramic vessels analysed in this study, 23 were assumed to have contained wine. In our analysis, none of these 23 samples yielded compounds that securely identify wine as a past vessel content. These samples did, however, yield abundant molecular evidence for conifer products. No visible lining was observed on any of the sherds prior to analysis, yet over 50% of the vessels yielded high quantities of conifer products, with the majority of these containing evidence for tar, rather than pitch, based on the presence of compounds that indicate heating in the presence of wood (Pollard and Heron, 1996, p. 257). It is well established that resinous products such as tar or pitch were used to line wine amphorae in the ancient world, from both archaeological remains and the works of ancient authors like Cato and Columella (Heron and Pollard, 1988). This is thought to have made the amphorae less permeable, as well as imparting anti-microbial properties to the wine.

The evidence for *Pinaceae* linings on wine amphorae is ample: the sticky linings of several amphorae recovered from the Tektas Burnu shipwreck had inclusions of waterlogged grape seeds adhered within, offering direct evidence for the past contents of the vessel (Carlson, 2004, p. 3). A modern wine product still consumed in Greece, *Retsina*, a white wine infused with pine products, is thought to be a continuation of this ancient tradition. In addition to wine amphorae, amphorae used to transport fish sauce were also lined with pitch or tar, which has been recently confirmed by analysis of ceramic vessels found in the Garum shop of ancient Pompeii, where evidence for both *Pinaceae* and macro-remains of fish bones were noted (Pecci et al., 2018, p. 494). However, in the shipwreck amphorae included in this study, no other acids considered to be biomarkers for fish sauce, such as isoprenoid fatty acids (Craig et al., 2007), were evident in our samples.

In addition to lining amphorae, *Pinaceae* products can be a type of vessel content in their own right. Nine Mendeian amphora from the Tektas Burnu shipwreck were filled nearly to capacity with pine tar (Carlson, 2004, p. 16), indicating that this product was either a commodity valuable enough to be shipped abroad, or of some use to sailors during the journey. Analysis of the outer hull material on wooden ships recovered from an Etruscan/Roman harbour near modern-day Pisa provided evidence that *Pinaceae* products were used to waterproof the hull of ancient ships (Colombini et al., 2003), while Beck and Borromeo (1990) have confirmed the presence of both pitch and tar on the Kyrenia shipwreck from samples of tarred areas of the wooden hull, and amphorae linings (1990, p. 53). These findings show the utility of conifer products on ancient shipwrecks beyond simply lining amphorae to inhibit a liquid cargo from being absorbed into the porous ceramic.

3.4.2 Mazotos shipwreck

For the Chian amphorae from the Mazotos shipwreck, thought to have contained wine, the detection of *Pinaceae* products may offer further evidence to support this assumption, as wine amphorae were known to have been lined. However, it is worth noting the possibility that the conifer products detected by GC-MS here constituted the actual content of some of the amphorae carried by the ship found at Mazotos, as they did on the Tektas Burnu ship, a wreck that shares similarities with both the Mazotos and Kyrenia, in terms of dates (5th – 4th century B.C.E.) and elements of cargo (primarily consisting of amphorae, with Chian and Samian vessels present). Only one of the 10 Chian amphora sherds analysed here derived from a whole vessel, the rest were recovered in a fragmented state which would have allowed the original vessel contents to be lost into the sea. Therefore, the detection of *Pinaceae* products in these Chian ‘wine’ amphorae, coupled with no molecular evidence for the presence of tartaric acid, does not definitely show that wine was the original, or indeed last, content. It remains an intriguing possibility that some of these vessels contained tar or pitch as a primary vessel content.

The discovery of thousands of whole olives in Chian ‘wine’ amphorae 426 from the Mazotos shipwreck provides direct evidence that these amphorae sometimes contained products other than wine, but neither molecular evidence of this known content, or a hypothetical previous content was detectable in the ceramic sherd from this olive-filled amphora. This result is not surprising if the amphora was full of whole olives (dried or in aqueous liquid), that would release very few lipids able to penetrate the ceramic walls. On the other hand, if one were to argue we are seeing reuse of a ‘wine’ amphora, it is worth noting that no *Pinaceae* products were detected in this sample, as the quantity of lipids preserved was too low to interpret, despite the interior of this sherd having a dark, coated appearance. Exploring evidence for the reuse of ceramic vessels in the archaeological record is important for the accurate interpretation of molecular findings, as palimpsests of residue may accumulate within amphorae over the course of their life cycle.

3.4.3 Kyrenia shipwreck

As with the Mazotos shipwreck, the cargo of the Kyrenia ship is thought to have been composed primarily of wine. No molecular evidence for wine was detected in these samples, but *Pinaceae* products were detected in all the Rhodian amphorae, as well as the Koan amphora and the torpedo jar. The hypothesis that pine pitch or tar constituted the primary contents of these vessels appears less likely for the sherds from the Kyrenia shipwreck site, as the samples included here derived from vessels that are partially or entirely whole, and thus would be more likely to retain pine resin inside, as was the case for the intact amphorae that contained pine resin as a primary content on the Tektas Burnu shipwreck. Therefore, the detection of conifer products in the Rhodian and Koan amphorae may strengthen the contention that wine was carried inside but cannot definitely confirm wine as a last content.

Fatty products consistent with plant oils were found in both of the two Samian amphorae thought to have contained wine from the Kyrenia shipwreck which were analysed here. Ancient authors praised the high quality of Samian olive oil (Bound, 1991, p. 186), and evidence gleaned from stamped Samian amphorae handles suggests that both wine and olive oil were

exported from Samos in such vessels (Grace, 1971). Such literary, epigraphic, and molecular evidence for diverse agricultural exports from Samos strengthens the argument that the traditional approach to amphorae interpretation may over-simplify the complex nature of agrarian industry and long-distance trade in the ancient world.

3.4.4 Babuljaš shipwreck

Plant oils were the expected content for both of the two *Africana III/Keay 25* amphorae from the Babuljaš shipwreck analysed here. One contained evidence of plant oil, confirming the normative approach in this case. A recent study found evidence for wine in numerous amphorae of the same type (Woodworth et al., 2015), suggesting that both wine and oil were exported from North Africa in *Africana III/Keay 25* amphorae. It has long been hypothesised that a resinous lining was unsuitable for amphorae that held an oily content (Heron and Pollard, 1988, p. 430), but this contention has been more recently questioned (Romanus et al., 2009). Interestingly, no diterpenes were detected in the amphorae included in this study that contained plant oil, from either the Samian amphorae of the Kyrenia wreck or the *Africana III/Keay 25* from the Babuljaš, lending support to the hypothesis that pitch or tar were unsuitable for lining oil amphorae.

3.4.5 Uluburun shipwreck resin

While one constituent of the contents of the Canaanite jars from the Uluburun shipwreck had long been established by the macro-remains of large chunks of resin preserved inside, it was asserted that resinated wine, and not merely resin, was carried on board (McGovern, 2003). This led to some debate in the literature (e.g. Stern et al., 2008; McGovern, and Hall, 2016), with Stern et al. (2008) contending that the tartaric acid detected by McGovern (2003) had not been detected through adequate methods. No biomarkers of wine were detected in the six resin samples from the Uluburun shipwreck included here. This may also result from the

solubility of tartaric acid but could also simply show further evidence that the significant volume of resin found within the over 150 Canaanite jars does indeed constitute their actual contents, rather than a content of resinated wine. A large variety of plant products appear to have been transported on the Uluburun, as evidenced by the recovery of almonds, fig seeds, wild pistachio nutlets, olives, coriander, sumac, pomegranate seeds and skins, and grape seeds, from virtually every context (Haldane, 1993, p. 352). It appears most likely that the presence of grape pips in the toe of Canaanite jars is due to the mixing of cargo post-deposition, rather than indicating a cargo of resinated wine. Evidence from Pompeii shows that jars and amphorae were washed prior to reuse (Peña, 2007), so it is unlikely that grape pips remained from a previous content of wine.

3.4.6 Detecting biomarkers of wine

As tartaric acid was not detected in any of the 23 shipwreck amphorae and domestic vessels assumed to have contained wine included in this study, it is necessary to explore why this is the case. The hydrophobic nature of oily substances would appear to explain the retention of these molecules in shipwreck amphorae, while the more soluble compounds found in wine may be lost. Perhaps it is these enhanced aspects of the diagenetic degradation processes found in the marine deposition environment that further complicate attempts to determine past vessel contents of shipwreck amphorae, as molecules that may be preserved in terrestrial depositional environments, such as tartaric acid, are more likely to be leached out of the ceramic matrix in marine environments.

Furthermore, syringic acid was detected, after alkaline treatment, in several environmental samples collected from the archaeological sites included in this study (seafloor sediment samples taken from the Uluburun, Babuljaš and Mazotos shipwreck). Importantly, this evidence that syringic acid is present as a bound compound in seafloor sediments surrounding ancient shipwrecks further complicates our ability to confidently use syringic acid as a wine biomarker in ceramic vessels. This also highlights that removing free syringic acid before performing chemical treatment to release it from malvidin, as suggested by some authors

(Barnard et al., 2011; Garnier and Valamoti, 2016), is not sufficient to assert that syringic acid originates from malvidin. However, the absence of syringic acid in all the amphorae we analysed demonstrates that the syringic acid in the environmental samples did not migrate into the ceramic matrix of the amphorae.

Additionally, the detection of several small acids such as succinic acid, vanillic acid, and 4-coumaric acid in the seafloor sediments analysed here suggests that these molecules, sometimes interpreted as ‘fruit biomarkers’ or ‘fermentation biomarkers’ can also be from another origin. These molecules could simply be from decaying plant matter becoming incorporated into seafloor sediment. However, similarly to syringic acid, they were not detected in the amphorae samples (except succinic acid), suggesting that despite their water solubility they did not migrate from the environment to the sherds during burial.

3.4.7 Sulphur as anthropogenic content or element of the marine environment?

Sulphur was identified in numerous ceramic samples and in seafloor sediment samples. While some authors suggest that sulphur may have been used to treat the surfaces of amphorae for antifungal or waterproofing purposes (Garnier, 2007), the identification of this compound in a marine context rather suggests that sulphur is the product of bacterial activity (Fors, 2008). Furthermore, quantities of sulphur were identified in the seafloor sediment samples from the Mazotos shipwreck, which were collected at both the very top of the stratigraphy and from just outside the main concentration of shipwreck artefacts. The seafloor sediment from excavated layers, however, did not yield appreciable amounts of sulphur, but did yield fatty acids (C_{16:0}-C_{18:0}), and plant sterols (sitosterol). This is consistent with the fact that sulphur-producing bacteria thrive in the interface between the water column and the seafloor, as well as in the interstitial space at the top of the seafloor, with burial below 50 cm representing a threshold below which fewer species of bacteria are present (Florian, 1987, p. 22). From these results, we can postulate that amphorae stowed at lower depths in the hold of the ship, and therefore

covered more quickly by the natural accumulation of seafloor sediments, which inhibit the propagation of sulphur-producing bacteria, are less likely to experience sulphur contamination. Further analysis of sulphur levels in shipwreck ceramics and seafloor sediment will help to clarify this supposition.

The seafloor sediment samples collected from the Babuljaš shipwreck in Croatia do not contain similar levels of sulphur as the seafloor sediment sample taken from the natural seafloor adjacent to the Mazotos shipwreck, Cyprus. The variability in sulphur levels found in the seafloor sediment sampled from these two shipwrecks highlights the necessity of characterising the deposition environment of archaeological sites, in order to make accurate assessments of which compounds and molecules are derived from past vessel contents, and which are not.

3.4.8 'Biomarkers' in seafloor sediment samples

While greatly informative for future research, our positive identification of syringic acid, vanillin, and succinic acid in each of the tested samples of seafloor sediment appears to frustrate, rather than aid, our initial research question which was concerned with detecting molecular evidence for past vessel contents, and by extension, ancient shipwreck cargoes. However, the discovery of these molecules in seafloor sediments highlights three key issues in the application of organic residue analysis to underwater archaeology.

First, the positive identification of these molecules in the deposition environment illustrates their relative ubiquity in underwater archaeological contexts, undermining their utility as a biomarker for anthropogenic activity. It is notable that sediments from both a very shallow and disrupted shipwreck in the Croatia (the Babuljaš shipwreck) and deep and relatively undisturbed wrecks in Turkey and Cyprus (the Uluburun and the Mazotos shipwrecks) contain the same 'biomarkers' is notable. Further analysis of seafloor sediment samples from additional submerged sites will provide greater insight into the ubiquity of these molecules in marine environments.

Second, these findings highlight the importance of collecting and testing sediments from both the general area of the shipwreck, and corresponding environmental samples from outside the underwater archaeological site. While both contained wine or fruit ‘biomarkers,’ the seafloor sediment collected from inside the excavation area of the Mazotos shipwreck had a higher concentration of lipids and a greater diversity of molecules than the natural seafloor sediment collected from outside the excavation area. The Babuljaš sediment samples from inside and outside the wreck had similar concentrations of lipids, but with some differences in the diversity of molecules present. That seafloor sediment samples from both inside and outside shipwreck areas on both the Mazotos and Babuljaš shipwreck sites appear to contain the same ‘biomarkers,’ but at different concentrations and with differing levels of molecular diversity, may indicate that the presence of these molecules is naturally occurring, but their accumulation and preservation may depend on complex site formation dynamics that vary from site to site.

Third, and perhaps conversely, that sediment collected from inside a Canaanite jar on the Uluburun shipwreck clearly contains triterpenes derived from *Pistacia* resin, known to have been contained in other jars from the same shipwreck, suggests that even in instances where jars or amphorae appear empty, it is possible that molecular evidence for their past contents migrates and is absorbed in to the sediments that is later found inside these jars. Our study suggests that the underwater environment may promote the exchange of molecules, even low water-soluble triterpenes, between the archaeological ceramics and accumulating sediment, which could complicate the interpretation of the molecules detected. While naturally occurring seafloor sediment from outside the Uluburun shipwreck area was not available for analysis in this research, the sediment recovered from jar KW 1553 was the only sample of seafloor sediment of the five included in this study found to contain triterpenes, a major constituent of *Pistacia* resin. This further illustrates the necessity of collecting sediment samples from both inside, and outside, ancient shipwreck amphorae.

Whether or not the small acids considered biomarkers of fruit, wine, or fermentation (syringic acid, succinic acid, vanillic acid) can easily migrate from seafloor sediment to buried ceramic material remains unclear, therefore the interpretation of these molecules on underwater sites

should be cautious, due to their solubility in water. Given the soluble nature of these molecules, and the dynamic nature of the marine environment, it appears that these issues could be unique to the specific conditions found on underwater archaeological sites.

3.5 Conclusions

Shipwreck amphorae that appear empty when discovered on the seafloor may contain molecular evidence for their past contents, which, in turn, can inform our understanding of what these ships were transporting in the past. As the numerous instances in which preserved macro-remains found within amphorae differ from their assumed contents, it is necessary to question our assumptions regarding amphora contents as being invariably related to amphorae shape and geographic origin. Establishing the utility of scientific methods for characterising shipwreck cargoes would allow for a vast and continually growing line of evidence for ancient trade to be fully utilised in our pursuit of a greater understanding of the past.

Our study has shown that tartaric acid, often cited as the primary biomarker of wine (Pecci et al., 2013, Woodworth et al., 2015) is difficult to detect. Its solubility in water may make it even more difficult to detect in underwater deposition environments, yet considerable success detecting these molecules in shipwreck amphorae using the same alkaline fusion protocol used here was demonstrated by Woodworth et al. (2015), indicating that it is indeed possible. The majority of the shipwreck amphorae included in this study thought to have contained wine revealed conifer products, a result that is not inconsistent with a wine content. Further research, and the testing of new extraction methods, may be necessary to enhance our ability to detect evidence for wine residues, such as tartaric acid, in ancient shipwreck amphorae. Evidence for plant oils and similarly oily products appears to be far easier to detect, due to the lower solubility, and hydrophobicity of these compounds. In addition, the detection of tartaric acid in one of the ceramic samples from the terrestrial site of Yeronisos may further indicate that

such water-soluble compounds may be more easily detected in archaeological material from terrestrial, rather than underwater, sites.

While seafloor sediment collected from inside and outside ancient shipwreck areas may appear to be a drab and uninteresting material to collect and analyse, when compared with the far more alluring artefacts found on ancient shipwrecks, this research has shown the numerous insights which can be gleaned from the study of these environmental samples. As ancient shipwreck sites can offer direct evidence for long-distance trade in the past, every opportunity should be taken to find increasingly effective ways of characterising the goods these ships carried. That organic molecules can so effectively bind to seafloor sediments may represent both potential future applications, and potential pitfalls of organic residue analysis of artefacts from underwater archaeological sites. Comprehensive sampling of sediments outside the ancient shipwreck area could easily mitigate these pitfalls, in order to establish what biomarkers are naturally present on these underwater archaeological sites, and what biomarkers may indeed be derived from past shipwreck cargo items.

Chapter 4 – DNA Analysis

TITLE

DNA from the Wine-Dark Sea: searching for ancient DNA in shipwreck ceramics, cargo items, and environmental samples from the Uluburun, Cape Gelidonya, Kyrenia, Mazotos, and Babuljaš shipwrecks

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Abstract

Finding a way to accurately characterise the contents of amphorae recovered from Mediterranean shipwrecks would provide invaluable insight into cargo compositions and trade dynamics. Is DNA the answer? There is tremendous potential for DNA studies to resolve long-standing questions in both terrestrial and underwater archaeology. Great optimism for the recovery of ancient DNA (aDNA) from maritime sites has spurred a series of studies claiming to have successfully extracted aDNA from a variety of artefacts recovered from underwater sites

including plant remains, human skeletons, and shipwreck amphorae. However, these studies have not adequately addressed the source of the DNA recovered, which may be a product of the underwater deposition environment rather than archaeological in origin. Extractions of ceramic transport vessels and organic cargo items from five ancient Mediterranean shipwreck are sequenced and analysed by shotgun metagenomics to determine what DNA can be recovered, and whether this DNA derives from past vessel contents or environmental contaminants. The DNA from several species of Mediterranean plants widely consumed and traded in the ancient world, including olive, grapevine, and pine DNA, have been identified in the environmental samples of seafloor sediment, seawater, and shelving-unit dust, calling into question the results from previous studies that claim to have recovered genuinely ancient DNA from within the ceramic matrix of shipwreck amphorae.

4.1 Introduction

4.1.1 Characterising shipwreck cargoes

On first inspection, most ancient shipwrecks are assumed to have carried one of two things: wine or olive oil. This is due to the fact that ceramic transport amphorae are most often associated with wine and olive oil (Pecci et al., 2013, p. 109), and most ancient shipwrecks consist of mounds of amphorae protruding from the seafloor. On several ancient shipwrecks, however, amphorae have been shown to contain preserved macro-remains that demonstrated the transportation of unexpected contents: vessels assumed to be wine or oil amphorae found on ancient shipwrecks have contained almonds (Swiny and Katzev, 1973), whole olives (Demesticha, 2011; Haldane, 1993; Santamaria, 1995), pomegranates (Haldane, 1993), butchered beef bones (Carlson, 2003), butchered pork (Bruni, 2000), and pitch (Carlson, 2003). Inorganic items found in shipwreck amphorae include glass beads (Pulak, 1998), orpiment (Bass, 1995), copper coins (Sharvit et al., publication pending) and broken pieces of metal statuary (Galili et al., publication pending).

These discoveries give us cause to reconsider the goods amphorae contained, which in turn is necessary to gauge the volume of trade in these goods (Dallongeville et al., 2011, p. 3053). The importance of accurately determining what amphorae contained is magnified on ancient shipwreck sites, as ceramic transport containers form the bulk of archaeological remains by which ancient shipwreck sites are discovered (Parker, 1992) and studied to understand patterns of ancient trade (Wilson, 2011). This research applies shotgun metagenomic DNA analysis to ceramics, cargo items, and environmental samples from five ancient Mediterranean shipwrecks, in order to characterise the DNA present, and determine if DNA from known contents can be detected through these methods.

4.1.2 DNA analysis and underwater archaeology

DNA analysis has been used to investigate several classes of archaeological material recovered from submerged sites, including plant remains (Elbaum et al., 2006; Manen et al., 2003), human skeletons (HersHKovitz et al., 2008) and shipwreck amphorae (Foley et al., 2012; Hansson and Foley, 2008). In the latter studies, the authors contend that genuinely ancient DNA derived from the past contents of shipwreck amphorae can be extracted from the ceramic matrix and amplified by Polymerase Chain Reaction (PCR), and further postulate that ancient DNA is more likely to survive inside the walls of pots from underwater sites than on land (Hansson and Foley, 2008, Foley et al., 2012). However, the methods of DNA analysis used in these studies may not be suitable for adequately addressing the source of the DNA recovered, and whether it derives from taxa present in the underwater deposition environment or the artefact itself. Without understanding the wide variety of taxa present on maritime archaeological sites it is not possible to establish whether or not any DNA detected genuinely derives from an ancient source. Recent improvements in DNA research methods can now be applied to similar archaeological material.

This is not to impugn the utility of PCR experiments in submerged environments: DNA from aquatic species living in proximity to the sample area can be successfully amplified through PCR to assess their presence and the propensity for free-floating DNA to breakdown in aquatic

environments (Ficetola et al., 2008). Advances in sequencing technology, however, have allowed us to move away from traditional Sanger sequencing and PCR amplification of preselected DNA, to new methods that provide an approximately 100-fold increase in throughput over the Sanger methods, which were the previous industry standard (Margulies et al., 2005). Shortly after the development of this new technology, its successful application on a well-preserved woolly mammoth mandible demonstrated the potential these advances held for the characterisation and analysis of ancient biological and archaeological remains (Poinar et al., 2006).

As this 'Next Generation Sequencing' (NGS) supplanted (PCR) as the predominant method for characterising the complex assemblages of DNA present in environmental samples and microbial communities in human remains (Key et al., 2017), a greater awareness of the potential for the preservation of DNA within environmental samples was realised. Metagenomics is the characterisation of the numerous genomes present within a sample (Zepeda Mendoza et al., 2015, p. 745), and recent applications to archaeological material have focused on environmental DNA recovered from submerged landscape sites and inland lake environments (Niemeyer et al., 2017; Sjögren et al., 2017; Smith et al., 2015).

A DNA study which amplified species present in a Greenland lake core found that macro-fossils, pollen, and DNA could be used together to present a more accurate picture of the total biodiversity present in both the aquatic lacustrine environment and the terrestrial environment nearby, as neither the DNA, macro-fossils or pollen represented the total range of species present in the sediment (Pedersen et al., 2013, p. 165). This further supports the argument that the examination of environmental DNA recovered from archaeological sites should be used as a complementary approach to the study of pollen, macrofossils, and micromorphology and other such traditional methods for characterising past environments (Pedersen et al., 2016, p. 1).

Here, the efficacy of recovering DNA from ancient shipwreck ceramics, cargo items, and environmental samples is explored. This research uses shotgun metagenomics which is

expected to amplify examine all manner of DNA present within the archaeological samples, be it bacterial, fungal or derived from marine organisms. The inclusion of environmental samples is therefore vital in order to characterise the deposition environment and determine what DNA may be derived from past vessel contents, and what is naturally present in Mediterranean seafloor sediments. Further, previous studies had postulated that ancient DNA was more likely to be preserved in ceramics recovered from underwater contexts than in artefacts excavated at terrestrial sites (Foley et al., 2012). To investigate this assertion, ceramic artefacts and environmental samples from one terrestrial site are also included in this analysis.

While a metagenomics approach through NGS is promising, a new issue may arise; as all DNA present in a sample will be amplified, it is understood that both modern, environmental DNA and any remaining ancient DNA will be recovered. The authentication of ancient DNA is therefore vital, as ancient DNA is expected to exist in low proportions in archaeological material, where DNA molecules derived from the environment are expected to dominate the DNA recovered (Key et al., 2017). Authentic ancient DNA has characteristic damage patterns of cytosine deamination (Briggs et al., 2007), and the extent of this deamination can be assessed with mapDamage 2.0, a program designed to detect and quantify these damage patterns in NGS datasets (Jónsson et al., 2013). However, research has shown that a minimum of around one thousand reads of the genome of interest are necessary for accurate alignment to a reference genome and assessment of the extent of cytosine deamination (Kim et al., 2017). The DNA detected here will be assessed by mapDamage2.0 to establish what patterns of nucleotide misincorporation are present at the terminal ends of the DNA fragments isolated, in order to determine either an ancient or modern origin for this DNA.

4.1.3 Organisms of Interest

Several ceramic vessels including in this research contained preserved macro-remains which established what they carried on their last journey, including olives, resin, pomegranate. In most cases these contents differ from what the amphorae were assumed to have contained, lending special relevance to their discovery. Here we investigate whether DNA derived from

these contents has penetrated the porous walls of ceramic vessels in antiquity, remained preserved, and can be detected by these methods. In addition, we investigate whether these species can be detected in environmental samples collected from the shipwreck sites. The detection of other commonly domesticated plants and animals including wheat, cat, dog, and sheep will also be assessed in order to evaluate levels of modern contamination in the archaeological samples.

Complementary approaches like those used by Pedersen et al. (2016) which used both palynological data and DNA data to characterise lake cores can provide insight into what might further information can be obtained from alternative methods. Here, the alternative method used to assess past vessel contents is organic residue analysis and results from a tandem study that analysed many of the same shipwreck ceramics, resin, and environmental samples (Briggs et al. in preparation) will be compared with results obtained through shotgun metagenomics in order to assess the suitability of these techniques in the determination of shipwreck amphorae contents.

4.2 Materials and methods

4.2.1 Sample descriptions

Three primary criteria were used to select ceramic artefacts for this study: first, the ceramic sherds tested were to be of known vessel type. Second, the vessel types selected have a clear presumed function (i.e. olive oil transport, wine container, et cetera). Third, if a vessel contained preserved macro-remains that showed empirically the nature of its' last contents, sherds or swabs from such a vessel were sought for inclusion in this study. It was possible to secure permission to perform destructive analysis on one such vessel: Chian amphora 426 from the Mazotos shipwreck contained several thousand waterlogged olive stones.

Destructive extractions were performed on 13 ceramic vessels, from the Mazotos, Kyrenia, and Babuljaš shipwrecks and the terrestrial site of Yeronisos (Table 10). Non-destructive swabs were taken from eight ceramic transport vessels from the Uluburun shipwreck and two ceramic transport vessels from the Cape Gelidonya shipwreck (Table 11). Four samples of vessel contents have also been extracted (resin recovered from Canaanite amphorae from the Uluburun shipwreck). Five samples of seafloor sediment have been extracted, variously from the Uluburun, Mazotos, and Babuljaš shipwrecks, as well as four samples of shelving unit dust swabbed in the storerooms of the Institute of Nautical Archaeology, Bodrum, three samples of seawater variously obtained from the Mazotos and Babuljaš shipwrecks, and one sample of soil from Yeronisos, Cyprus (Table 12).

Previous studies contended that the matrix of ceramic artefacts stored for decades in museum conditions yielded ancient DNA derived from past vessel contents through a non-destructive collection method of swabbing the interior surface of the vessel with a lysis buffer-soaked swab (Foley et al., 2012). In order to test whether such a non-destructive method is replicable with shotgun metagenomics through NGS, several transport vessels from the Late Bronze Age Uluburun and Cape Gelidonya shipwrecks were swabbed for analysis at the Institute of Nautical Archaeology in Bodrum, Turkey (Table 11).

Four of the vessels investigated through non-destructive analysis preserved their original contents: Uluburun shipwreck Canaanite jar KW 1565 contained thousands of whole olives (Haldane, 1993), two of the many Canaanite jars that contained resin were sampled, (KW 36, KW 169), and one jar that contained some pomegranate seeds, but also faience beads, bits of wood, and some olive stones (KW 1553) (Pulak, 1988). Establishing whether DNA derived from the known contents of a vessel can be detected through the methods used here would greatly aid in our development of methods for determining past vessel contents. Starting from a known entity (i.e. the nature of the vessel contents) will allow us insight into the success rate of vessel content detection through shotgun DNA metagenomic analysis.

Four resin samples recovered from the inside of Canaanite jars found on the Uluburun shipwreck have also been analysed by destructive analysis. These derive from Canaanite jars KW39, KW144, KW169, and KW605. Including these samples will allow us to determine if endogenous DNA can be recovered from this archaeological resin, and whether or not matching DNA can be detected in the vessels from which they came. In addition to ceramic artefacts and resin samples, where possible environmental samples have been collected in order to establish a baseline of what DNA is present in the deposition environment (Table 12).

For the shipwreck sites of Babuljaš, Croatia and Mazotos, Cyprus, seafloor sediments samples have been taken from among the artefacts, as well as outside of the shipwreck area in order to further establish what differences may exist in the DNA present on the wreck itself and its greater deposition environment. Finally, samples of the water column were also taken in order to establish what DNA is present in the ether that surrounds these sites. For the terrestrial site of Yeronisos, soil samples have been taken as a proxy for seafloor sediment, in order to illuminate what DNA can be present in the strata of this site.

As the Uluburun shipwreck excavation concluded prior to the genesis of this project, it was not possible to test seafloor sediment from this site. Samples of sediment were, however, recovered from the interior of one of the Canaanite jars (KW1553) and offered by the principal investigator as a pseudo-proxy for Uluburun seafloor sediment. Further, in order to establish what DNA is present in the storeroom of the Institute of Nautical Archaeology in Bodrum, swabs were taken of the shelving units on which the transport containers are stored. These could establish if arboreal pollen or other plant matter settling onto stored artefacts could be the origin of the olive DNA found in the previous studies (e.g. Foley et al., 2012).

Table 10. Ceramic artefacts analysed by destructive methods.

Site	Artefact	Assumed contents
<i>Mazotos shipwreck</i>		
	<i>Chian amphora</i>	
	426	Wine
	P0824	Wine
	P0784	Wine
<i>Kyrenia shipwreck</i>		
	<i>amphora</i>	
	069	Wine
	<i>Samian amphora</i>	
	A97	Wine
<i>Babuljas shipwreck</i>		
	<i>27 amphora</i>	
	A114	Olive oil
	A115	Olive oil
	<i>Bronze Age Cooking Pot</i>	
	A118	-
<i>Yeronisos Island</i>		
	<i>Lagynos</i>	
	P.15.17	Wine
	P.05.03	Wine
	<i>Spouted jug</i>	
	P.05.07	-
	P.94.18	-
	<i>Chamber pot</i>	
	P.93.12	Coprolites

Table 11. Artefacts analysed by non-destructive swabbing.

Site	Artefact	Contents	Assumed contents
<i>Uluburun shipwreck</i>			
	<i>Canaanite jar</i>		
	KW 1565	Olives	Resin
	KW 1553	Pomegranate	Resin
	KW 39	Resin	Resin
	KW 169	Resin	Resin
	<i>Pilgrim flasks</i>		
	KW 747	-	-
	KW 1183	-	-
	KW 3686	-	-
	KW 4496	-	-
<i>Cape Gelidonya shipwreck</i>			
	<i>Canaanite jar</i>		
	P2	-	-
	<i>Stirrup jar</i>		
	1.2.95	-	Olive oil

Table 12. Environmental samples analysed.

Site	Sample	Sampled from -	DNA library
<u>Mazotos shipwreck</u>			
	<i>Seafloor sediment</i>		
	A120	Outside shipwreck area	S36, S44
	A121	Inside shipwreck area	S37, S45
	<i>Seawater</i>		
	A123	Above shipwreck area	S48
<u>Babuljaš shipwreck</u>			
	<i>Seafloor Sediment</i>		
	A116	Inside shipwreck area	S27, S42
	A117	Outside shipwreck area	S28, S43
	<i>Seawater</i>		
	A122	Above shipwreck	S47
	A124	Above shipwreck	S66, S67
<u>Yeronisos Island</u>			
	<i>Soil</i>		
	A25	P.93.12	S24, S40
<u>Uluburun shipwreck</u>			
	<i>Seafloor Sediment</i>		
	L3561	KW 1553	S52
	<i>Shelving unit dust</i>		
	A91.a	INA Uluburun storage	S18
	A91.b	INA Uluburun storage	S19
<u>Cape Gelidonya shipwreck</u>			
	<i>Shelving unit dust</i>		
	A92.a	INA Cape Gelidonya storage	S20
	A92.b	INA Cape Gelidonya storage	S21

4.2.2 Extraction, Library building, Sequencing

Undertaken at the Allaby Laboratory, University of Warwick, all sample preparations, extractions, and library building were conducted in a dedicated, chambered laboratory, which is physically separated from post-PCR laboratories, and utilised protocols developed by the

Allaby Group for the extraction of DNA from submerged sites (Smith et al., 2015; Ware, 2016).

Precautions were taken against the introduction of any sources of DNA, including fungal and bacterial DNA. Ceramic samples were cleaned with ethanol wipes to remove external contaminants. Steel scalpels sterilised with bleach, ethanol, and UV light were used to pulverise the ceramic matrix and 1g of material was mixed with 4 mL of CTAB and incubated for one week at 37°C on a sample agitator at 400 rpm. In experiments involving recalcitrant DNA, CTAB extractions have been shown to yield higher concentrations of DNA than MoBio, Qiagen, and Epicentre kits (Jedlicka et al., 2013; Ware, 2016, p. 53). CTAB can stabilise DNA and facilitates the separation of polysaccharides in high sodium chloride concentrations (Ware, 2016, p. 53). As the majority of these samples come from a marine environment, this method is preferable to deal with the high sodium chloride levels that are likely present in these samples. Incubation at 37°C for one week was selected as a method, as incubation at low temperatures for an extended period (as opposed to the typical procedure for modern DNA extractions of incubation at 67°C for one hour) is less likely to cause heat-induced damage to what is already likely highly fragmented DNA (Rohland and Hofreiter, 2007; Ware, 2016).

After incubation, sediments were re-suspended, then spun at 25,000 xg for five minutes. The supernatant was removed and placed into 4 mL chloroform, manually shaken for five minutes, then spun at 25,000 xg for an additional five minutes. The supernatant was removed and placed in 20 mL AW1 binding buffer (Qiagen), mixed and incubated manually for one hour in the dark. Samples were then filtered through Qiagen DNeasy columns and washed with 500 mL AW2 buffer (Qiagen). Columns were centrifuged at 6,000 xg for one minute. 300 mL acetone was added as a wash, spun at 6,000 xg for one minute, after which the column was removed and left to dry for five minutes. To elute the extracted DNA, 65 mL of elution buffer was added to the columns, the columns were placed on a heating block at 37°C for five minutes, then spun at 15,000 xg for two minutes. Eluted DNA extractions were labelled and frozen.

For the non-destructive analysis, sterile swabs were soaked in lysis buffer then rubbed vigorously on the interior surface of ceramic transport vessels from the Uluburun and Cape Gelidonya shipwrecks. The swabs were stored in sterile containers and transported to the UK. Sterilised scissors were used to cut the swabs directly into 1.5 mL Eppendorf tubes and mixed with 1 mL of CTAB and allowed to incubate at 37° C for one week.

After incubation, sediments were re-suspended, then spun at 25,000 xg for five minutes. The supernatant was placed into 400 mL chloroform, manually shaken for five minutes, then spun at 25,000 xg for an additional five minutes. The supernatant was removed and placed in 1,600 ml of AW1 binding buffer (Qiagen), gently shaken manually for five minutes before being placed in the dark to incubate for one hour. Samples were then filtered through DNeasy columns to trap any DNA, then washed with 500 ml AW2 buffer (Qiagen). Columns were centrifuged at 6,000 xg for one minute. 300 ml acetone was added as a wash, spun at 6,000 xg for one minute, then the column was removed and left to dry for five minutes. To elute the extracted DNA, 65 ml of elution buffer was added to the columns, the columns were placed on a heating block at 37°C for five minutes, then spun at 15,000 xg for two minutes. Eluted DNA extractions were labelled and frozen at -20°C.

In order to establish what DNA is present in the seawater surrounding shipwreck artefacts, three samples of seawater were analysed in this study, two from the Babuljaš shipwreck (Sample 122 and 124), and one from the Mazotos shipwreck (Sample 123). A modern DNA extraction protocol was first attempted. The seawater samples incubated for one hour at 37°C were spun at 6,000 xg for 10 minutes at 4°C to create a pellet at the bottom of the sample tube. Supernatant was removed and the pellet was re-suspended into 400 ml AW1 binding buffer (Qiagen), gently shaken, then transferred into DNeasy columns and incubated at room temperature for two minutes. Samples were then spun at 6,000 xg for one minute, and the flow-through was discarded. 500 mL of AW2 wash buffer was added to columns and spun at 6,000 xg for one minute, and flow-through was again discarded. Samples were then spun again at 6,000 xg for one minute to dry. Next, 65 ml of elution buffer was added, and samples were left to incubate at room temperature for two minutes, after which samples were spun at 15,000 xg for two minutes, then quantified using HS Qubit fluorometric quantitation.

No DNA was detected in the HS Qubit fluorometric quantitation, so a new protocol was devised based on the protocol used at the Allaby Laboratory used to extract DNA from underwater cores. 1 mL of seawater from the Babuljaš shipwreck was mixed with 4 mL of CTAB and allowed to incubate at 37°C for one hour on a sample agitator at 400rpm. After incubation, the seawater was re-suspended in the CTAB, then spun at 25,000 xg for five minutes. The supernatant was placed into 4 mL chloroform, manually shaken for five minutes, then spun at 25,000 xg for an additional five minutes. The supernatant was then removed and placed in 20 mL AW1 binding buffer (Qiagen), gently shaken manually for five minutes before being placed in the dark to incubate for one hour.

Samples were then filtered through DNeasy columns to trap any DNA, then washed with 500 mL AW2 buffer columns (Qiagen) were centrifuged at 6,000 xg for one minute. 300 mL acetone was added as a wash, spun at 6,000 xg for one minute, then the column was removed and left to dry for five minutes. To elute the extracted DNA, 65 mL of elution buffer was added to the columns, the columns were placed on a heating block at 37°C for five minutes, then spun at 15,000 xg for two minutes. This new protocol, based on protocol for extracting ancient DNA but with a shorter incubation (one hour as opposed to one week), was far more effective: 4.21 ng/mL of DNA was detected using the HS Qubit fluorometric quantitation. Indexing of all DNA libraries prior to sequencing followed established protocols (Meyer and Kircher, 2010).

4.2.3 Bioinformatics

After sequencing on an Illumina NexSeq, resulting sequence files were analysed through a series of software programs in order to assess the number, quality and content of reads (Figure 39).

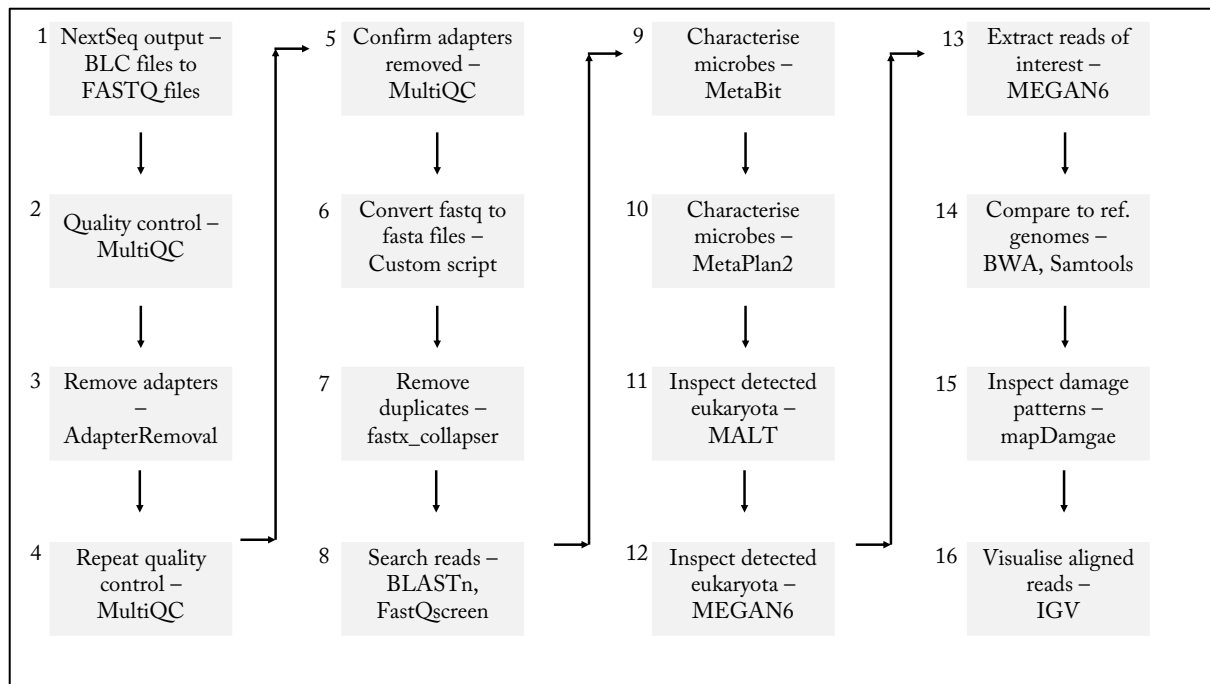


Figure 39. Bioinformatics process details of software used during each step.

Quality assessment was conducted with FastQC, and the resulting reports collated with MultiQC v1.5 (Ewels et al., 2016), adapters were removed with AdapterRemoval and quality assessment repeated. Fastq files were transformed into Fasta files, and duplicate reads collapsed with fastx_collapser. Filtered and collapsed Fasta files were searched via a Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) and the resulting fasta.txt files were visualised with MEGAN6 (Meta Genomic ANalyzer) to extract relevant phyla for further analysis (Huson et al., 2016). In order to compare results from different bioinformatic means of assessment, a metagenomics pipeline (Louvel et al., 2016), developed at the University of Toulouse was utilised at the same time.

With assistance from the Orlando group at the University of Toulouse, further bioinformatics analyses were conducted, consisting of a FastQscreen that allows alignment to multiple genomes at once, allowing reads to be mapped to common organisms such as humans and domesticates (Wingett and Andrews, 2018), analysis with metaBit which enables taxonomic profiling, clustering, Principal Coordinate Analysis (PCoA), Shannon alpha-diversity indices, MALT analysis which produces rma files for viewing in MEGAN6, and MetaPhlan2 which characterises bacterial communities within the sample.

4.3 Results

4.3.1 General description of DNA results

Of the 67 DNA libraries prepared for sequencing, 61 yielded a sufficient number of reads (1,000+) to be interpretable. The number of reads produced ranges from +30,000,000 to 53. No clear pattern emerges as to what type of sample consistently produced the greatest number of reads; while environmental samples top the chart (S67, seawater from the Babuljaš shipwreck), archaeological samples are not far behind (S33, Mazotos Chian amphora sherd P0824). It remains unclear exactly why some of the 67 libraries prepared failed, but unfortunately both of the two libraries built from Kyrenia shipwreck sherd extracts were uninterpretable.

When the calling of a base is unclear during sequencing, an 'N' is assigned in its position. Figure 40 shows the percentage N-count is low for these samples, indicating that for the majority of bases the calling of the base was clear.

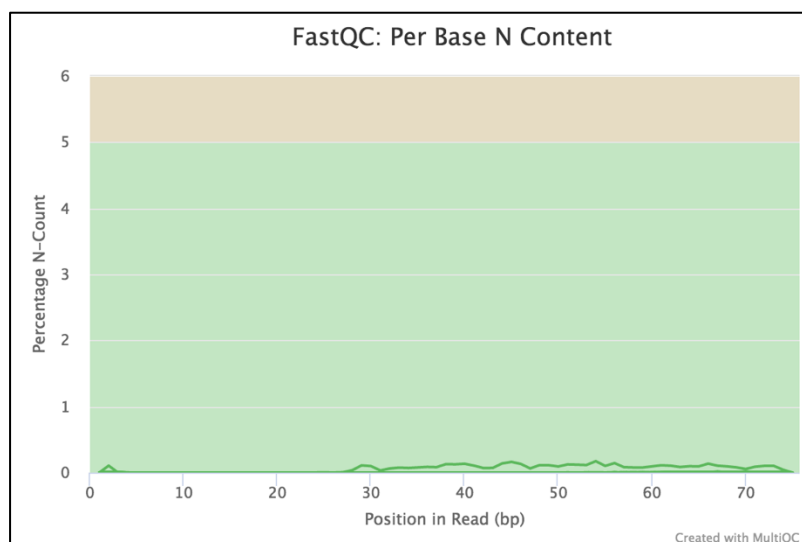


Figure 40. N - count for all samples, from MultiQC.

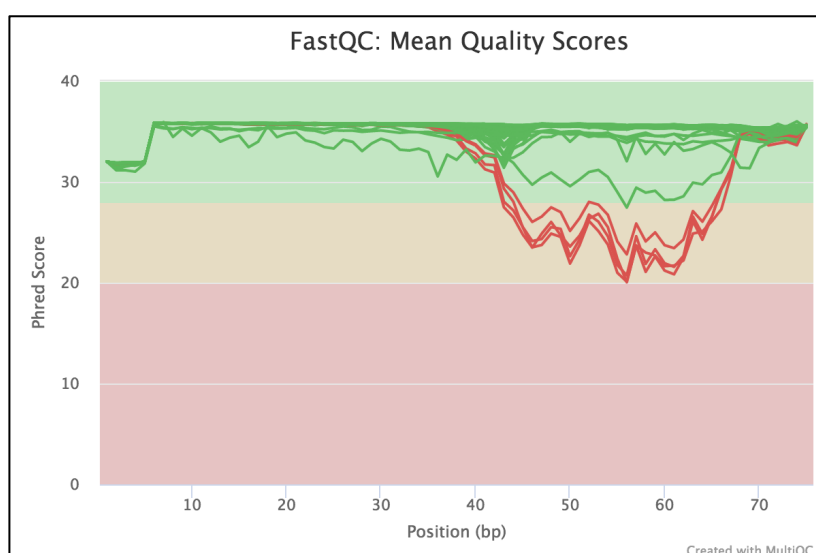


Figure 41. Mean quality scores for all samples, from MultiQC.

Figure 41 shows the mean quality score per base position for all samples. Four of the 67 libraries show a clear fall off in the quality scores for their base pairs (bp) in positions beyond position 40, these are Libraries 29 (BA cooking pot, Babuljas), 38 (laboratory Blank), 47 (Seawater sample, Babuljas), and 56 (duplicate run of the BA cooking pot, Babuljas). The fact that both extractions for the BA cooking pot have a low mean quality score above position 40 suggests that this results accurately represents the quality of Libraries 29 and 56. While the mean quality score per base position (Figure 41) shows that four samples were of lower quality than desired,

a report that determines quality score across the entire sequence, rather than per base position, shows that all 67 libraries have an average or above average Phred score when the quality of the entire sequence is appraised (Figure 42).

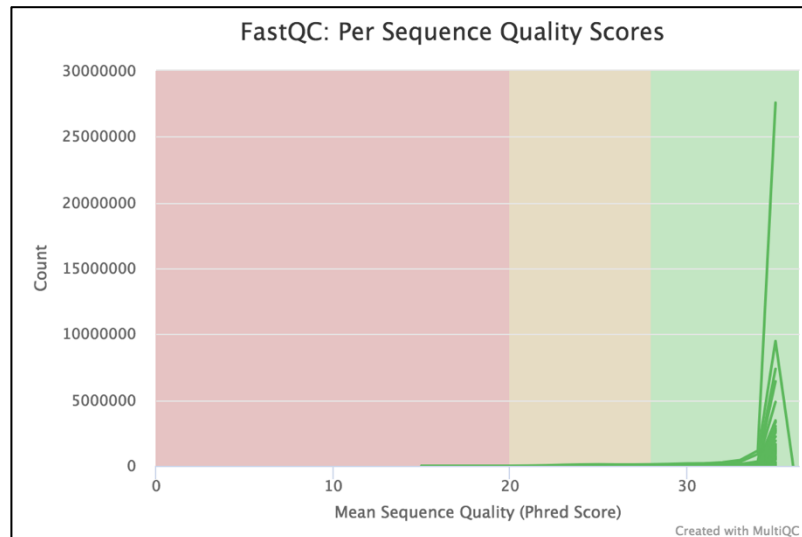


Figure 42. Mean sequence quality (Phred score) for all samples, from MultiQC.

Six of the DNA libraries were uninterpretable due to the low number of reads. Any DNA library with fewer than 1,000+ reads is likely to have malfunctioned during preparation. This could be a result of laboratory errors during the library building process, or during the sequencing of the assembled libraries. Sixty-one libraries were interpretable, representing a success rate of 91%.

The reads produced from the successful libraries predominantly align with bacterial, fungal and human genomes, with some domesticates present. Analysis through metaBit and MetaPhlan2 has characterised the composition of bacterial communities within all the samples included in this study, and demonstrates clusters are present among the genera of bacteria detected (Figure 43).

In this dendrogram the 'Approximate Unbiased' p-value (AU) is written above clusters in red, while the 'Bootstrap probability' p-value (BP) is written above clusters in green. The AU value

amplified from the environment would differ from the profile of bacterial communities detected in laboratory reagents and enzymes. The DNA results for these samples are therefore no better than blanks in what they demonstrate archaeologically.

The potential for contaminant DNA to be present in laboratory reagents has been explored in detail (Salter et al., 2014), and when the composition of bacterial communities has been ignored it can lead to skewed and inaccurate conclusions, as has been shown by Eisenhofer and Weyrich (2018) in their critique of a study that concluded the gut microbiome of Italian and Incan mummies was substantially different (Santiago-Rodriguez et al., 2017). Eisenhofer and Weyrich showed that the differences found in the original study are driven primarily by the composition of laboratory contaminants that are also present in the ‘blanks,’ and do not reflect a difference in the mummies’ gut microbiome (Eisenhofer and Weyrich, 2018). These issues lie at the heart of metagenomics research, and it is within this theoretical framework that the composition of the bacterial communities present in the samples analysed here is appraised. For example, the samples of seafloor sediment from both the Mazotos and Babuljaš shipwreck site cluster together, indicating that they share similar genera of bacteria (Figure 43, circled in green).

Another two clusters contain four of the resin samples from the Uluburun shipwreck, in addition these are duplicate samples, showing clearly that any amplified DNA (bacterial or otherwise) is indeed a product of the archaeological resin and the environmental contamination it contained, rather than being a similarity derived from contaminants in the laboratory reagents (Figure 43, circled in yellow). It does not appear that the clustering shown here is dependent on the number of reads produced: we find the seawater sample S67 sharing a cluster with a sample with far fewer reads (S3, a spouted jug from Yeronisos). This suggests that it is not only the number of reads that has affected the clustering, but rather the actual genera represented.

The genera of bacteria and fungus detected are visualised by bar plot in Figure 44. Genera are colour-coded, and once again patterns emerge among types of archaeological material and

environmental samples that contain similar genera of bacteria of fungus. In Figure 45, seafloor sediment, dust swabs, resin, and blanks are circled.

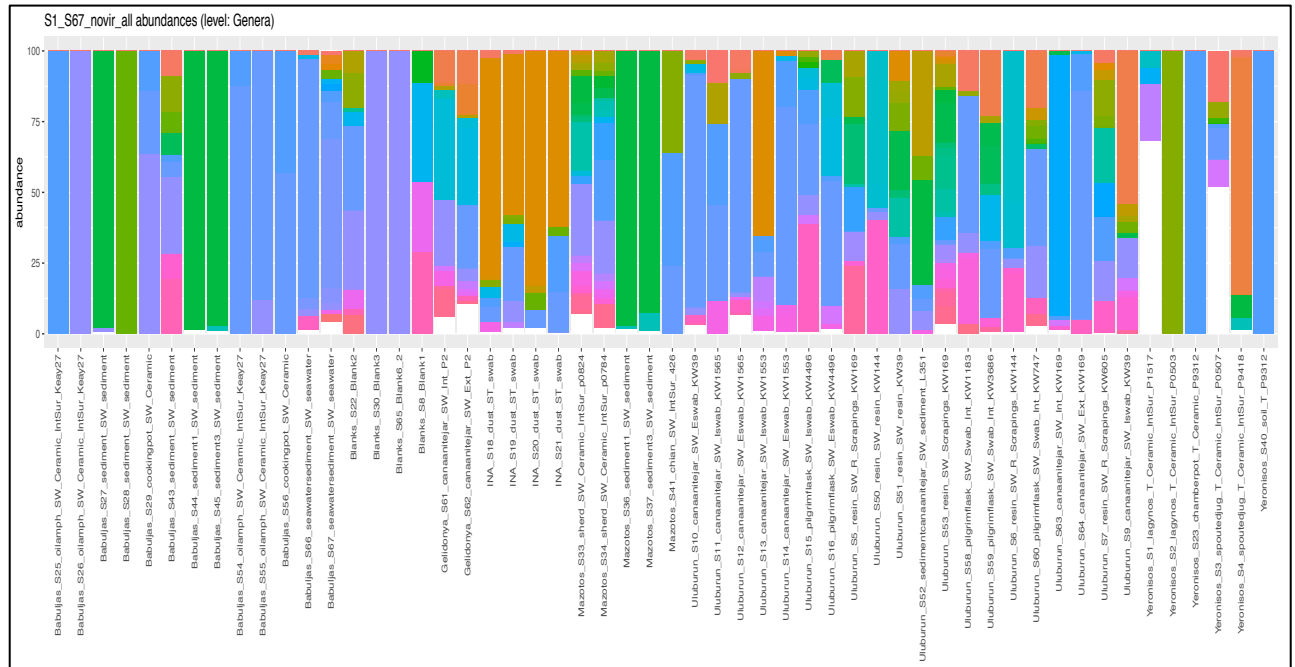


Figure 44. Bar plot showing abundances of bacterial genera, with colour key to names of bacterial genera below.

Actinomyces	Corynebacterium	Leptotrichiaceae unclassified	Polaromonas	Sphingopyxis
Aflipia	Deinococcus	Leuconostoc	Propionibacteriaceae unclassified	Spiribacter
Alcanivorax	Dermabacteraceae unclassified	Limnobacter	Propionibacterium	Spirochaeta
Alicyclophilus	Dermatophilaceae unclassified	Limnobacter	Pseudoalteromonadaceae unclassified	Staphylococcus
Alteromonas	Eimeria	Marinobacter	Pseudoalteromonas	Stenotrophomonas
Arcobacter	Erythrobacteraceae unclassified	Massilia	Pseudomonas	Streptococcus
Arthrobacter	Escherichia	Mesorhizobium	Pseudonocardia	Thauera
Aspergillaceae unclassified	Geodermatophilaceae unclassified	Methanofollis	Pusillimonas	Thioalkalivibrio
Aspergillus	Granulicella	Methanoplanus	Reinekea	Thiobacillus
Asticcacaulis	Haemophilus	Methylbium	Rhodobiaceae unclassified	Thiomicrospira
Azoarcus	Hahellaceae unclassified	Methylophaga	Rhodococcus	Thiomonas
Blautia	Halomonas	Methyloversatilis	Rhodopirellula	Variovorax
Brachybacterium	Hydrogenophilaceae unclassified	Micrococcus	Rhodospseudomonas	Veillonella
Brevibacterium	Hyphomicrobiaceae unclassified	Mycobacterium	Rhodospirillum	Vibrio
Brevundimonas	Kocuria	Neisseria	Rickettsia	unclassified
Burkholderia	Labrenzia	Neptuniibacter	Roseomonas	
Capnocytophaga	Lactobacillus	Nocardioides	Rothia	
Caulobacter	Legionellaceae unclassified	Paracoccus	Shewanella	
Cellvibrio	Leifsonia	Penicillium	Sinobacteraceae unclassified	

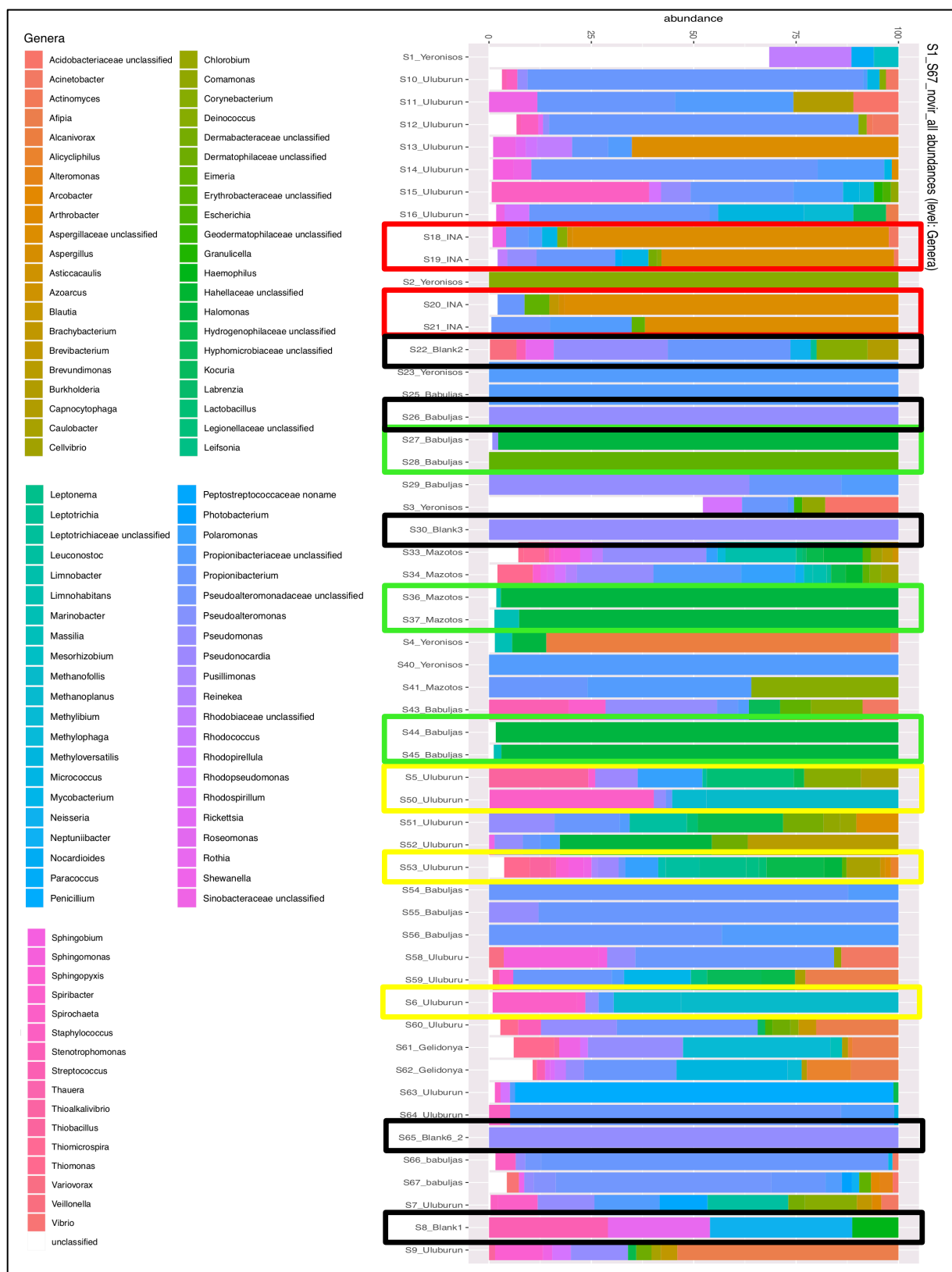


Figure 45. Bar plot showing composition and abundance of bacterial communities. Seafloor sediment circled in GREEN. Resin samples circled in YELLOW. Dust swabs circled in RED. Laboratory 'blanks' circled in BLACK.

The similarity in composition and abundance of bacteria genera is readily apparent in the samples of seafloor sediment collected from the Mazotos and Babuljaš shipwrecks (Figure 45, circled in green). This indicates that both the composition and abundance of bacterial genera found for these samples of seafloor sediment are very similar, despite being collected from shipwreck sites in different part of the Mediterranean. *Halomonas*, a salt-tolerant genus of proteobacteria (Vreeland, 2015), dominate these samples. Dust swab samples from the storage areas of the Uluburun and Cape Gelidonya artefacts also show clear similarities (Figure 45, circled in red), and are dominated by *Aspergillus*, a genus including a few hundred species of mold (Osmani and Goldman, 2008).

Duplicate runs of resin from the Uluburun shipwreck have detected a similar composition of bacteria and fungus (Figure 45, circled in yellow). That duplicate runs share similar genera of bacteria is promising, as it indicates a minimal amount of contaminant bacteria was introduced during the extraction and library building laboratory work, as the duplicate extractions were completed on different days and with different laboratory ‘blanks.’ The resin samples have a mixed composition which includes *Halomonas* and *Methanobacterium*, a methane-producing genus that can be found in wetlands and seafloor sediments (Boone, 2015).

With the bar-plot it is also possible to show the relationships among samples and laboratory ‘blanks’ (Figure 45, circled in black). Samples with bacterial communities that are indistinguishable from blanks are unlikely to provide any useful archaeological data as this is an indication that no DNA from the samples was successfully extracted and sequenced. Here we see that the relationship between Blank 2 and library S60 does not appear to share the same level of similarity as that shown among Blank 3, Blank 6 and library S26. It is quite clear that S60 contains levels of *Alcanivorax alicyclophilus* not shared by Blank 2. While library 26 is identical to Blank 3 and Blank 6, it is reasonable to eliminate further analysis of this sample entirely. Library 60, however, while sharing many genera of bacteria with Blank 2, has been shown to contain differing levels of *Alcanivorax alicyclophilus*, undermining our security that this sample is indistinguishable from a laboratory ‘blank.’ Therefore, this sample will be analysed further, but with caution.

In addition to the clustering analysis that produced cluster dendrograms and bar plots, Principal Coordinates Analysis (PCoA) has been completed. This analysis attempts to represent the theoretical ‘distance’ in the composition of variables within the archaeological samples, in this case the variables are the genera of bacteria. This analysis was performed with metaBit, and the following scatter plots demonstrate the components detected and where affinities lie among them (Figure 46). From the PCoA we can also see a cluster of DNA libraries in the right-hand side of the diagram that share similar bacterial communities with seawater collected from directly above the Babuljaš shipwreck site. Ceramic samples from the Babuljaš shipwreck are present within this cluster, perhaps indicating that bacterial communities present in the seawater have also been detected in the ceramics due to their long submergence in the very same seawater. Interestingly, the PCoA does not show as close an affinity between Blank 6, Blank 3, and Library 26 (Africana III/Keay 25), as was shown by the clustering.

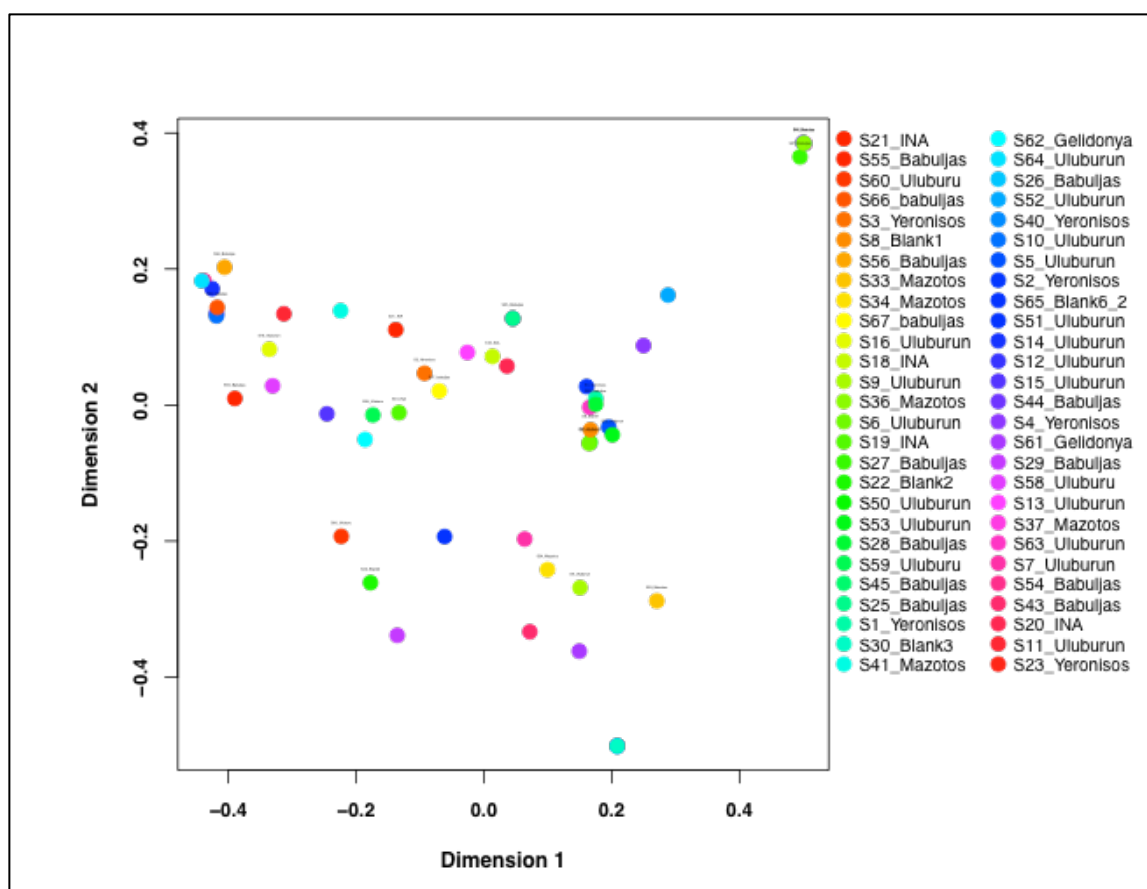


Figure 46. Scatter plot showing PCoA of bacteria genera for all samples.

All laboratory blanks were closely scanned for the detection of organisms of interest, including olive, grapevine, *Pistacia*, pomegranate, and pine. This is to ensure that the detection of these species in archaeological samples is not simply due to their presence in laboratory reagents. The species have not been detected in the laboratory blanks in any meaningful numbers. The laboratory blanks were dominated by human and bacterial DNA, and also each revealed high number of reads (1,500+) assigned to the plant species *Dioon edule*, a cycad native to Mexico and Honduras that has been analysed for transcriptome-derived microsatellite markers (Prado et al., 2016). This was also detected in the archaeological and environmental samples in high numbers, but as it occurs in all the laboratory blanks, has been excluded as having any archaeological relevance for this study.

To conclude the general description of results from the DNA analysis, the extraction and sequencing appears to have been successful based on the number of libraries with sufficient reads to be interpretable (91%) and the similarity in bacterial communities among duplicate extractions. The fact that seafloor sediment samples, dust swab samples, and resin samples, form discrete clusters is also indicative of the fact that bacterial communities specific to these material types have been successfully extracted and detected. For all sequenced DNA libraries, it is clear that bacterial, fungal and human DNA dominates the results from all archaeological samples. However, in certain samples, some domesticated organisms of interest have been detected in low numbers of reads. These organisms of interest have not been detected in the laboratory blanks in similar numbers.

4.3.2 Detection of Organisms of Interest

Organisms of interest to this study are those that have been detected through macro-remains within the shipwreck amphorae analysed here, or through molecular evidence obtained from organic residue detected in the tandem study (Briggs et al., in preparation) of these artefacts. These include *Olea* (olive), *Vitis* (grapevine), *Punica* (pomegranate), *Pistacia* (pistacia). Beeswax

was detected by GC-MS in *lagynos* P.15.17, as were conifer products in the majority of the shipwreck amphorae, so *Apis* (bees) and *Pinus* (pine) are added to the organisms of interest. However, in any sample where a relevant number of reads (>1,000) was assigned to any eukaryotic species, this has been investigated to determine its archaeological merit, regardless of whether or not this species was an assumed vessel content, a known content, or something else entirely.

One method used to tease out the archaeological meaning from shotgun metagenomic datasets is the practice of calculating the percentage of eukaryotic reads assigned to a given species. This method thus excludes the bacterial DNA present in a sample, and instead focuses on the plants and animals that are represented. In addition, rather than looking at absolute numbers of reads, a calculation of the percentage assigned to a given species can be more informative given the varying absolute number of reads across the samples analysed here. Weyrich et al. (2017) used this method in their analysis of Neanderthal dental calculus, and found in one sample that 62% of the eukaryotic reads assigned to *Ovis*, or sheep, and this was reported as evidence for the inclusion of sheep in the Neanderthal diet, with implications for their behaviour and oral health (Weyrich et al., 2017, p. 359). Following this method, the percentage of reads assigned to eukaryotic organisms has been calculated from all samples. The results for reads mapping to organisms of interest and commonly domesticated organisms in the ceramic vessels of known contents are presented in Figure 47.

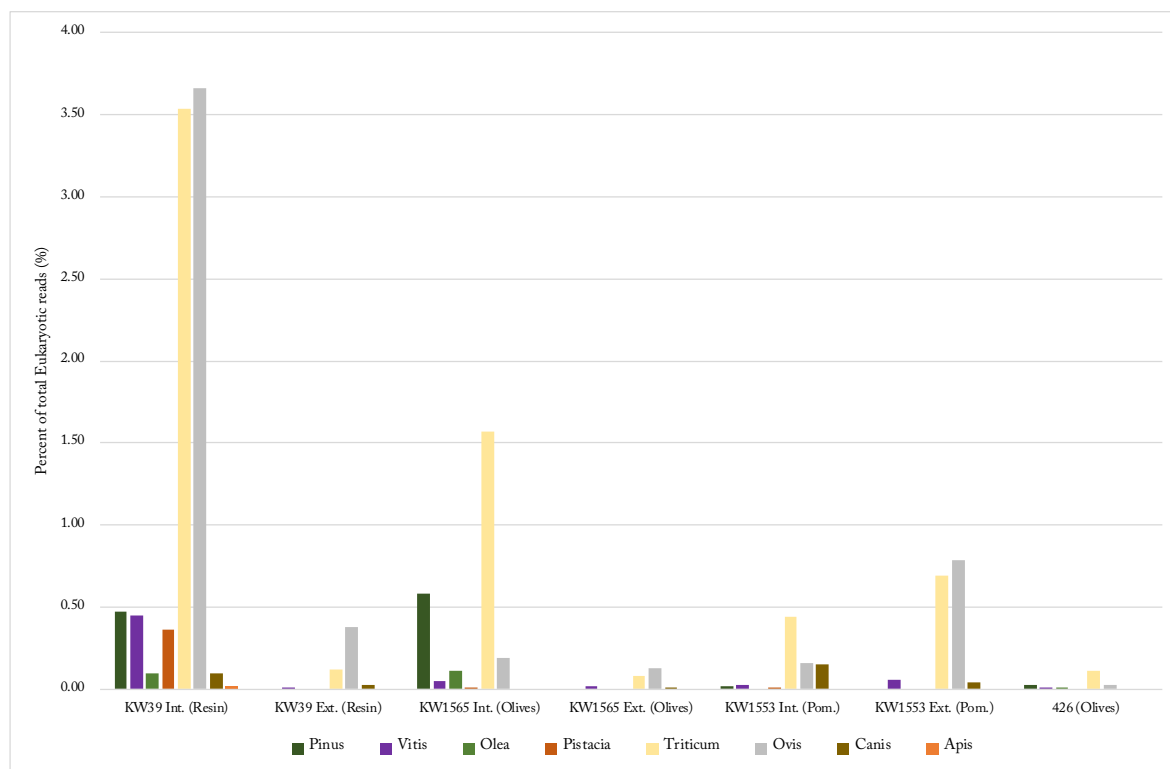


Figure 47. Cluster column chart showing the percentage of eukaryotic reads that match to organisms of interest in DNA results from shipwreck amphorae of known contents. Artefacts are displayed as follows: Artefact code (Known contents). "Pom" = pomegranate.

Canaanite jar KW39 from the Uluburun shipwreck is known to have contained *Pistacia* resin, and *Pistacia* DNA was detected in a low number of reads (18/5,056 eukaryotic reads), as was grapevine, pine, olive and dog DNA, but far higher numbers of reads were assigned to wheat (179/5,056 eukaryotic reads) and sheep (185/5,056). Found full of whole olive stones preserved by the marine environment, Canaanite jar KW1565 also yielded a small number of reads that matched to its known contents (44/38,896 eukaryotic reads). This number of reads is too few to authenticate, however, and far more reads in this sample matched to wheat (609/38,896) and sheep (1,363/38,896) than to olive. The source of the sheep and wheat DNA is likely to be modern contamination.

For Canaanite jar KW1553, no DNA from the pomegranate seeds found inside were detected by these methods. Incidentally, more DNA from the grapevine plant (a possible past content) was detected in the swab taken from the outside of the vessel (KW1553 Ext), than from found in the swab from the interior surface (Figure 47), further reducing the possibility that this grape

DNA is archaeological in origin. Chian amphorae 426 from the Mazotos shipwreck was full of olives when it was recovered, like KW1565, but similarly did not appear to retain DNA derived from its known contents (olives), that can be detected by these methods.

To gauge our ability to recover DNA associated with organisms of interest in our archaeological samples by the methods used here, the samples with the highest percentage of eukaryotic reads that match to these organisms have been tabulated. Figure 48 shows that samples analysed that have yielded the highest percentage of eukaryotic reads that match to *Vitis vinifera*, or grapevine. This was the assumed content for the majority of the shipwreck amphorae analysed in this study.

Analysis by MALT and MEGAN6A of a swab taken from the interior of Uluburun shipwreck pilgrim flask KW747 revealed 1,248 out of 79,387 eukaryotic reads that map to the *Vitis vinifera*, or grapevine, genome. Pilgrim Flasks are vessels of unknown contents, leaving the possibilities as to what they contained in the past very much open for debate. Significantly, however, four of the remaining top nine samples are modern environmental samples, confirming that grapevine DNA is present in seafloor sediment (A120 and A121) and shelving unit dust (A91.b, A91.a). In order to authenticate the *Vitis vinifera* as ancient DNA, it is necessary to investigate what damage patterns are present on the isolated sequences.

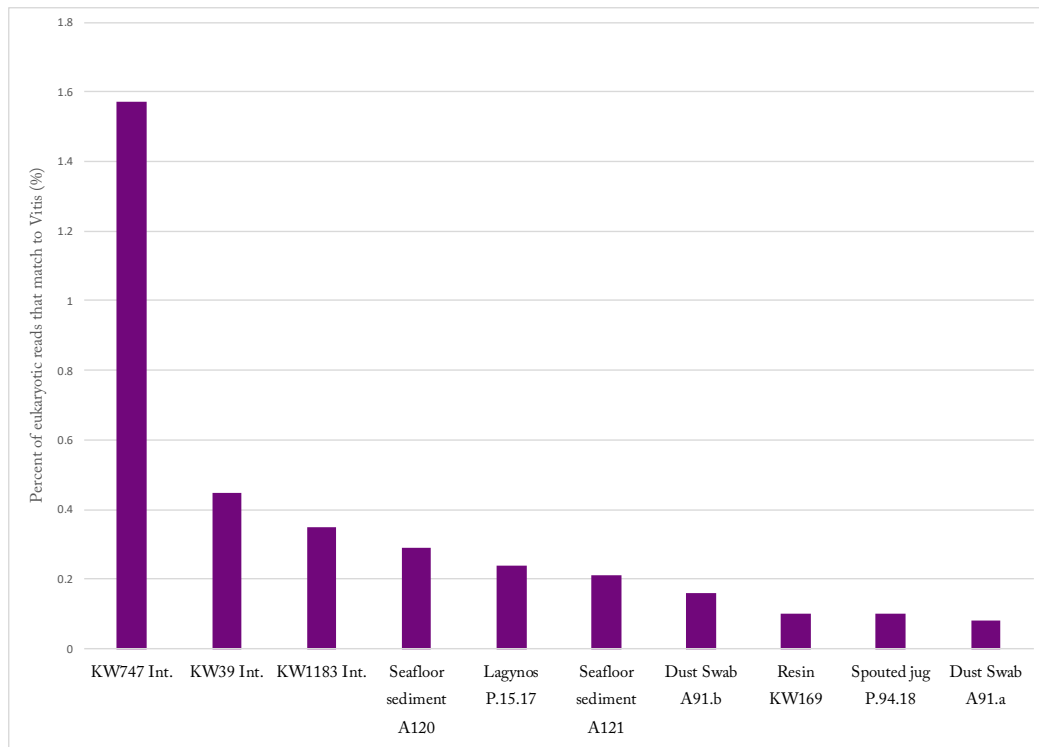


Figure 48. The ten samples with the highest percent of eukaryotic reads matching to *Vitis*.

Briggs et al. (2007) propose that ‘cytosine residues close to the ends of ancient DNA molecules are more susceptible to deamination than cytosine residues more internal in the molecule’ (Briggs et al., 2007, p. 14619).

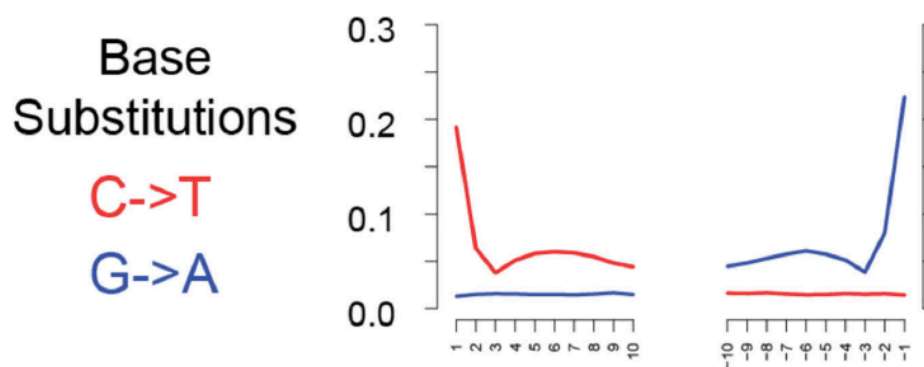


Figure 49. Characteristic damage pattern for ancient DNA, after Ziesemer et al. 2015.

As has been discussed by Briggs et al. (2007) and is shown in the image above from Ziesemer et al. (2015) ancient DNA has a greater frequency of substitutions at the terminal ends of the DNA fragment, and this pattern of nucleotide misincorporation produces a characteristic ‘smile’ pattern in the C- >T and G- >A substitutions, as shown in Figure 49 (Ziesemer et al., 2015).

Of the ten samples with the highest percent of eukaryotic DNA that matched to *Vitis vinifera*, only Pilgrim Flask KW747 had sufficient reads to assess the nucleotide misincorporation in MapDamage2.0 (>1,000). According to Eisenhoffer and Weyrich (2018) only a few thousand reads are necessary to analyse nucleotide misincorporation, but the greater the number the more robust results can be expected. Here a threshold of >1,000 reads is used.

The *Vitis vinifera* reads identified in MEGAN 6 were exported as a FASTA file. These reads were then mapped against the *Vitis vinifera* reference genome obtained from the NCBI database, GCF_000003745.3_12X_genomic.fna, with the BWA back-track (ALN) aligner. The outputs were converted into SAM files then used as input into mapDamage 2.0, for comparison with the *Vitis vinifera* genome. Figure 50 shows the frequency of nucleotide misincorporation found for the grapevine reads in KW747.

S60_vitis

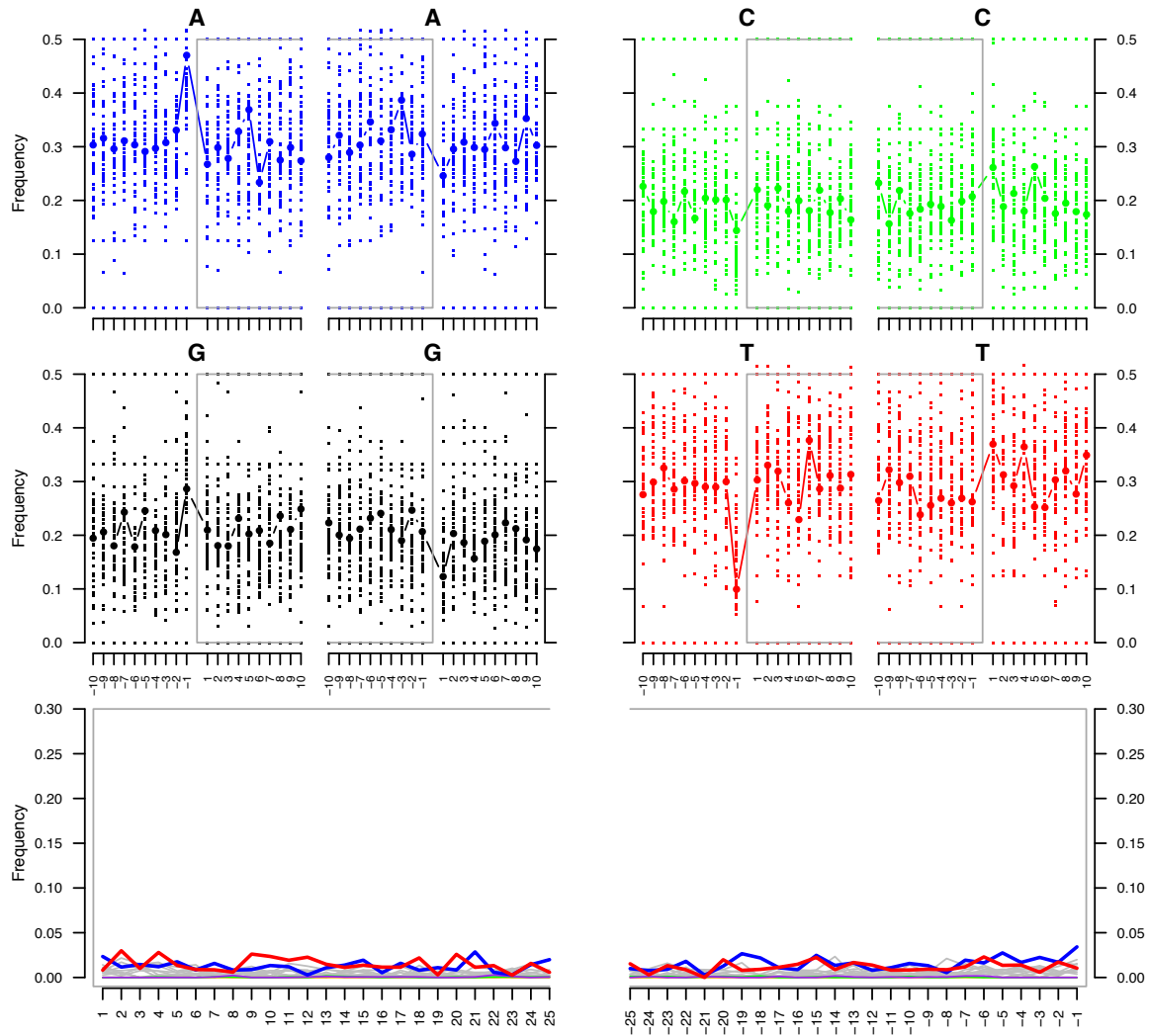


Figure 50. Substitution patterns for *Vitis vinifera* DNA found in Pilgrim Flask 747.

The lack of nucleotide misincorporation at the terminal end of DNA fragments found for the *Vitis vinifera* DNA recovered from Pilgrim Flask 747 is indicative of modern DNA. Another interesting aspect of these grape-DNA reads is their base-pair fragment length. Foley et al. (2012) argued that the short base-pair fragment length of the DNA recovered from inside the analysed shipwreck amphorae was sufficient evidence that the DNA was ancient in origin: ‘Because our test runs amplified no long nucleotide fragments, we concluded that modern DNA contamination in these amphorae was unlikely’ (Foley et al., 2012, p. 393). Yet, base-pair fragment length alone is not sufficient for the authentication of ancient DNA. The

fragment length of the grape DNA reads obtained from Pilgrim Flask 747 are shown in Figure 51.

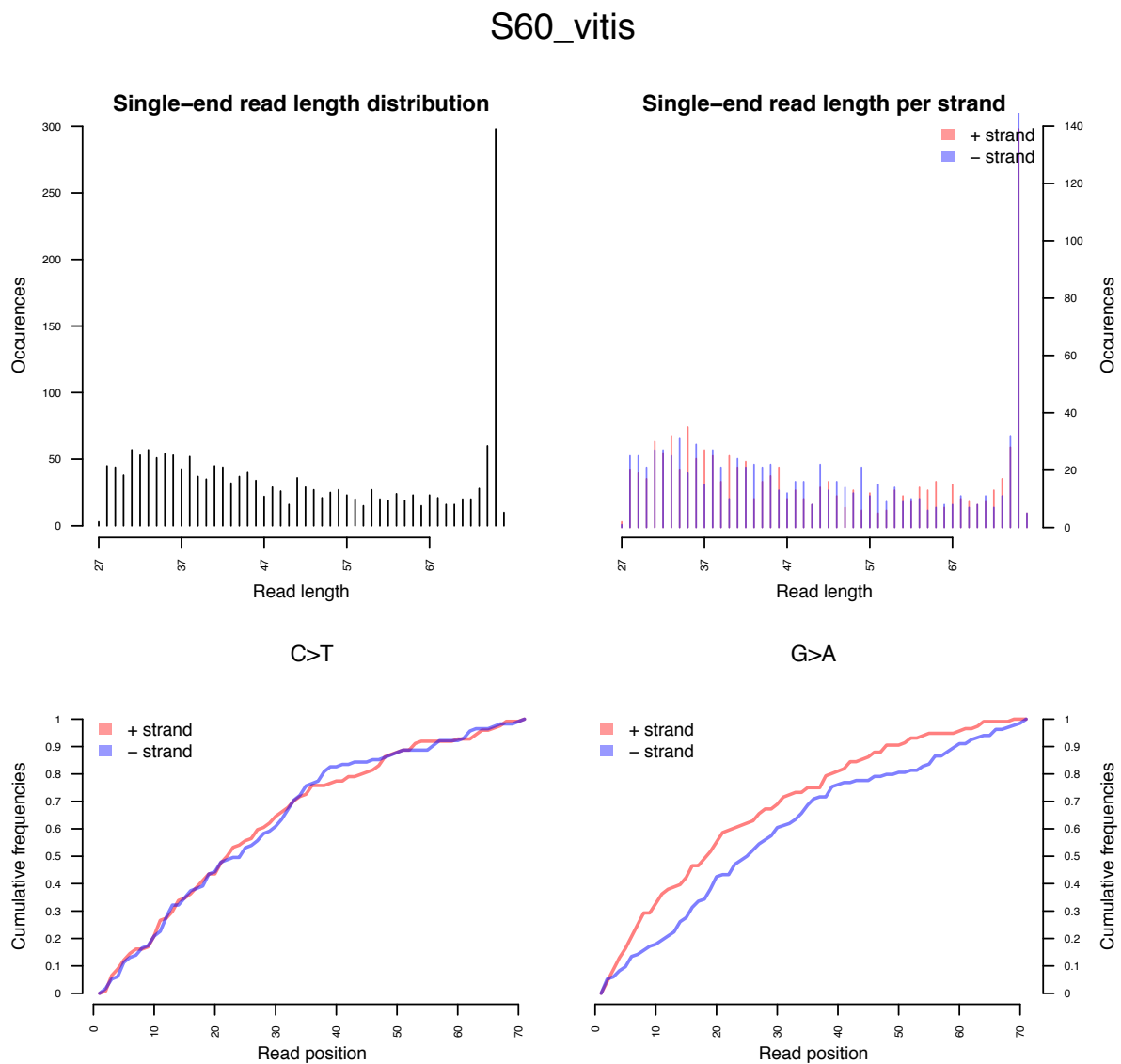


Figure 51. Base-pair fragment lengths for grape DNA recovered from Pilgrim Flask 747.

Here we see that a number of fragments contain fewer than 100 base pairs, but the greatest number of fragments have over 67 base pairs, further showing that this is not highly fragmented grapevine DNA. The lack of nucleotide misincorporation does not support an ancient origin for this DNA, and this illustrates the robust analysis needed to properly authenticate DNA recovered from archaeological samples.

Olives were known to have been contained in at least two of the shipwreck amphorae analysed here (Chian amphorae 426 and Canaanite jar KW1565). Olive oil is assumed to have been contained in the Africana III/Keay 25 amphorae from the Babuljaš shipwreck and the Stirrup jar from the Cape Gelidonya shipwreck. Figure 52 shows the ten samples with the highest percent of eukaryotic reads that match to *Olea*, the olive tree.

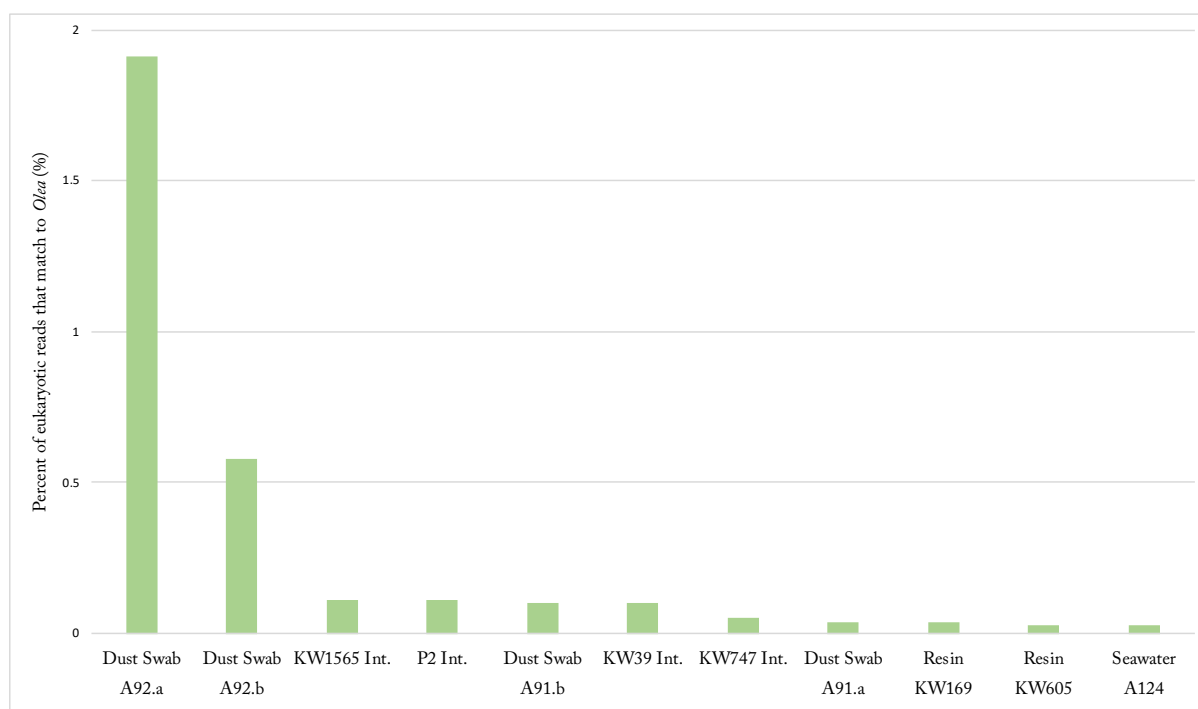


Figure 52. The ten samples with the highest percent of eukaryotic reads that match to *Olea*.

Five of the ten samples with the highest percent of eukaryotic reads that match to *Olea* are modern environmental samples, one seawater sample and four samples of shelving unit dust. One vessel known to have contained olives is represented here, Canaanite jar KW1553 from the Uluburun shipwreck. Yet, the absolute number of reads is still quite low, with only 44/38,896 eukaryotic reads matching to the known contents of this vessel. This is not sufficient coverage for analysis with MapDamage, but enough *Olea* DNA was recovered from Dust Swab A92.a to assess its origins. As a modern environmental sample, the results were expected to show that the DNA was modern, but as ancient olive stones are present and analysed in the Institute of Nautical Archaeology, it bears investigation into what the origin of this DNA is.

The same process of analysis undertaken for the reads assigned to *Vitis* was completed for the *Olea* reads, but with alignment to genome GCF_002742605.1_O_europaea_v1_genomic.fna from the NCBI database. Results from MapDamage2.0 for the *Olea* reads found in Dust Swab A92.a (S60) are shown in Figure 53.

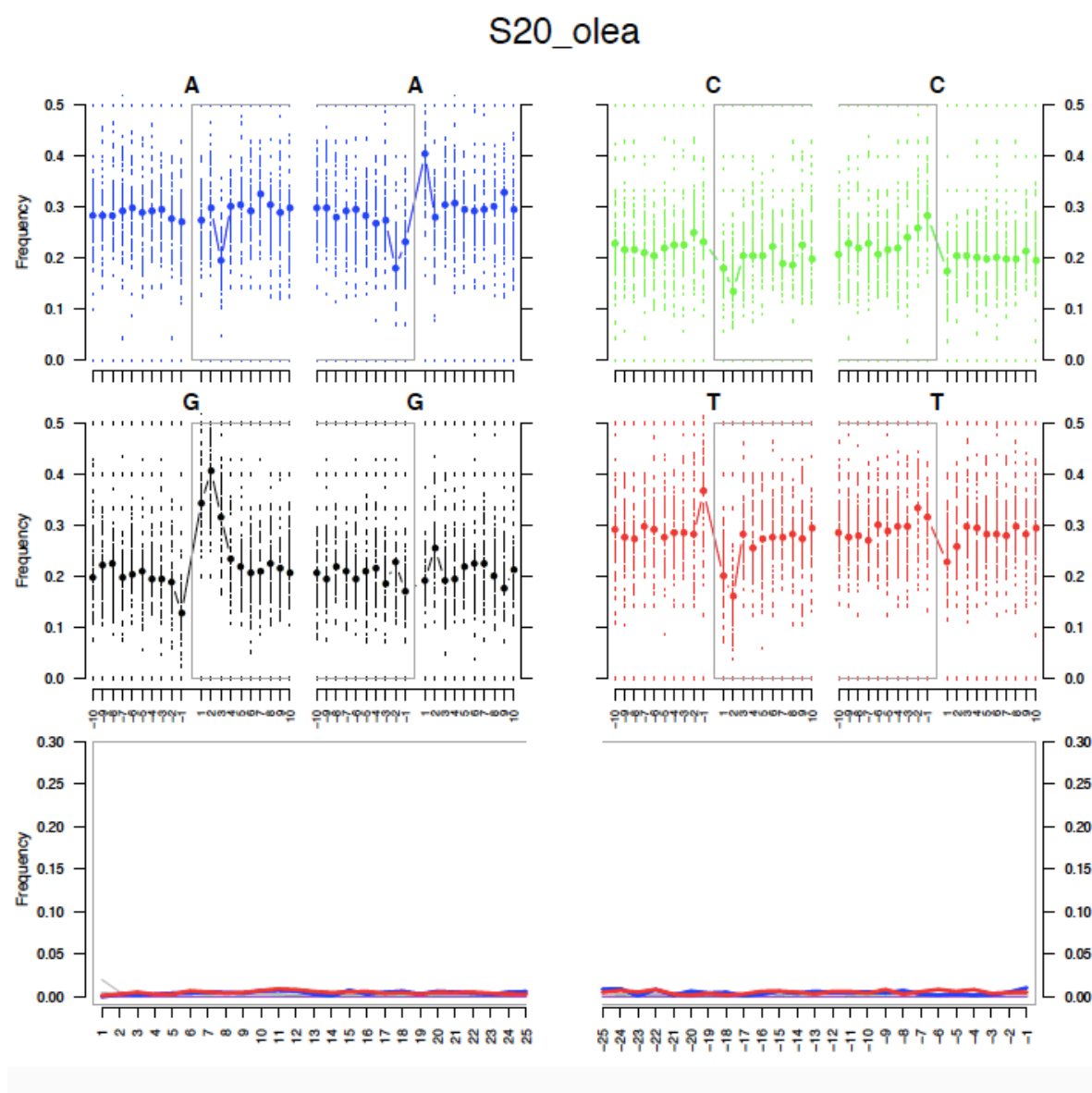


Figure 53. Results from mapDamage2.0 for the *Olea* DNA detected in shelving-unit dust from the Cape Gelidonya shipwreck artefacts storage, (S20).

The olive DNA detected in this sample is modern DNA based on the lack of nucleotide misincorporation patterns that are characteristic of ancient DNA. Even more acute than the

results for the *Vitis* DNA, the fragment length for the olive DNA detected is very high for Dust Swab A92.a (S20), with over 2,000 occurrences of fragments with more than 67 base pairs, further confirming that this is modern olive DNA (Figure 54).

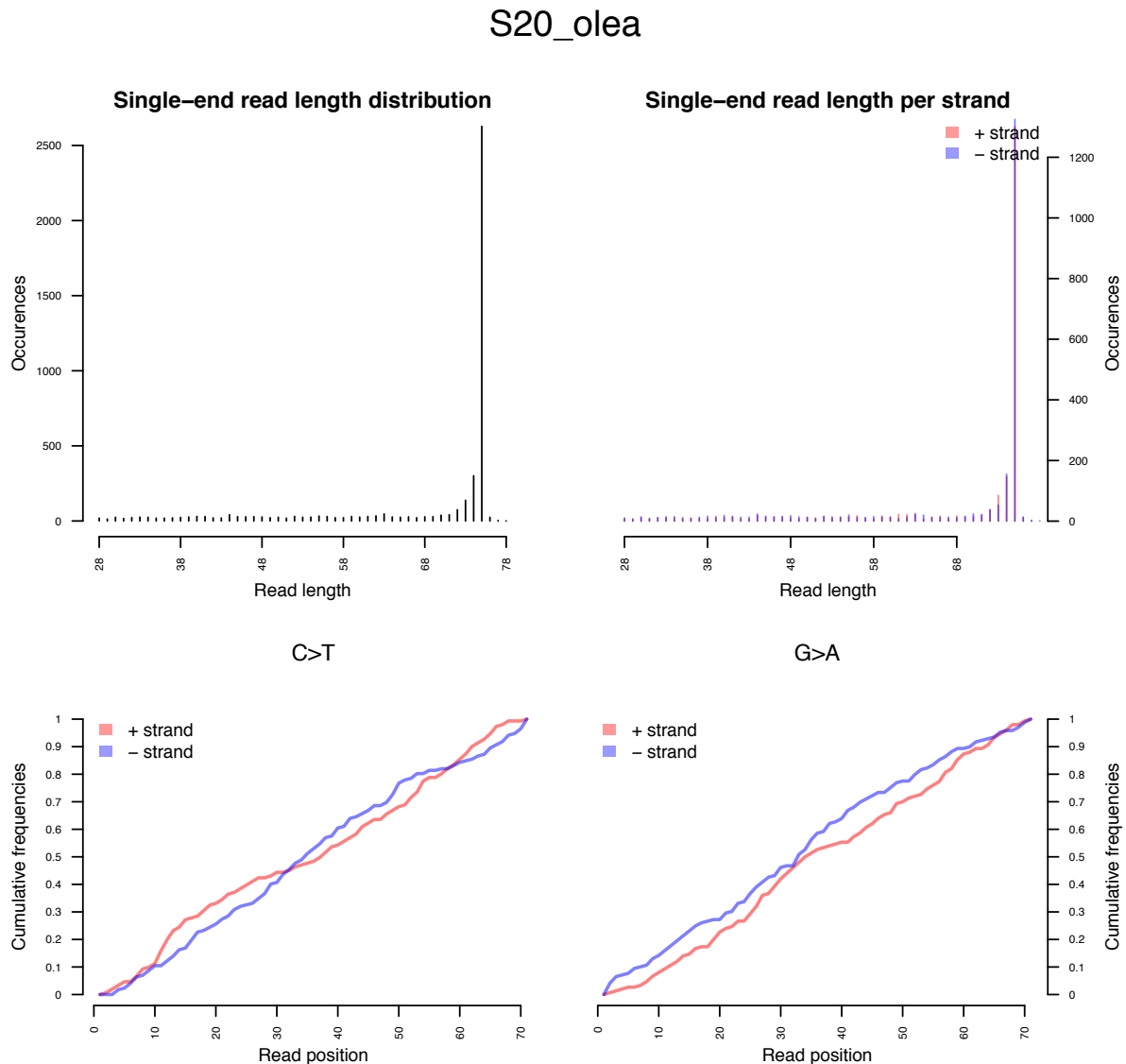


Figure 54. Fragment length of olive DNA detected in dust swab S20.

The majority of the Canaanite jars from the Uluburun shipwreck contained an amber-coloured resin identified as *Pistacia* resin by Mills and White (1989) and Stern et al. (2008) through organic residue analysis. Four resins samples were analysed here, and at least small numbers of reads that matched to *Pistacia* were detected in each sample. The percentage of eukaryotic reads

that matched to *Pistacia* was calculated for each sample, and every resin sample is represented in the ten samples with the highest percent of eukaryotic reads that match to the tree that produces this resin (Figure 55).

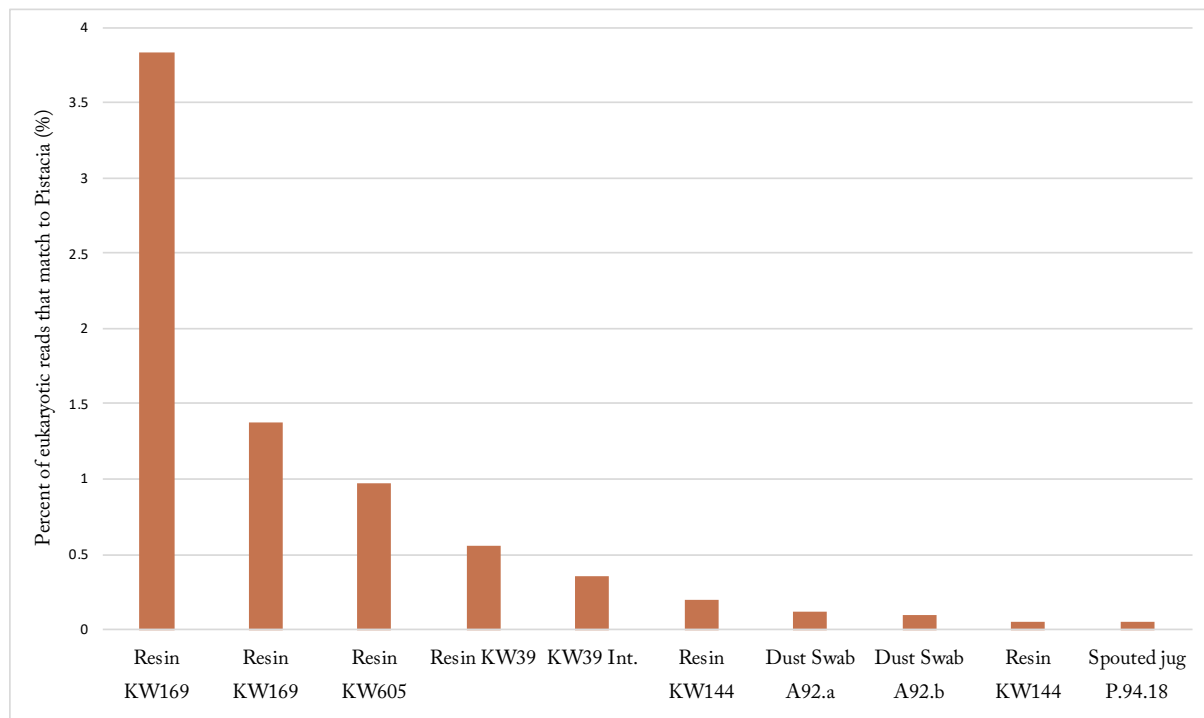


Figure 55. The ten samples with the highest percent of eukaryotic reads that match to *Pistacia*.

In each of these ten samples there is still a very low absolute number of reads that match to *Pistacia*, the highest being 357/ 25989 eukaryotic reads in resin sample KW169. This is too low for assessment of characteristic damage patterns, but the detection of *Pistacia* DNA from this resin may indicate that more endogenous DNA is present within, and adaptation of protocols and methods may be able to recover greater amounts of *Pistacia* DNA from this material. The detection of *Pistacia* DNA in the Dust Swabs complicates the picture, but as living *Pistacia* trees grow around the Institute of Nautical Archaeology, more reads are necessary to determine if the *Pistacia* DNA detected in the resin is a result of environmental contamination.

The percent of eukaryotic reads that match *Pistacia* DNA varies within duplicate runs of the same material. In Figure 56, the eukaryotic reads that match to organisms of interest detected within the resin samples are shown, with duplicate runs displayed for KW169 and KW144. In the first run of resin sample KW169 3.83% match to *Pistacia*, but in the second run only 1.37% match to *Pistacia*. As this material had been stored at room temperature for approximately two decades, it is unlikely that these values reflect degradation that occurred in the 10 days between extractions from the same material. What is more likely is that the amount of endogenous DNA varies considerably within archaeological material, even within the same sample.

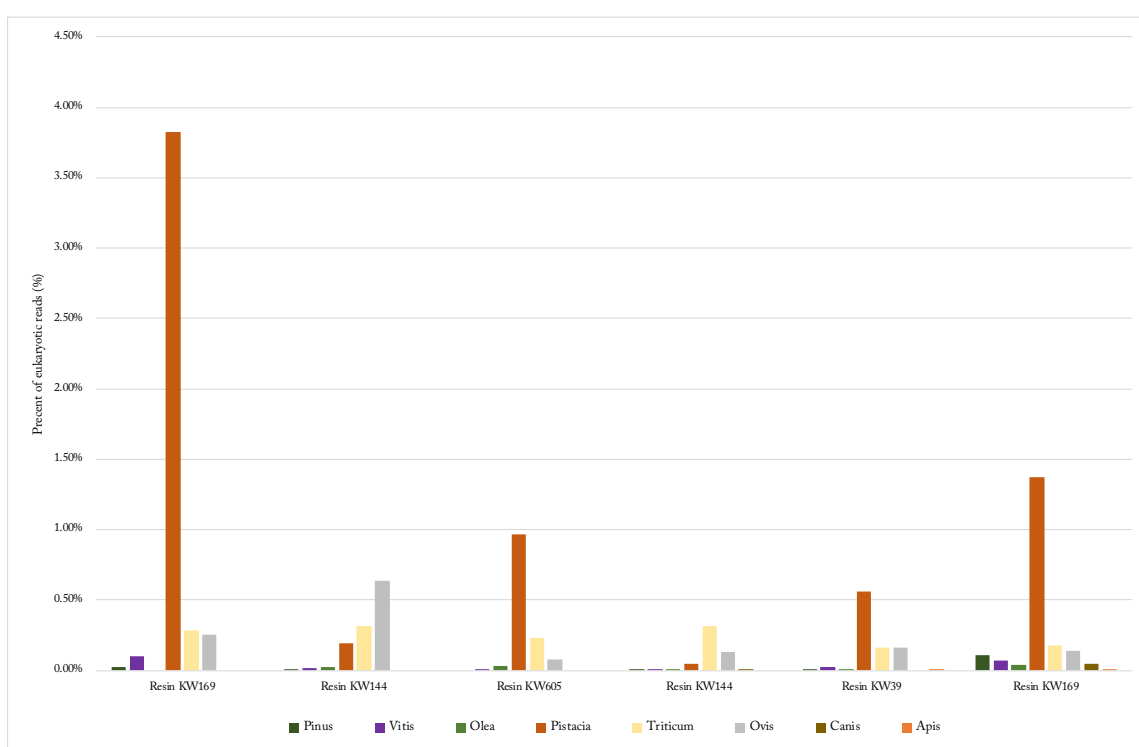


Figure 56. Clustered column chart showing percent of eukaryotic reads that match to organisms of interest in the resin samples from the Uluburun shipwreck.

Pine DNA was detected in relatively high amounts in some of the samples analysed, with 12.23% of the eukaryotic reads mapping to pine in the swab taken from the interior of Canaanite jar P2 from the Cape Gelidonya shipwreck (Figure 57). Amphorae on the Tektaş Burnu shipwreck were found filled to their brims with pine tar (Carlson, 2003), and conifer products have been detected by GC-MS in over 50% of the shipwreck sherds analysed in a tandem study (Briggs et al., in preparation), nevertheless it is necessary to consider whether

this *Pinus* DNA is ancient or modern in origin. Figure 57 shows the ten samples with the highest percent of eukaryotic reads that match to *Pinus*.

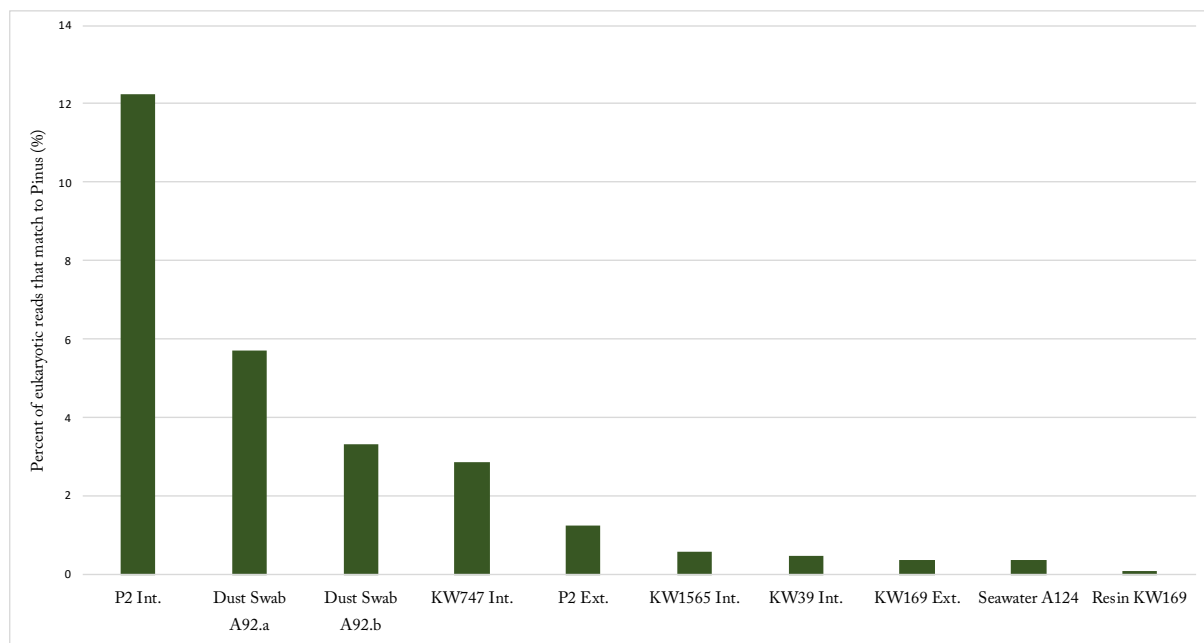


Figure 57. The ten samples with the highest percentage of eukaryotic reads that match to *Pinus*.

The first indication that the pine DNA detected in these samples is the result of environmental contamination is the fact that pine DNA has been found in high amounts in two of the four dust swabs analysed here, as well as in the seawater. In fact, as none of the shipwreck ceramics analysed by GC-MS that were found to contain conifer products were among the ten samples with the highest percent of eukaryotic reads assigned to pine, it appears that seawater is more likely to contain pine DNA than archaeological ceramics that still retain pine residues.

Results from the non-destructive swab of the interior of Canaanite jar P2 from the Cape Gelidonya shipwreck yielded 6,002/49,095 eukaryotic reads matched to *Pinus taeda* from the interior swab (S61). The swab of the exterior of the vessel (S62) produced 1,834/49,095 reads that matched to the same species. Native to North America, *Pinus taeda*, or Loblolly Pine, was

one of the first species of pine to have its complete genome sequenced, and at 22 billion base pairs is seven times the size of the human genome (Zimin et al., 2014). Species that are well represented in the NCBI database, such as *Pinus taeda*, are more likely to have false positive identifications aligned to their genomes, but it is equally likely that if DNA of a related species is recovered a match will be made to a closely related species of pine. *Pinus halepensis*, or Aleppo pine, is a species native to the Mediterranean, but this species has not been fully sequenced and thus does not appear in the NCBI for alignment with our samples.

Assessment of the damage patterns for the pine DNA detected was performed as above, but with alignment to the Loblolly Pine genome GCA_000404065.3_Ptaeda2.0_genomic.fna downloaded from the NCBI database. Figure 58 shows results for the interior swab of P2 (S61).

Here again we see minimal nucleotide misincorporation, suggesting that the pine DNA detected in Canaanite jar P2 from the Cape Gelidonya wreck is modern. Assessment of the fragment length shows a high number of fragments above 65 base pairs in length (Figure 59).

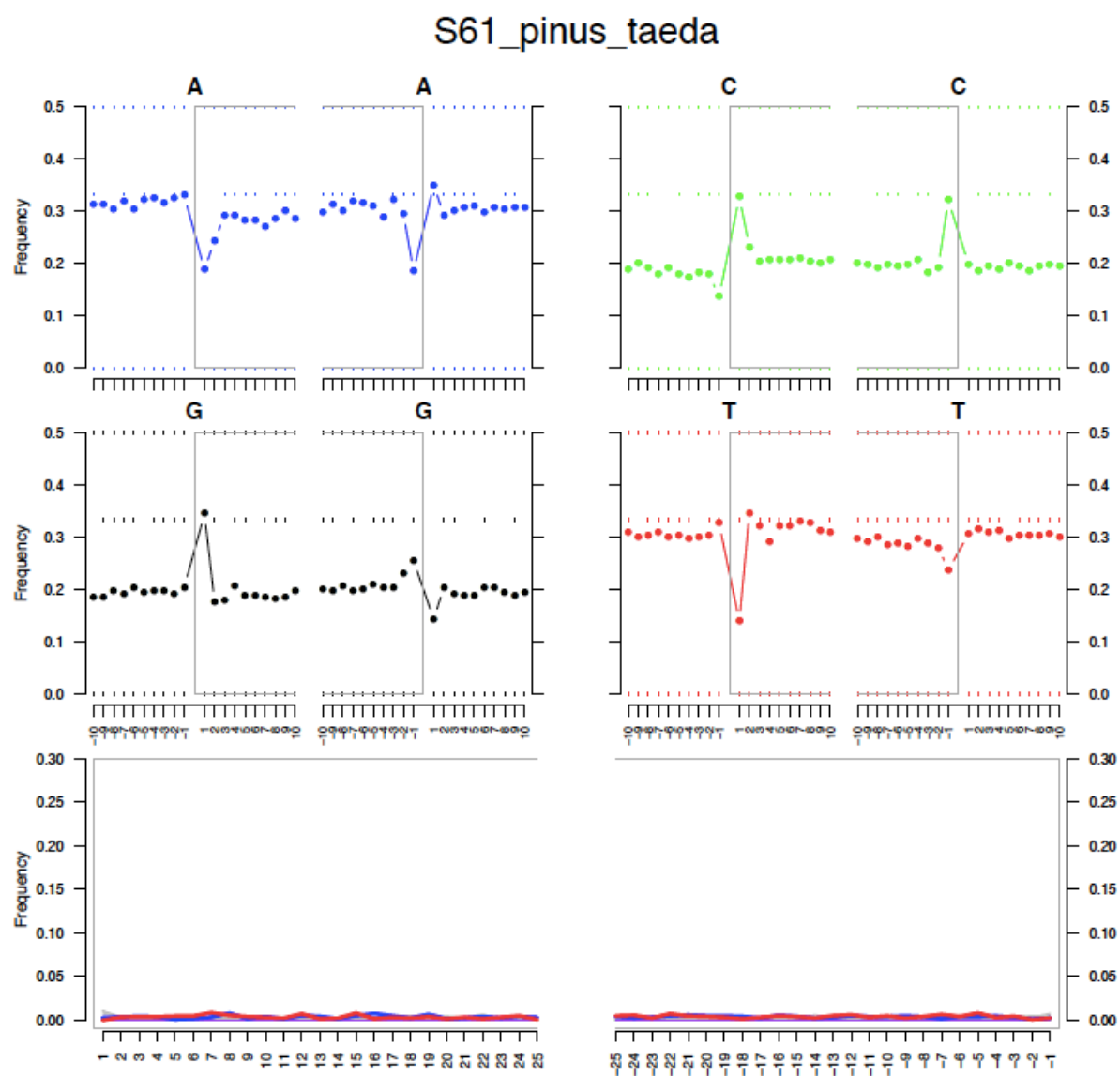


Figure 58. Results from mapDamage2.0 for the *Pinus* DNA detected in Canaanite jar P2, interior surface.

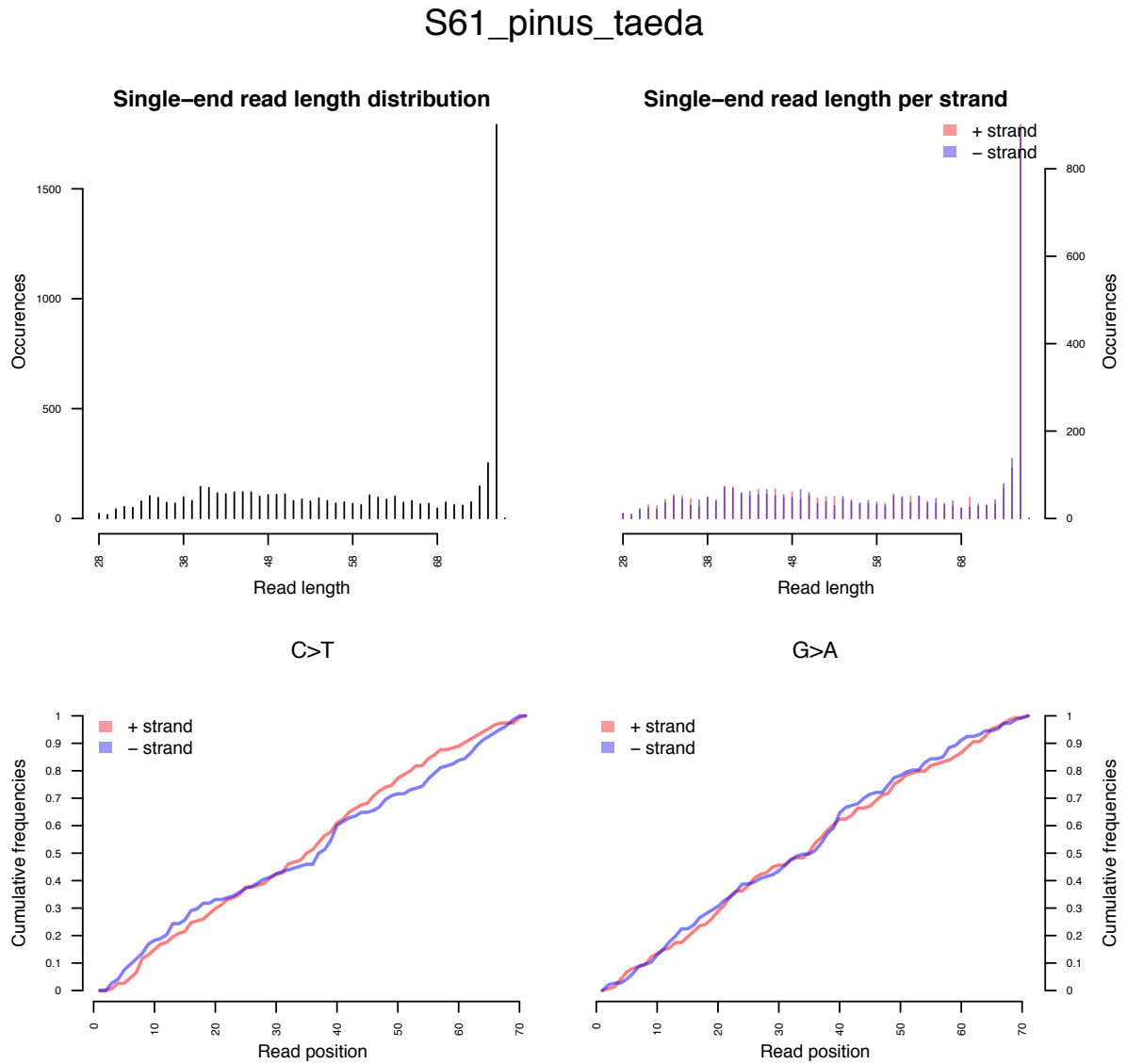


Figure 59. Fragment length of *Pinus* DNA detected in Canaanite jar P2, Cape Gelidonya shipwreck.

Analysis of the *Pinus* DNA detected in a swab of the exterior of the vessel indicates that this, as well, is modern pine DNA (Figure 60).

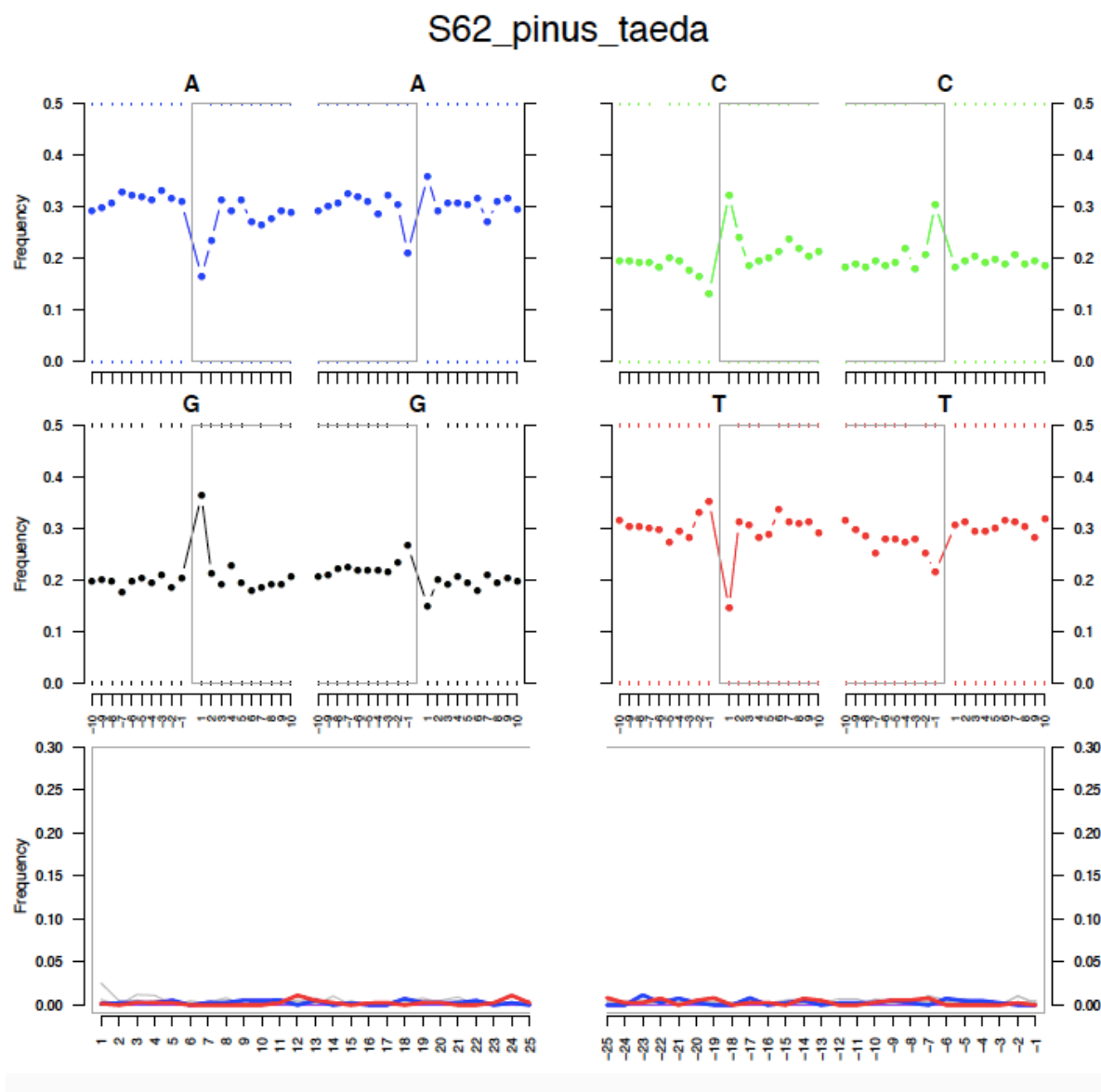


Figure 60. Results from mapDamage2.0 for the *Pinus* DNA detected in Canaanite jar P2, exterior of vessel.

Pilgrim flask KW747 yielded 2,264 reads that match to the *Pinus taeda* genome. As with the *Vitis* and *Olea* reads, the *Pinus* DNA detected in these samples does not demonstrate the nucleotide misincorporation patterns characteristic of ancient DNA (Figure 61). By analysing the average fragment length for the *Pinus* DNA detected in this sample, we again see a high number of long fragments (Figure 62). This is further reason to believe that this is modern pine DNA and does not derive from ancient pine DNA trapped inside the vessel.

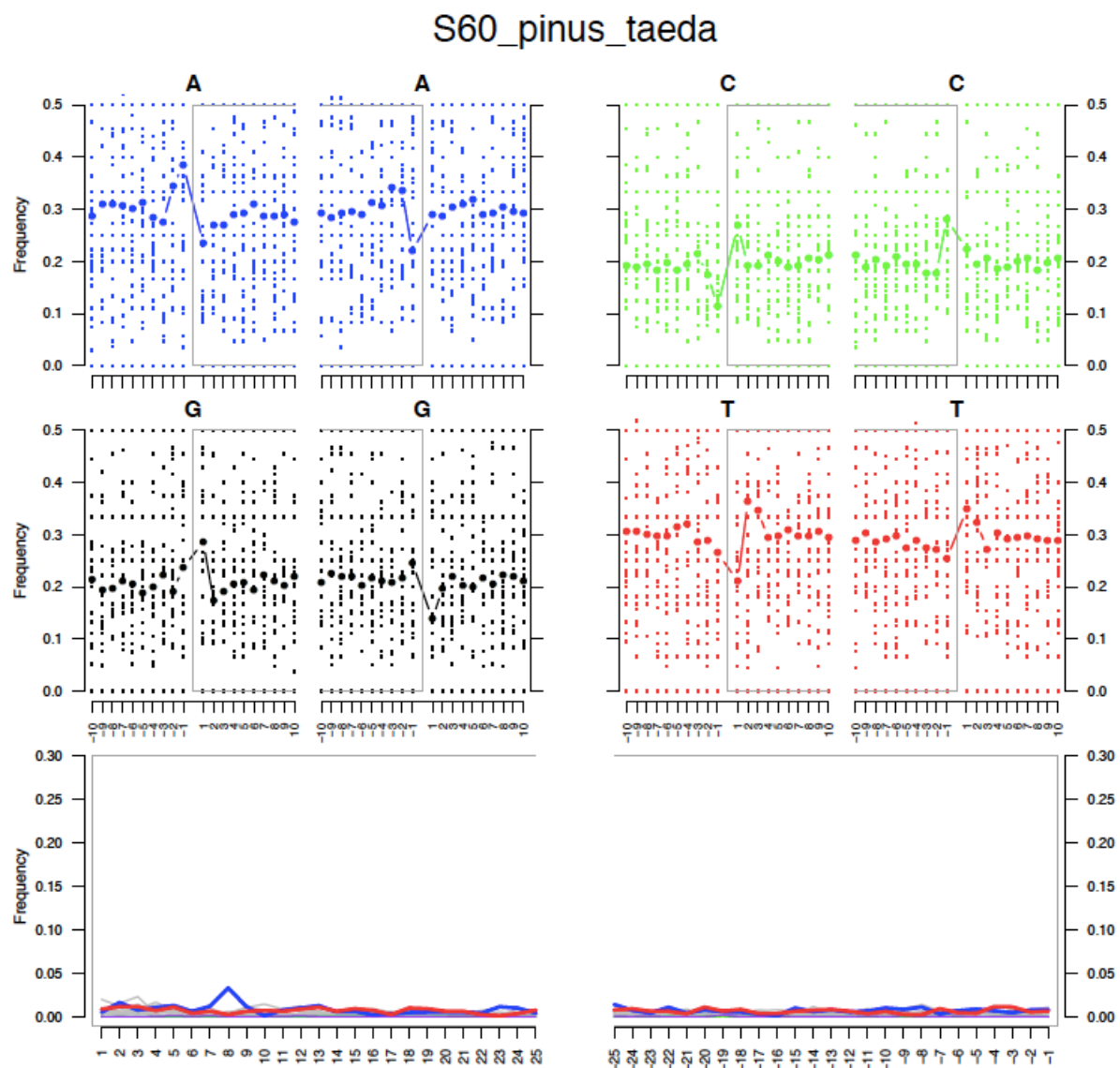


Figure 61. MapDamage2.0 results for the Pinus DNA detected in pilgrim flask 747.

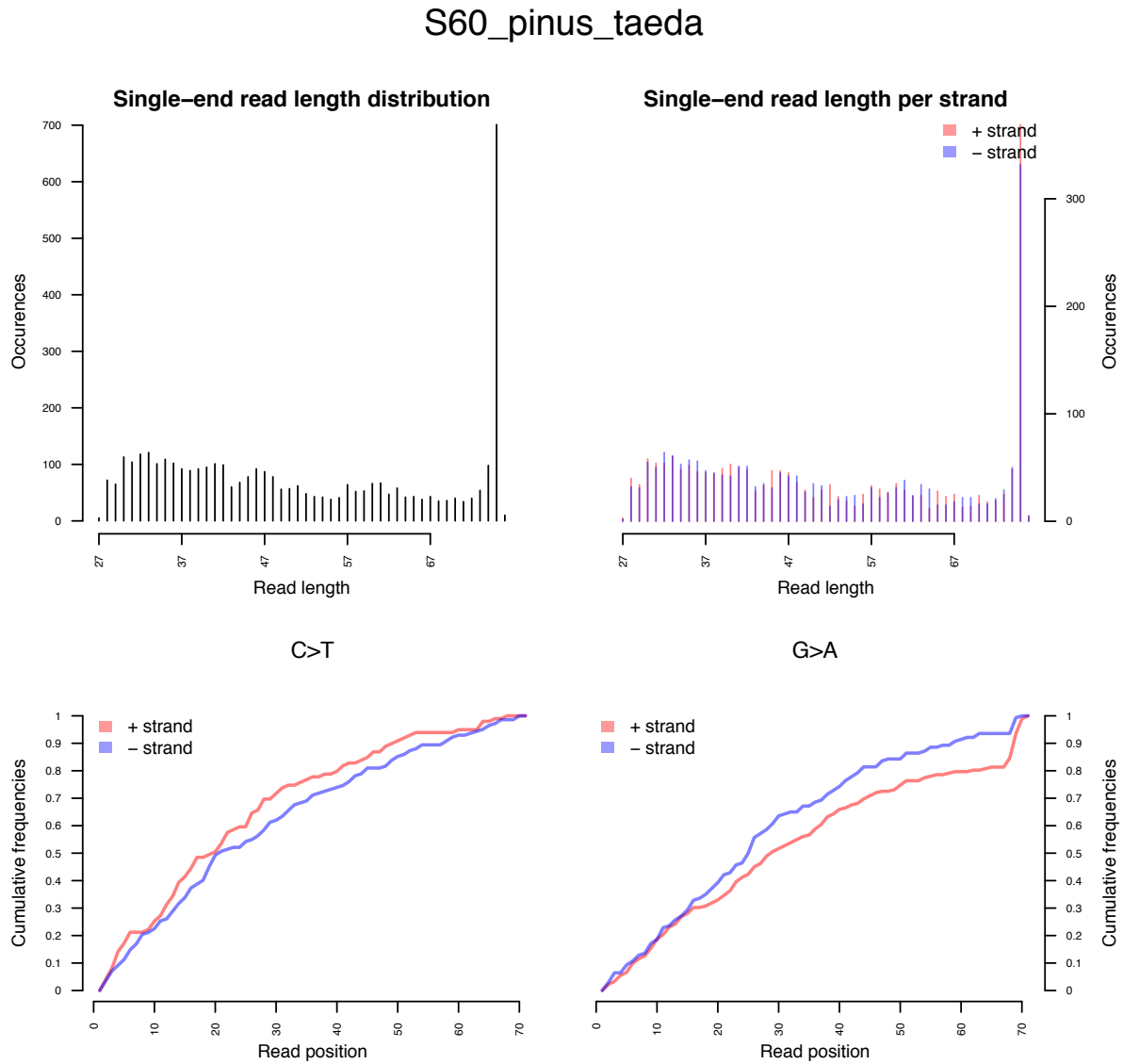


Figure 62. Fragment lengths for *Pinus* DNA in Pilgrim Flask KW 747.

As with the Uluburun storage space samples discussed above, the surfaces on which the Cape Gelidonya artefacts are stored have been swabbed and included in order to establish a baseline of what DNA may be present on these surfaces. Swabs A92.a (S20) and A92.b (S21) were taken from a table surface on which Cape Gelidonya artefacts are stored. This table surface is proximal to a large set of French doors that open on to a terrace at the Institute of Nautical Archaeology building, Bodrum, Turkey. This could account for the prevalence of terrestrial plant DNA on both the storage spaces and the artefacts stored within them.

The olive DNA detected in Dust Swab A92.a (S20), has already been assessed. The pine DNA detected in this sample was analysed by MapDamage2.0 and the results are shown in Figure 63.

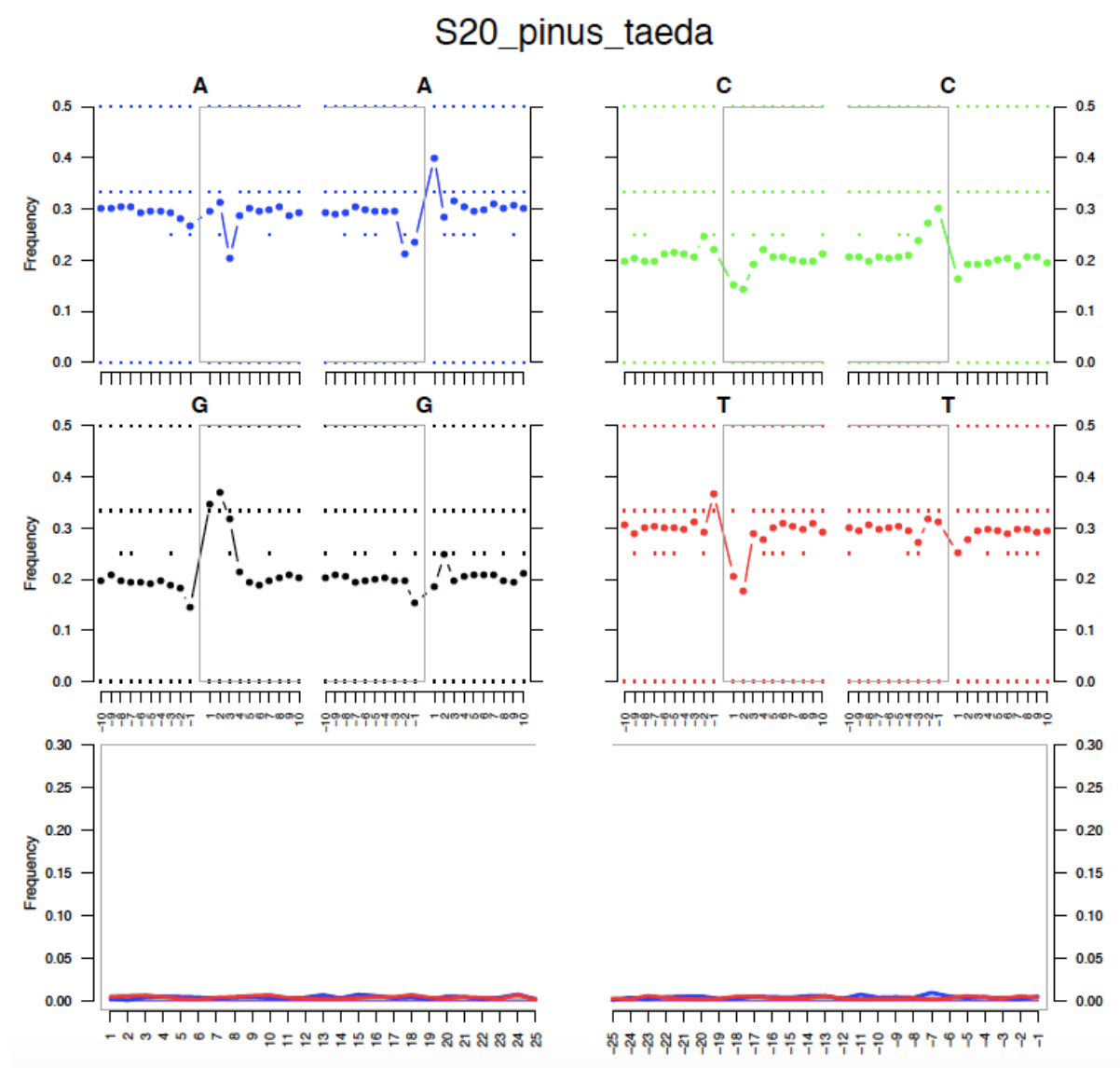


Figure 63. MapDamage2.0 results for Dust Swab A92.a (S20) taken from the Cape Gelidonya artefact storage area.

As with the olive DNA also detected in this sample, the pine DNA does not exhibit any cytoside deamination that is characteristic of ancient DNA degradation patterns. Figure 64 shows the fragment lengths found for the *Pinus* DNA detected, and as with the other examples of pine DNA analysed, in most occurrences the fragment length is over 67 base pairs.

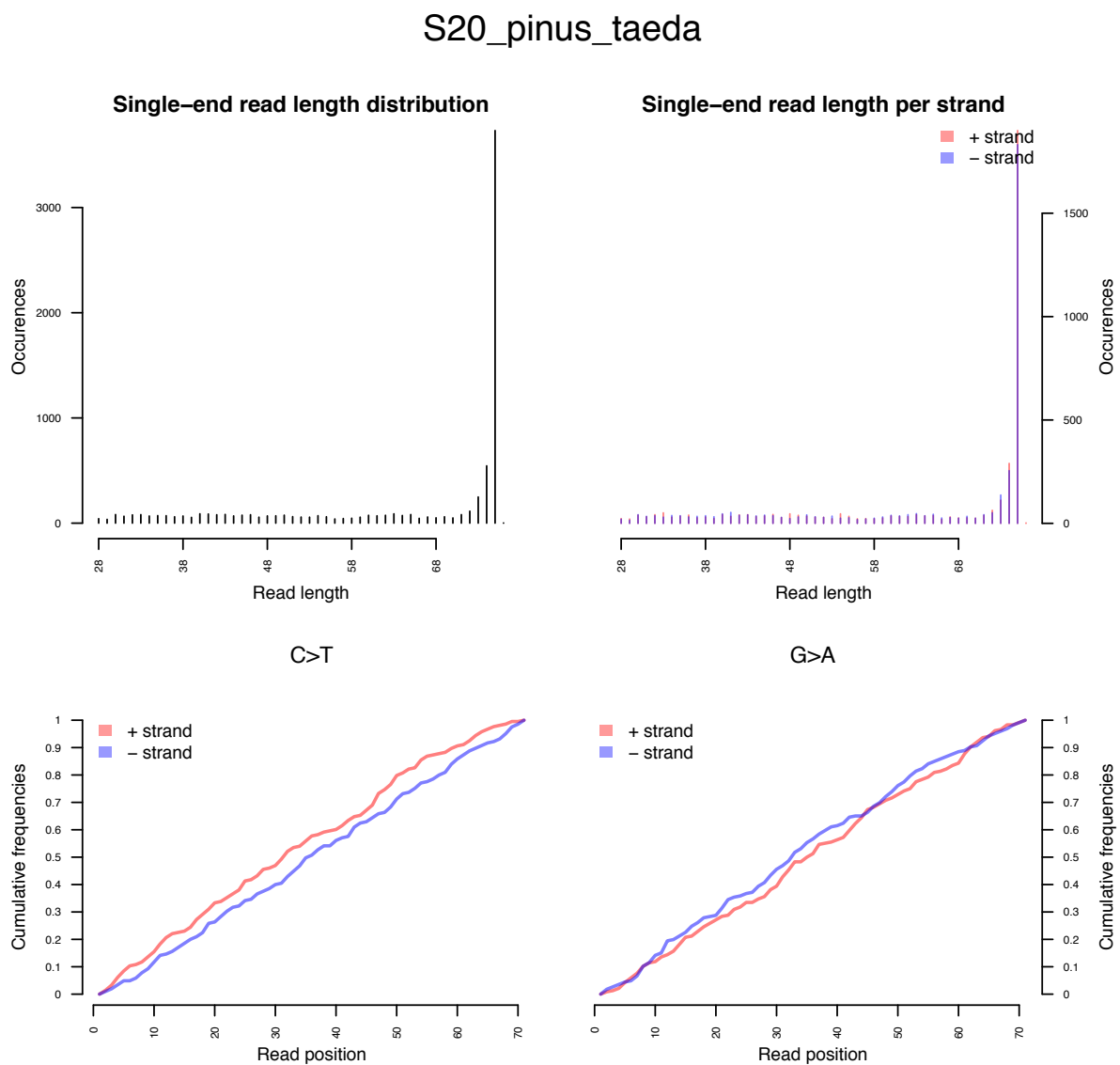


Figure 64. Fragment length for pine DNA detected in Dust Swab A92.a (S20).

Pine DNA was also detected in Dust Swab A92.b (S21) taken from the Cape Gelidonya artefacts storage area. Analysis by mapDamage2.0 was undertaken for this sample as well and the results are shown in Figure 65.

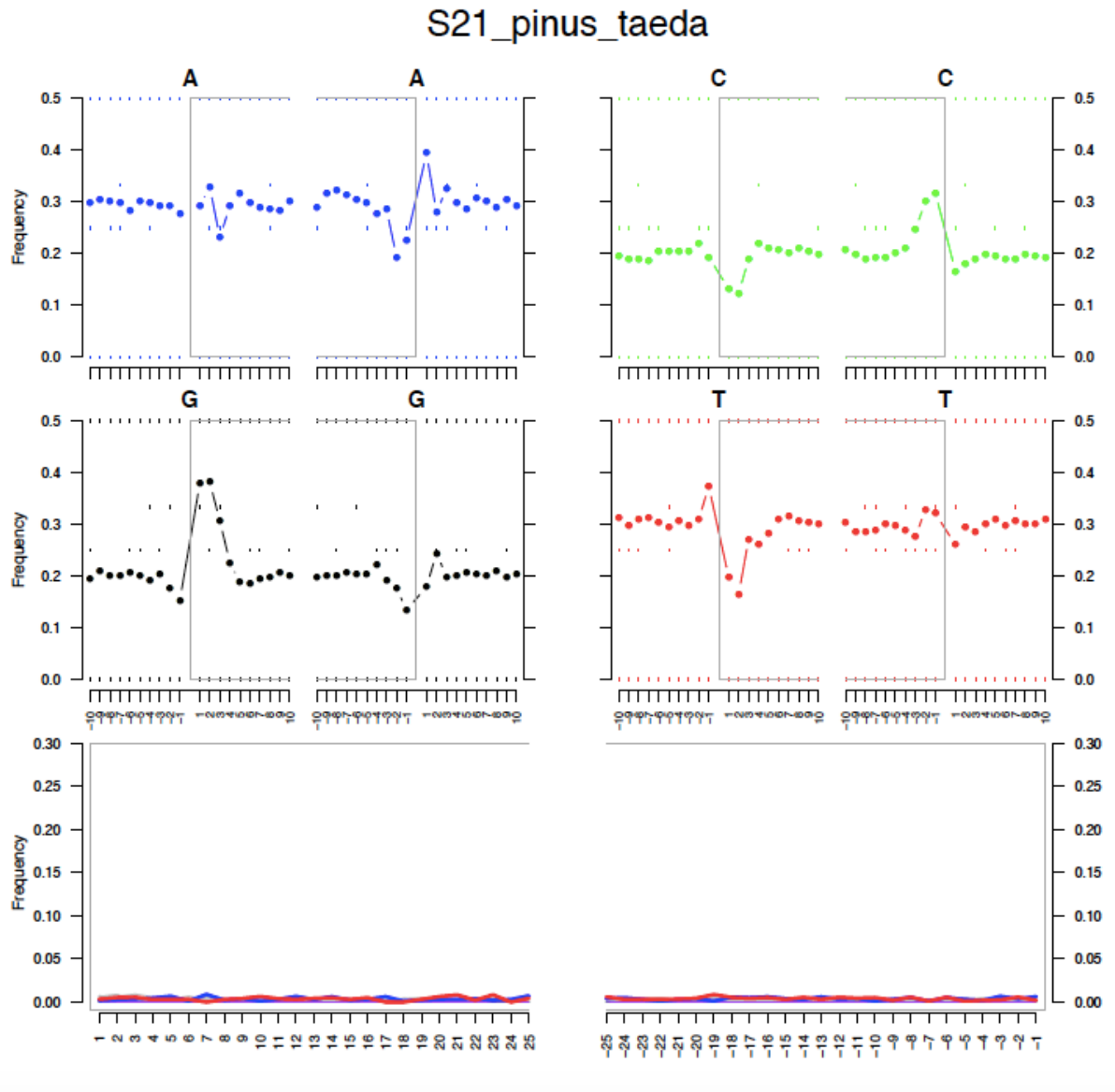


Figure 65. Results from mapDamage2.0 for Dust Swab A92.b (S21) collected from Cape Gelidonya artefact storage area.

Both the lack of cytosine deamination patterns, and the fragment length found for this sample (Figure 66), support a modern origin for this pine DNA. The results of this analysis show that the pine DNA detected in both archaeological samples and samples taken from the storage spaces in which these artefacts are housed, are modern in origin.

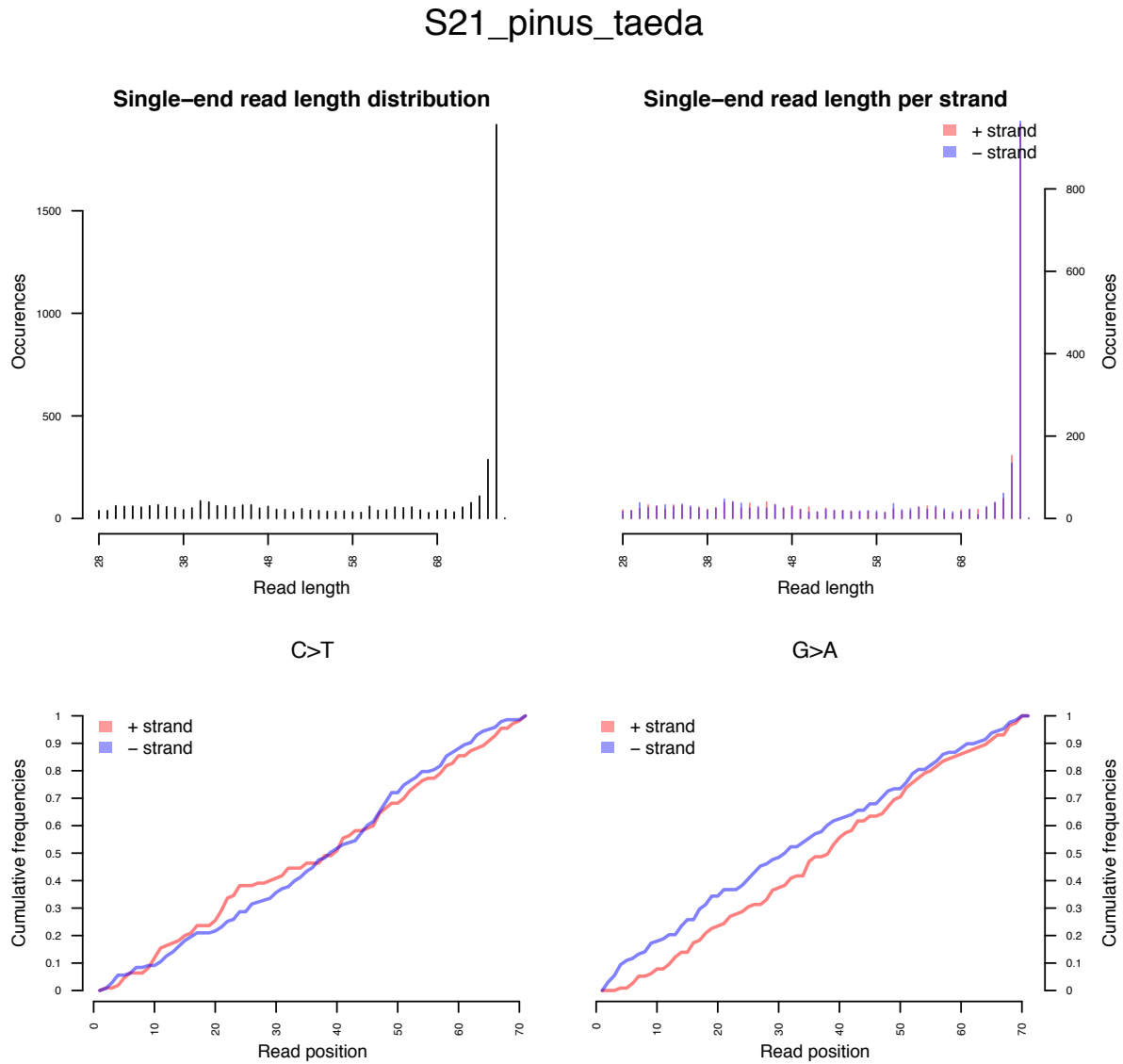


Figure 66. Results from mapDamage2.01 showing fragment length found for Dust Swab A92.b (S21).

To conclude, results indicate that DNA from terrestrial plants commonly consumed, used, and traded in the ancient world including grapevine, olive, pine and Pistacia have been detected in shipwreck samples, samples of seafloor sediment, samples of seawater, and in dust swabs taken from shipwreck artefact storage areas. Analysis of the frequency and position of cytosine deamination in the DNA sequences isolated indicates a modern origin for the DNA from organisms of interest detected here.

4.3.3 Detection of common domesticated organisms

In addition to the organisms of interest known to have been transported in shipwreck amphorae, it is useful to assess the levels of DNA detected from a group of randomly selected domesticated plants and animals.

Figure 67 shows that DNA from domesticated dogs (*Canis*) has been detected in the dust swabs from artefact storage areas, artefacts, and samples of seawater. It is worth noting that far higher levels of *Canis* DNA were detected in KW1553, a Canaanite jar found to contain pomegranate seeds, than DNA derived from the known contents of the vessel.

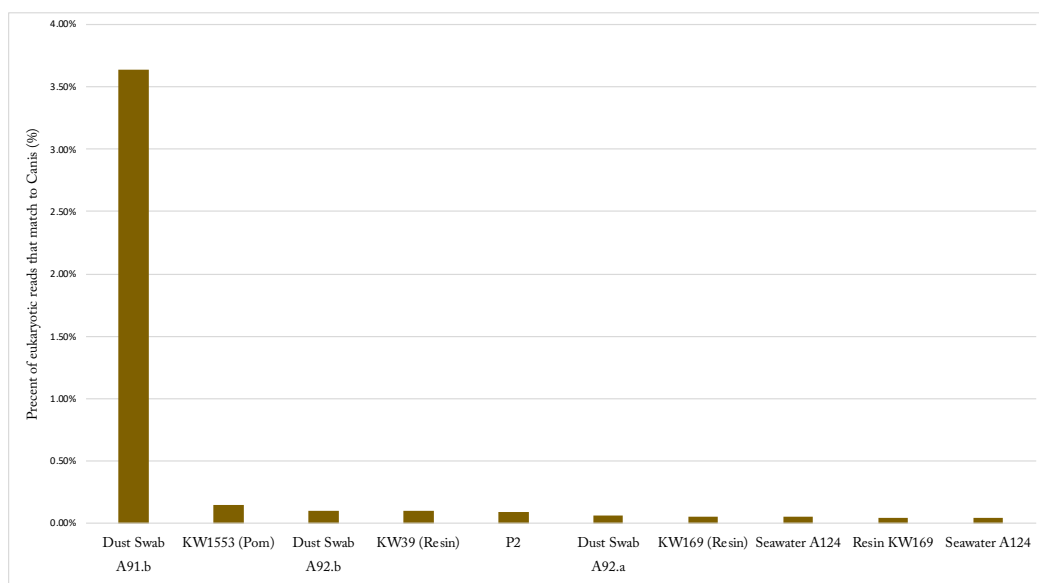


Figure 67. The ten samples with the highest percent of eukaryotic reads that match to *Canis*.

Over 3.5% of the eukaryotic reads assigned in Dust Swab A91.b matched to *Canis*. This sample was taken from a windowless storage area holding Uluburun shipwreck artefacts. By contrast, Dust Swab A92.b was taken from the storage area where the Cape Gelidonya shipwreck artefacts are stored, directly in front of a large outdoor terrace, and only 0.1% of eukaryotic reads match to *Canis*. This demonstrates the variability of potential contaminant present in artefact storage areas.

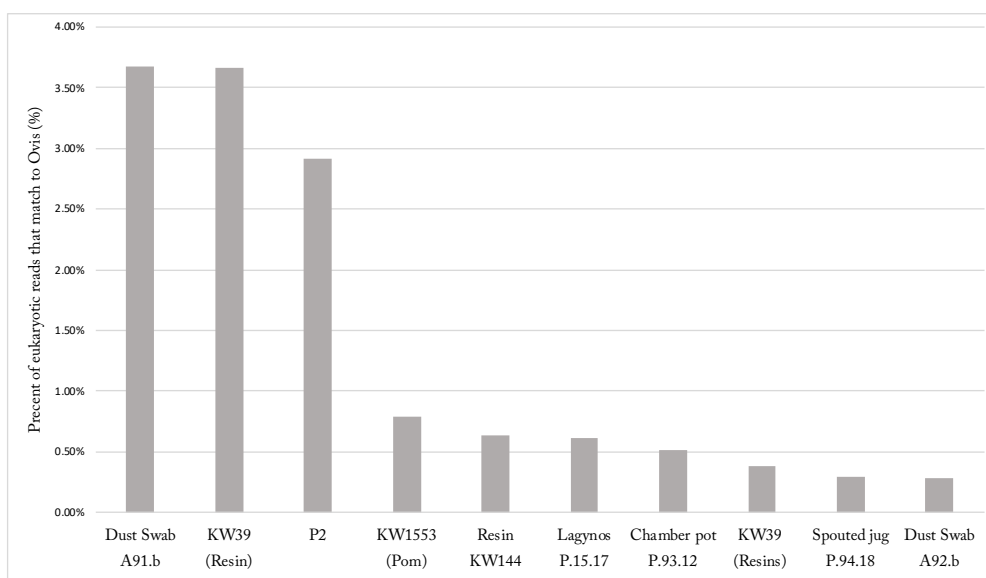


Figure 68. Ten samples with the highest percent of eukaryotic reads that match to *Ovis*. Vessels of known contents are shown: Artefact (Known contents). Pom = pomegranate.

Ovis, or sheep, DNA has been detected in two dust swabs from the artefact storage areas, three Canaanite jars, a resin sample, and three of the ceramic vessels included from the terrestrial site of Yeronisos (Figure 68). As sheep are utilised both in present times and in the ancient Mediterranean, the detection of sheep DNA in archaeological samples bears further research. However, its prevalence in dust swab samples indicates that sheep DNA is present in the general environment, therefore the authentication of detected DNA is necessary.

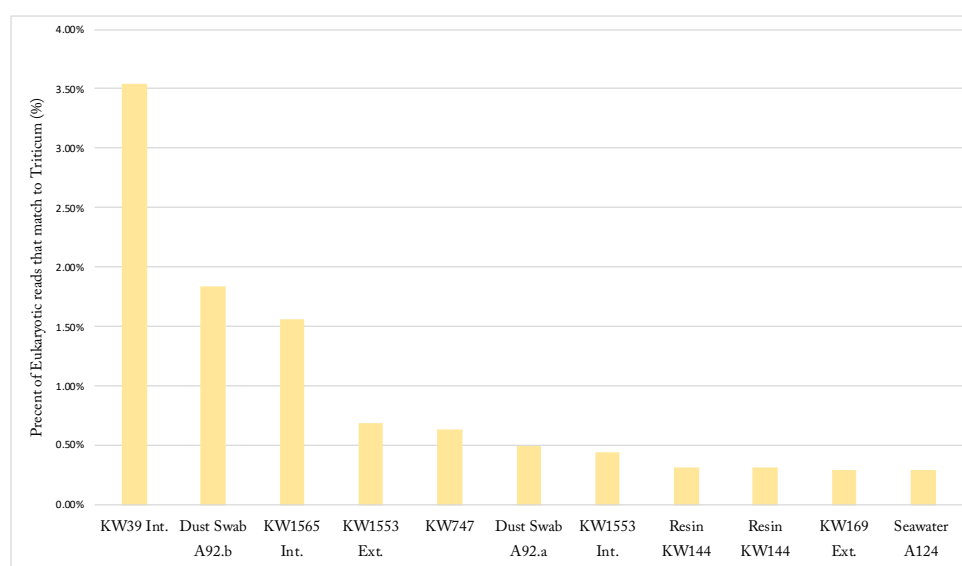


Figure 69. Ten samples with the highest percent of eukaryotic reads that match to *Triticum*.

Wheat (*Triticum*) has great agricultural value in modern (and ancient) times, and accordingly a great deal of DNA research has focussed on sequencing the wheat genome (Appels et al., 2018). As such, wheat may be preferentially represented in gene and whole genome databases. The detection of wheat DNA in the samples analysed here could either be a result of the presence of modern wheat in the environment, or the tendency for small DNA fragments to align to a species so well-represented in genomic databases. Figure 69 shows that wheat has been detected in both archaeological and environmental samples. Comparing amounts of wheat DNA found on the interior and exterior of previously excavated shipwreck amphorae may be informative, in the case of Canaanite jar KW1553 more wheat DNA was detected from the swab of the exterior than the swab of the interior. This informs us that there is a recognisable level of contamination on the exterior of this vessel. This is also true for the discovery of higher levels of *Pinus* DNA on exterior swabs of Canaanite jars than on interior swabs: if high levels are found on the exterior it makes it highly unlikely that this DNA derives from past vessel contents which would be expected to be found in higher proportions on the interior, rather than the exterior of vessels.

Shotgun metagenomics will inevitably amplify DNA from the environment, so the investigation of the source of this DNA is important to understanding what archaeological meaning this data provides for understanding amphorae contents in the past. Modern contaminant DNA is unavoidable, but how it is treated and appraised defines the integrity of metagenomics research on archaeological material.

4.4 Discussion

4.4.1 Detection of known contents and organisms of interest

Five ceramic transport containers of known contents recovered from ancient shipwreck sites were analysed by shotgun metagenomic analysis. One was analysed destructively, and three were analysed by a non-destructive swabbing method proposed by Foley et al. (2012). The contents included whole olives in two of the vessels (426 and KW 1565), *Pistacia* resin in two of the vessels (KW 39 and KW 169), and a mixed matrix of pomegranate seeds, bits of wood, and a small number of olive stones in the final vessel (KW 1553). No DNA from the species of known contents was detected in any meaningful amounts in these samples by the MALT and MEGAN6 analysis conducted here.

The *Pistacia* resin from several of the Uluburun vessels was analysed to investigate the potential for recovering endogenous DNA from this material. The number of reads assigned to *Pistacia* DNA was too low to investigate further. While the CTAB extraction method used here has been shown to be more effective in the recovery of olive and grapevine DNA than other commercial kits available (Kalogianni et al., 2015, p. 3123; Raieta et al., 2015, p. 598), it seems likely that due to the relatively insoluble nature of the resin this material was not sufficiently lysed during the one week incubation in CTAB, and a pre-treatment designed specifically for resinous material may be more suitable than the methods used here. Further investigation into DNA extraction methods for resinous material is necessary.

Vessels of assumed contents analysed included wine amphorae from the Mazotos and Kyrenia shipwrecks, olive oil amphorae from the Babuljaš shipwreck, and wine pitchers from Yeronisos. No DNA from the species of assumed contents was detected in any meaningful amounts in these samples by the MALT and MEGAN6 analysis conducted here.

Pilgrim Flask 747 from the Uluburun shipwreck yielded a sufficient number of *Vitis vinifera* reads to be analysed further. Alignment to the reference genome and analysis by mapDamage2.0 showed that the grapevine DNA detected did not display the damage patterns characteristic of ancient DNA. The non-destructive swab taken from this pilgrim flask also

yielded reads that aligned to a species of *Pinus*, in double the number that aligned to *Vitis vinifera*.

Wine amphorae were often lined with an interior coating of conifer product, and pine pitch or pine tar were also a vessel content in their own right, as evidence from the Tektas Burnu shipwreck demonstrates (Carlson, 2003). A species of *Pinus* was detected in several of the samples in numbers of reads large enough to warrant further investigation.

Detected in Pilgrim Flask 747 from the Uluburun shipwreck, and Canaanite jar P2 from the Cape Gelidonya shipwreck, the *Pinus* DNA analysed did not show the nucleotide misincorporation patterns characteristic to ancient DNA. Similar amounts of *Pinus* DNA were detected in the dust swabs from the surfaces on which these artefacts are stored, and analysis showed this was also modern.

Environmental samples from these sites almost invariably yielded a higher number of reads for species of known vessel contents and species of assumed vessel contents. Samples of seafloor sediment, seawater, and shelving unit dust contained DNA reads that match to species of interest. Similar numbers were not detected in the laboratory blanks. Species of olive and pine were detected in their greatest number of reads from the samples of shelving unit dust, indicating the level to which modern plant DNA can be present in museum storage conditions. The only exception is the detection of *Vitis vinifera* DNA in pilgrim flask 747, which produced the highest number of reads to match to the grapevine genome of any sample analysed here, archaeological or environmental. After investigation, this grapevine DNA appears to be modern.

4.4.2 DNA in vessels contents

In order to evaluate the preservation of DNA in artefacts and environmental samples, it is necessary to establish what levels of DNA one would expect to find in the assumed vessel contents. Studies have gauged the amounts of *Vitis vinifera* DNA (ng/ml) recovered from grape leaves, must, and wine (Işçi et al., 2014, p. 242), the amount of DNA recovered throughout the wine fermentation process (Siret et al., 2002), and from bottled wine between two and six years old (Pereira et al., 2011). The young leaves of the grapevine remain the preferred material as they yield the greatest amount of endogenous DNA (Lodhi et al., 1994), but significant amounts can also be recovered from grape must, with wine yielding less endogenous DNA than must (Işçi et al., 2012, p. 242). An earlier study showed that the decrease in recoverable *Vitis vinifera* DNA was notable throughout the fermentation process, with the disappearance of alleles necessary to determine the grape varietal of one sample as early as four days into fermentation (Siret et al., 2002, p. 244), yet results differ depending on the extraction protocols used, as contrary reports suggest that *Vitis vinifera* DNA can be recovered and amplified even from bottled wine up to six years old (Pereira, 2011, p. 550).

Of particular relevance to the present study are results that show the proportion of grapevine DNA decreases over the fermentation process, with the DNA present in fermented and bottled wine being primarily composed of DNA derived from yeast and bacteria (Drábek et al., 2008, p. 494). This study also analysed the sediment that collects at the bottom of a wine bottle, and found that from the termination of fermentation, the maximum amount of DNA, be it yeast, bacterial, or grapevine DNA, was recovered from the sediment, rather than the liquid wine (Drábek et al., 2008, p. 494). If sediment at the bottom of a wine bottle, or amphora, is more likely to retain DNA, this could have implications for future sampling strategies of archaeological material. In addition, yeast DNA should be sought in future studies, as this may help identify the past vessel contents where grapevine DNA has not survived.

Several of the same issues present with grapevine DNA are also a concern in the recovery of *Olea europaea* DNA from olive oil; the amount of endogenous DNA recovered from olive oil is often low, the quality poor, and degraded by nucleases (Raieta et al., 2015, p. 598). Extraction protocols that are based on the CTAB-hexane-chloroform method appear to have more success

in recovering both olive DNA from olive oil (Raieta et al., 2015, p. 598; Kalogianni et al., 2015, p. 3123) and grapevine DNA from wine, when compared to other protocols and commercially available plant DNA extraction kits (Pereira et al., 2011, p. 549). Based on this research that has shown the difficulties in recovering endogenous DNA from modern wine and modern olive oil, the recovery of endogenous grapevine, or olive, DNA from the ceramic matrix of ancient shipwreck amphorae seems unlikely. The source of any recovered and amplified DNA is likely derived from the deposition environment, but it is necessary to test both in order to further elucidate the source of the DNA recovered that relates to species of interest for our known or assumed amphorae contents.

The resin analysed here was extracted with the CTAB method, but did not yield significant amounts of endogenous DNA, further illustrating the complexity involved in recovering ancient DNA from plant exudates. The type and extent of processing a plant exudate is subjected to will also affect the degradation of endogenous DNA in this material. Tree resins like the *Pistacia* and *Pinus* products utilised in antiquity could be heated to produce tar. Both pine pitch and tar were detected through GC-MS analysis of an amphora lining and planking element of the hull of the Kyrenia shipwreck, indicating that one of the conifer products detected had been heating in the presence of wood (Beck and Borromeo, 1990). A prolonged heating process during production in antiquity could damage endogenous DNA, making the detection of endogenous DNA in heated resinous material less likely after the intervening millennia.

4.4.3 DNA results compared to results from organic residue analysis

A number of ceramic transport containers, resin samples, and environmental samples analysed here by shotgun metagenomics were also subjected to organic residue analysis (Briggs et al. in preparation). Table 13 compares results from the organic residue analysis to the detection of associated species in the DNA results. Contents detected through organic residue analysis by

solvent extraction and alkaline fusion include degraded beeswax, plant oil, *Pistacia*, and conifer exudate (both pitch and tar), and numerous small acids thought to be biomarkers of wine or fruit products (syngic acid, succinic acid, vanillin). The MALT and MEGAN6 output have been searched for species associated with the vessel contents detected through organic residue analysis including honeybees (*Apis mellifera*), olive, *Pistacia*, and species of pine.

The species associated with the molecules identified by organic residue analysis are not similarly detected by the shotgun metagenomics methods used here. As shown in Table 14, in many cases, a greater number of reads matched to species in a randomly selected group of domesticated species, than to the species associated with the identified contents.

The results from the organic residue analysis are compared to results from the MALT and MEGAN6 analysis for the resin samples from the Uluburun shipwreck, and the environmental samples of seafloor sediment and soil are shown in Table 15.

Table 13. Results from organic residue analysis compared to results from shotgun metagenomics.

Artefact / DNA library	Assumed contents	ORA results	<i>Vitis</i> <i>vinifera</i>	<i>Olea</i> <i>sylvestris</i>	<i>Pistacia</i> <i>vera</i>	<i>Pinus</i>	<i>Apis</i> <i>mellifera</i>	Single reads total
Mazotos shipwreck								
<i>Chian amphora</i>								
p0824 / S33	wine	conifer	6	1	0	0	3	5,897,537
p0784 / S34	wine	conifer	6	0	0	7	3	3,316,856
426 / S35	wine	-	1	1	0	5	0	48,483
Kyrenia shipwreck								
<i>Rhodian amphora</i>								
069 / S31	wine	conifer	-	-	-	-	-	-
<i>Samian amphora</i>								
A97 / S23	wine	plant oil	-	-	-	-	-	-
Babuljaš shipwreck								
<i>Africana III/Keay 25 amphora</i>								
A114 / S25	olive oil	plant oil	0	0	0	0	0	57,792
A115 / S26	olive oil	-	0	0	0	0	0	30,514
<i>Bronze Age cooking pot</i>								
A118 / S29	-	fatty product, conifer	1	0	0	1	0	95,086
Yeronisos island								
<i>Lagynos</i>								
P.15.17 / S1	wine	beeswax	2	0	0	0	0	1,377,437
P.05.03 / S2	wine	conifer	0	0	0	0	0	777,718
<i>Spouted jug</i>								
P.05.07 / S3	-	fatty product	9	0	4	1	1	1,517,540
P.94.18 / S4	-	fatty product	2	0	1	1	0	907,161
<i>Chamber pot</i>								
P.93.12 / S23	coprolites	contamination - cosmetics	1	1	0	2	0	71,776

Table 14. Comparison between organic residue analysis results and number of reads assigned to a group of domesticated species.

Artefact / DNA library	Assumed contents	ORA results	<i>Triticum aestivum</i>	<i>Sus scrofa</i>	<i>Ovis</i>	<i>Felis catus</i>	<i>Canis familiaris</i>	<i>Homo sapiens</i>
Mazotos shipwreck								
<i>Chian amphora</i>								
p0824 / S33	wine	conifer	19	10	13	8	3	4,248
p0784 / S34	wine	conifer	322	362	100	0	1	159,856
426 / S35	wine	-	21	22	6	0	0	14,454
Kyrenia shipwreck								
<i>Rhodian amphora</i>								
069 / S31	wine	conifer	-	-	-	-	-	-
<i>Samian amphora</i>								
A97 / S23	wine	plant oil	-	-	-	-	-	-
Babuljaš shipwreck								
<i>Africana III/Keay 25 amphora</i>								
A114 / S25	olive oil	plant oil	8	20	5	0	3	17,273
A115 / S26	olive oil	-	3	2	2	0	0	1,176
<i>Bronze Age cooking pot</i>								
A118 / S29	-	fatty product, conifer	13	28	5	0	0	18,767
Yeronisos island								
<i>Lagynos</i>								
P.15.17 / S1	wine	beeswax	2	4	5	0	0	235
P.05.03 / S2	wine	conifer	8	23	10	0	1	12,876
<i>Spouted jug</i>								
P.05.07 / S3	-	fatty product	11	8	15	2	2	14,381
P.94.18 / S4	-	fatty product	3	7	6	0	0	924
<i>Chamber pot</i>								
P.93.12 / S23	coprolites	contamination - cosmetics	17	17	143	0	0	13,085

Table 15. Comparison between organic residue analysis results and number of reads assigned to associated species.

Artefact / DNA library	ORA results	<i>Vitis vinifera</i>	<i>Olea sylvestris</i>	<i>Pistacia vera</i>	<i>Pinus taeda</i>	<i>Apis mellifera</i>	Single reads total
<i>Uluburun resin</i>							
<i>Resin</i>							
KW169							
/S5	pistacia	7	0	271	2	0	1,177,949
KW 144							
/S6	pistacia	2	3	27	1	0	1,417,531
KW 605							
/S7	pistacia	2	10	316	0	1	655,065
KW 39							
/S51	pistacia	4	2	88	1	1	1,143,034
<i>Babuljas seafloor sediment</i>							
A116	syringic acid, vanillic acid,						
/S27	plant sterols	3	0	0	0	0	1,363,328
A117	syringic acid, vanillic acid,						
/S43	plant sterols	7	12	0	14	28	3,315,077
<i>Mazotos seafloor sediment</i>							
A120	syringic acid, succinic acid,						
/S36	lactic acid	39	3	2	3	0	2,167,515
A121	fatty acids,						
/S37	plant sterols	42	4	0	21	0	3,781,334
<i>Uluburun sediment</i>							
	<i>Pistacia</i> triterpenes, vanillin,						
L3561	syringic acid,						
/S52	vanillic acid	3	0	0	6	0	792,385
<i>Yeronisos soil</i>							
P.93.12	Plant sterols, fatty acids,						
/S24	alcohols	-	-	-	-	-	-

Numerous small acids frequently associated with fruit and wine ‘biomarkers’ in archaeological ceramics were detected in the samples of seafloor sediment from the Mazotos, Babuljaš and Uluburun shipwreck (Briggs et al., in preparation) The seafloor sediment collected from the toe of Canaanite jar KW 1553 contained triterpenes characteristic of *Pistacia* resin, the content of the majority of the other Canaanite jars in the cargo of the ship. This suggests that as the

resin degraded triterpenes coalesced with accumulating sediment inside the jar and were preserved amongst the mixed matrix of sand, shell and decaying organics. While residues from *Pistacia* resin were identified through organic residue analysis, *Pistacia* DNA was not detected in this seafloor sediment.

4.4.4 Organisms of interest in environmental samples

Terrestrial plant species detected in seawater are likely to be locally deposited and modern. A study that gauged the detection of recorded fish species in Danish waters through environmental DNA analysis also included a controlled aquarium study that showed fish DNA quickly degraded beyond detectability in between 0.9 and 6.7 days (Thomsen and Willerslev, 2015, p. 2). Depending on currents, salinity, temperature, and wind direction, deposited DNA may travel considerable distances in that time, but will likely be consistently degrading as it does. Combined with the fact this environmental fish DNA will be continuously diluted as it drifts further suggest any DNA detected is locally deposited (Thomsen and Willerslev, 2015, p. 4).

While aimed at detecting modern fish species, that study may have relevance for the instances in which terrestrial plant DNA has been detected in the seawater samples analysed here. It seems increasingly likely that the DNA from grapevine, species of pine, and domesticated animals in the seawater samples collected from the shipwreck sites analysed here represents DNA that has entered the water column recently and had not yet degraded beyond detection. To what extent this seawater, containing recently deposited DNA, circulates around the artefacts on ancient shipwrecks, potentially permeating any porous material, remains unclear.

Seawater and seafloor sediment samples collected from two shipwreck sites analysed in this research have yielded DNA derived from grapevine, olive, wheat, pine, sheep, dog, and bees (*Apis*). Establishing this has been fundamental to this research, as the mere detection of DNA derived from common amphorae contents, including grape and olive, on ancient shipwreck

sites cannot be reliably assigned as having an origin in archaeological material. Similarly, seafloor sediment collected from both inside and outside of the ancient shipwreck site contain a collection of terrestrial plant DNA that could potentially skew results of studies conducted on ancient shipwreck sites.

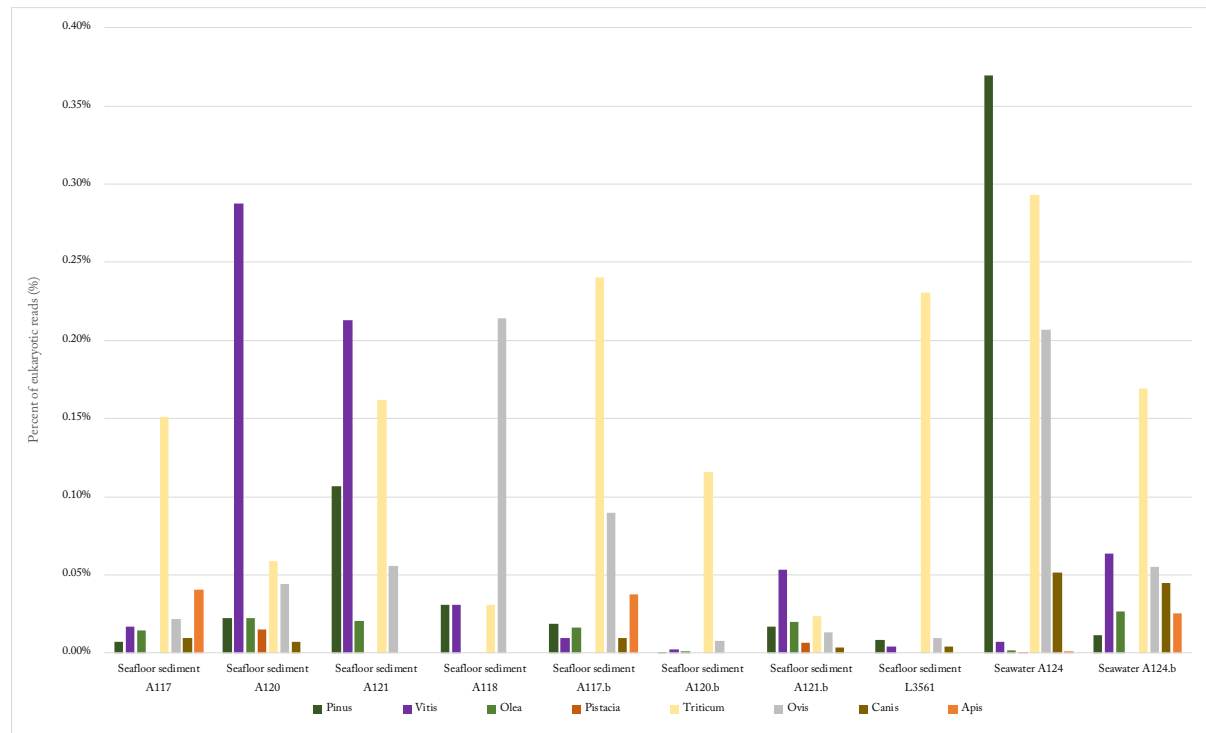


Figure 70. Samples of seafloor sediment and seawater collected from the Babuljaš and Mazotos shipwreck, with percent of eukaryotic reads that match to organisms of interest and common domesticated species.

As shown in Figure 70, a wide variety of DNA derived from organisms transported in amphorae can be found in seafloor sediment, including olive, grape, and pine. Interestingly, more *Apis* DNA was detected in samples of seafloor sediment and seawater than was detected in *lagynos* P.15.17 that was found to contain beeswax through organic residue analysis.

Two species of animal yielded relatively high percentages of eukaryotic reads in the samples analysed here, species of sheep (*Ovis*), and dogs (*Canis*). The detection of *Ovis* is worth noting as the detection of DNA derived from sheep species through shotgun metagenomic analysis had previously been interpreted as constituting evidence for an element of the diet of Neanderthals (Weyrich et al., 2017). Here, *Ovis* DNA was detected in numerous

archaeological and environmental samples, including three ceramic vessels from the terrestrial site of Yeronisos.

Foley et al. (2012) postulated that shipwreck amphorae would be more likely to preserve ancient DNA derived from vessel contents than ceramic artefacts found on terrestrial sites. Figure 71 shows the percent of eukaryotic reads assigned to organisms of interest in the terrestrial ceramics from Yeronisos island analysed here.

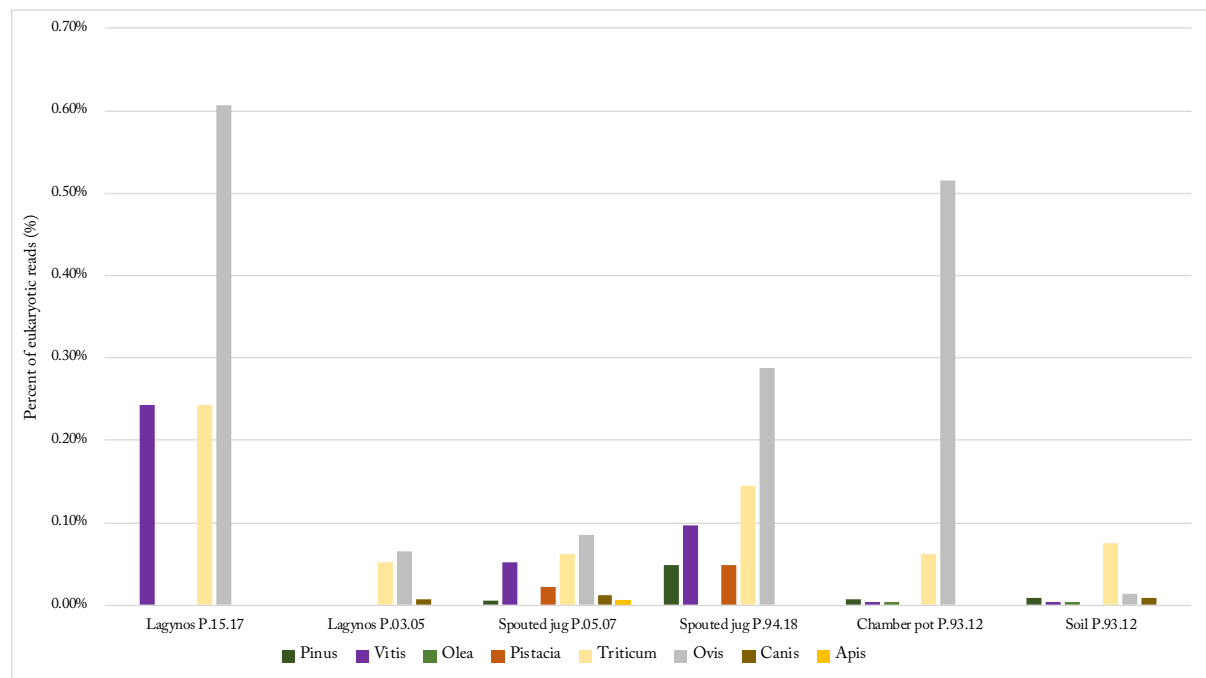


Figure 71. Percent of eukaryotic reads that match to organisms of interest and commonly domesticated plants and animals in the ceramics from the terrestrial site of Yeronisos.

While *lagynos* P.15.17 and both spouted jugs yielded *Vitis* DNA (Figure 71), the absolute number of reads was too few to assess whether this was genuinely ancient DNA. One pattern that has emerged in the terrestrial samples is the propensity for *Ovis* DNA to be detected.

It is interesting to reflect on the data reported by Weyrich et al. (2017) regarding the *Ovis* DNA detected in Neanderthal dental calculus. The authors report that from the samples from Spy II, 532 eukaryotic reads were assigned and 62% matched to *Ovis aries* (Weyrich et al., 2017, p. 359), which is expected to be 329.84 reads, rounded up to 330 reads matching to *Ovis*.

This remains a very low absolute number of both eukaryotic reads and reads matching to species of *Ovis*. This raises concern as sheep DNA may be able to migrate through terrestrial stratigraphy, as was shown in the detection of *Ovis aries* DNA in strata containing Moa bird remains in New Zealand, a species that went extinct long before the introduction of domesticated sheep to New Zealand (Haile et al., 2007).

Based on the evidence for the leeching of sheep DNA in terrestrial strata (Haile et al., 2007), it seems possible that the *Ovis* DNA detected in the Yeronisos ceramics may have resulted from sheep grazing on the surface layers and is unlikely derived from past vessels contents. Furthermore, that shelving-unit dust can yield 5,050 reads that match to *Ovis*, or 3.68% of the total eukaryotic reads for this sample, demonstrates again the prevalence of *Ovis* DNA detection in metagenomic datasets.

While not a metagenomic dataset, the data presented in the reports of Hansson and Foley (2008) and Foley et al. (2012) may suffer from the same issue discussed above. With the power of PCR amplification, it is possible that tiny amounts of environmental contaminant DNA can be amplified to the point that it appears to have some archaeological relevance. It is argued here that in both cases, the olive DNA Hansson and Foley (2008) claim to have isolated, and the *Ovis* DNA purported to be part of the Neanderthal diet in the Weyrich et al. study (2017), are both instances of environmental contaminant DNA masquerading as archaeological DNA.

4.5 Conclusion

Shotgun metagenomic analysis was performed on artefacts, environmental samples, and non-destructive swabs from five ancient Mediterranean shipwrecks and one Hellenistic terrestrial site. Of the 23 ceramic vessels analysed either through destructive extraction or non-destructive swabbing, five contained macro-remains that empirically show the nature of their past contents. The majority of the remaining shipwreck ceramics are vessels for which their past contents are assumed to have either wine or olive oil.

No DNA evidence for the known contents of these vessels has been detected by these methods. Evidence for the species associated with these contents, however, has been detected in numerous environmental samples including swabs of shelving unit dust, seafloor sediment, and seawater. The swabs of shelving-unit dust in particular yielded a broad range of DNA derived from organisms of interest, indicating the potential for contamination of artefacts post-excavation.

The resin samples from the Uluburun shipwreck analysed here showed the most promising result, yielding the highest percentage of *Pistacia* DNA of all the sample types. As a type of plant exudate, rather than a physical part of the plant such as a grape seed or olive stone, it remains unclear how much endogenous DNA can be expected to be preserved within this material. As discussed above, studies that have attempted to recover endogenous DNA from modern wine and olive oil encounter numerous difficulties from nucleases and polysaccharides present in the material inhibiting the recovery of grapevine or olive DNA.

Elements of the production process such as fermentation in the case of wine, or heating in the case of resinous plant material, compound the issue of endogenous DNA recovery. Future research on the extent to which endogenous plant DNA is preserved within other cargo items such as grape seeds, olive stones, pomegranate seeds, et cetera, may help to elucidate to what extent ancient DNA analysis can illuminate aspects of ancient trade. Skeletal remains, such as the butchered beef bones found inside amphorae on the Tektaş Burnu shipwreck (Carlson, 2003), and pork shoulders found on the shipwrecks near Pisa (Bruni, 2000), seem far more likely to yield endogenous DNA than processed plant products like pine tar, wine, or even a relatively unprocessed product like olive oil. The tissues derived from the actual plant or animal contain millions of cells that could yield endogenous DNA, whereas plant effluents like resin, or processed fruit oils and juice like wine and olive oil that have been subjected to chemical alterations through heating, fermenting, or pressing, will have already lost DNA during the process of transformation into a commodity.

Comparison between results from organic residue analysis through solvent extraction and alkaline fusion suggest that these more traditional methods of ceramic analysis may continue to be more effective at illuminating past vessel contents of ancient shipwreck amphorae than the shotgun metagenomic methods used here. Previous studies that have used DNA methods as a way to determine the past contents of ceramic vessels found on ancient shipwrecks did not include analysis of the deposition environment, or assess what DNA may be present in museum storage conditions where artefacts are housed post-excavation (Hasson and Foley, 2008; Foley et al., 2012). The identification of terrestrial plant DNA in the seafloor sediments, seawater, and museum storage conditions analysed here suggest that the results from these previous studies may have been due to environmental contamination, rather than constituting genuinely ancient DNA derived from past vessel contents.

The results shown here may prove to be a cautionary tale for archaeological scientists hoping to incorporate ancient DNA research into the work on artefacts recovered from underwater sites or stored in ‘museum’ conditions prior to analysis. Our results indicate that DNA from a variety of Mediterranean species widely used and transported throughout the region, including grapevine, olive, and pine, can also be detected in samples of seafloor sediment, seawater, and shelving-unit dust. In order to determine the source of this DNA, be it from environmental contamination, or derived from past vessel contents, authentication of the DNA sequences recovered has been essential. Here, only modern DNA has been positively identified. However, some *Pistacia* DNA has been recovered from the amber-coloured resin transported on board the Uluburun shipwreck, but the number of reads is currently too few to authenticate as ancient or modern. Work will continue on this material, as new methods and protocols are tested to increase our success of retrieving endogenous DNA from shipwreck cargo items.

Despite the challenges identified, there remains great promise for ancient DNA research of shipwreck cargo items to yield interesting and relevant information for the broader archaeological and historical community. A greater change for success in recovering genuinely ancient DNA will come from further analysis of the cargo items themselves, including plant and animal remains, rather than the ceramics in which they were held. From shipwrecks to harbours to sunken cities and submerged settlement sites, artefacts recovered from underwater

sites may hold genetic information that can illuminate aspects of the human past that, until now, have been hidden under the waves.

Chapter 5 – Pandora’s Pithos

5.1 Hope, anticipation, and deceptive expectations

Readers may be familiar with the myth of Pandora, as told by the archaic Greek poet Hesiod in his book *Work and Days*. In brief, Prometheus steals fire from the gods, Zeus seeks to punish him and all mankind for this transgression, Hephaestus is instructed by Zeus to build the first-ever mortal woman from clay, she is brought to life and bedecked in finery by the goddesses, but Zeus gives her a ‘thievish’ mind. She is given to the brother of Prometheus and settles among the mortals. Driven by curiosity, she opens a container of unknown contents, which unleashes misery and anguish for mankind (Montanari et al., 2009). After evil, fury, and anguish have been unleashed, only one thing remains inside the container. Often translated as *hope*, it has variously been translated as *hope*, *anticipation* (Loeb Classics) or *deceptive expectation* (Montanari et al., 2009). This is not, however the only questionable translation in the myth: the modern versions of this myth have preserved a renaissance-era mistranslation: it was never a box that Pandora opened at all; it was a *pithos* (πίθος).

A pithos, or *pithoi* in the plural form, is a ceramic storage jar. While larger than the amphorae analysed here, pithoi and amphorae share several principal characteristics: they are both made from clay, extremely durable, and thought to have contained primarily agricultural products such oil, wine, or fish sauce. Why is the correct translation of this myth so important? The fact that it is a pithos, not a box, that is referred to in the myth of Pandora offers several relevant things to consider as we resurface from the world of ancient shipwrecks, and the cargoes they contained.

First, ceramic storage jars, be they pithoi or amphorae, are a common item to be found in domestic contexts in the ancient world. Their ubiquity in the ancient world could only be expressed by comparing them to plastic boxes, bins, take away containers, and barrels we find in our world today. Undoubtedly, an element of drama in this myth is derived from the fact that a ceramic storage jar should be an unobtrusive, innocuous, everyday object. Amphorae, the

smaller cousins of the pithos, were similarly ubiquitous in the ancient world, and are especially prevalent on ancient shipwrecks. Found under the sea or on land, ceramic storage jars are extremely common in the archaeological record, and what they contained must be understood.

Second, in the myth of Pandora the contents of the ceramic storage jar appear to have been unclear. It was necessary for Pandora to open the pithos to see what was inside. This suggests that a variety of things could be stored inside, with anguish and misery being admittedly unexpected, and indeed unwelcome contents. In our work, it is necessary to ‘look’ inside of ceramic storage jars for the contents to be empirically known. Once the type of contents has been established, further investigation is possible, but that first crucial step must be taken in order to determine if we are dealing with macro-remains that are easily identified, or if we must look closer to find invisible molecular evidence. While seemingly trite, this point is often overlooked. On ancient shipwreck surveys and excavations, where one may be confronted with a mountain of identical amphorae, all too often assumptions are made regarding the content of these storage jars, without actually looking inside.

Finally, when we look inside a ceramic storage jar from the ancient world will it be *hope*, *anticipation* or *deceptive expectation* that we find? In this thesis, we have ‘opened’ storage jars, cargo items, and environmental samples found on ancient Mediterranean shipwrecks by analysing macro-remains, barely visible residues, and invisible DNA. Reflecting on the results from the analysis conducting here, perhaps all three translations have emanated from the shipwreck ceramics investigated here: *hope*, *anticipation*, and *deceptive expectations*.

Here, the results and conclusions from each data chapter are reviewed, research limitations are considered, and future directions for the molecular characterisation of ancient shipwreck cargo items are explored. The *hope* we have for the application stable isotope analysis of waterlogged organic remains is discussed, and ideas on how to expand on this preliminary study are explored. The *anticipation* of robust results from the organic residue analysis did not disappoint, and in some instances demonstrated unexpected content types in so-called ‘wine’ or ‘olive oil’ amphorae that appear to challenge the traditional approach to amphorae interpretation. The

deceptive expectations set for the ancient DNA analysis of shipwreck amphorae are discussed last, and suggestions are given for the best way to use the powerful tools of ancient DNA analysis on shipwreck artefacts in the future.

5.2 Stable isotope analysis of shipwreck olive stones - *hope*

There is great hope that stable isotope analysis of waterlogged organics can provide meaningful insights into the conditions under which these crops were grown, and possibly even their origin. The isotopic ratios found the ancient shipwreck olive stones analysed here are not significantly different from modern olive stone material, suggesting these ratios have not been substantially altered by the marine environment. We hope to apply stable isotope analysis to many more examples of waterlogged shipwreck cargo items, in order to paint a clearer picture as to where these items came from and how they were grown.

Ancient shipwreck excavations have yielded preserved olive remains in the tens of thousands, offering a unique opportunity to study an agricultural product that is often overlooked in the academic literature. While olive oil has been explored in detail, the consumption and trade of whole olives in the ancient world remains unclear. Through further investigations into the stable isotope composition, and whether or not the extraction of remnant lipids is successful, we are hopeful that a wealth of information regarding the trade, transport, and consumption of the fruits from this emblematic plant can be uncovered.

Mounting evidence for the trade in whole olives gleaned from shipwreck evidence undermines arguments made by Runnels and Hanson (1986) as well as Hamilakis (1996) that oil processing equipment on terrestrial archaeological sites is necessary to infer widespread use and consumption of olives by a large societal demographic (Hamilakis, 1996; Runnels and Hansen, 1986). Whole olives were far easier to produce and acquire than olive oil in the ancient world due to their lower cost of production (Foxhall, 2007, p. 132). Increasing evidence from ancient

shipwreck excavation shows that whole olives, not just olive oil, were traded and distributed widely. Yet the question remains as to where the olives found on ancient shipwrecks originated, and what purpose they served on board.

Haldane (1993) argued that the large average size of the Uluburun shipwreck olives indicated a trade function (Haldane, 1993). Others have argued that olives were a prestigious greeting gift in the Late Bronze Age used to cement relationships among elites (Kelder, 2009). The function of whole olives is likely to have changed over the millennia, however, as we read reports in the Zenon papyri of the olive trade from Chios, Knidos and Hellenistic Egypt (Demesticha, 2011, p. 290; Kruit and Worp, 2000, p. 81). Perhaps the prestige of whole olives as greeting gifts had diminished by the Classical period, in which olives were a common food item, or trade good. It is also possible that whole olives found on shipwrecks could have served many different purposes; providing food for the crew during the journey, with the potential to sell, barter, consume, or give them as a gift upon arrival. Perhaps seeking one simple function for the thousands of olive stones recovered from ancient shipwrecks is overly simplistic and does not accurately demonstrate the many uses they could serve, including greeting gift, trade item, or food for the crew.

In this thesis, the very first application of stable isotope analysis to waterlogged organic remains has been presented. Waterlogged olive stones were found in several Chian amphorae on the Mazotos shipwreck, a surprising discovery given the fact these Chian amphorae were assumed to have contained wine. Whole olive remains were also found in additional amphorae that originated in the southern Aegean, and this called into question whether the olives originated in one or multiple locations, as the amphora they were found in certainly had. As carbon and nitrogen stable isotope analysis of botanical remains has frequently been used to argue for either a single or multiple origins for ancient agricultural products (Fiorentino et al., 2012; Heaton et al., 2009; Lightfoot and Stevens, 2012), a decision was made to attempt this method on the waterlogged olive stones from a Classical shipwreck. For the olive stones from the Mazotos shipwreck, understanding whether they were grown in either one or multiple regions would in turn inform our understanding of amphorae use and reuse, given the fact that these olive stones were recovered from several types of amphorae on the wreck.

Results from the stable isotope analysis show that the olive stone samples from Mazotos have a far larger standard deviation and absolute range in $\delta^{13}\text{C}$ values than the modern Chios olives. This range also exceeds those found for botanical remains grown in one geographical location examined in previously published studies that investigate geographic origins through isotope analysis. Admittedly, no previously published work on the stable isotope analysis of waterlogged organics existed, so all comparisons have been drawn between published data sets that analysed carbonised organic remains from terrestrial sites. Further complicating any attempt to compare the results of our study with others is the fact that no carbonised olive stones from terrestrial sites have been analysed and published by this method. While no published data on the $\delta^{13}\text{C}$ of olive stones could be found, comparisons have been drawn between the ranges in isotopic values found for modern olive oil (Angerosa et al., 1999; Gumus et al., 2017; Portarena et al., 2014), and charred archaeobotanical remains including durum wheat (Heaton et al., 2009), barley (Fiorentino et al., 2012), and tree resin (Stern et al., 2008) grown in the Eastern Mediterranean. While the large range in isotopic values found for the Mazotos shipwreck olive stones exceeds that found for previously published results from charred remains recovered from archaeological sites in the same region (Eastern Mediterranean) in these studies, the difference between the values found for the modern sample set from Chios and the shipwreck olive stones were shown to be statistically insignificant.

A lack of statistically significant difference between the ancient and modern sample set may not provide definite answers regarding geographic origin; however, it may provide several insights into the archaeological material, and how this method can be applied in the future. First, it appears to indicate that waterlogging by seawater has not significantly changed the isotopic composition of the carbon in the ancient olive stones. Otherwise, we would expect to see drastically different values for the ancient and modern samples sets. Second, a lack of a statistically significant difference between the ancient and modern sets may suggest that the olive stones recovered from the bow and stern of the Mazotos shipwreck, as well as from inside two different amphorae types (a southern Aegean and a Chian), may share a common geographic origin. That a southern Aegean style amphora (P0450) and an amphora from Chios

(P0392) may have contained whole olives from the same geographic location is an interesting possibility as it could have implications for the use and re-use of amphorae in the ancient world (Peña, 2007), ship provisioning strategies, and cargo compositions in general.

Evidence for lipid preservation in ancient olive stone material could have interesting future implications for pinpointing the geographic origin of ancient olive stone remains. If lipids extracted from ancient shipwreck olive stones could be recovered in large enough amounts to be collected, it may be possible to analyse the lipids themselves in order to establish where ancient shipwreck olives were grown. Attempts to analyse artefacts waterlogged in the marine environment by oxygen isotope analysis may prove difficult as the internal standard of ^{18}O isotope analysis is the Standard Mean of Ocean Water (SMOW). Artefacts waterlogged by seawater would have, in essence, been soaking in the internal standard used to calibrate the Mass Spectrometer for thousands of years. If, however, the lipids from olive stone endocarp could be extracted and isolated, it may be possible to perform oxygen isotope analysis on this material. A dual approach of using oxygen and carbon isotopes to pinpoint geographic origin may likely prove far more effective (Scheidegger et al., 2000).

It is intriguing that two amphorae from different parts of the Aegean (P0392 and P0450) contained olive stones, rather than the wine with which these amphorae shapes are associated. This appears to support research that argues for a complex relationship between transport amphorae and particular contents in antiquity (Lawall and Lund, 2011, p. 32). Perhaps wine was the first content of the Chian amphorae on board, but as at least three of the Chian amphorae on board carried olives, it remains an interesting possibility that far more amphorae on the Mazotos shipwreck are also full of ancient olives, as the vast majority of the amphorae have not yet been raised or thoroughly investigated on the seafloor.

Additional study of whole olive remains from ancient shipwreck sites is necessary to better understand their use place and purpose in sea-borne trade, and to establish whether or not whole olives formed an important part of the export economy of ancient Mediterranean states.

Subjecting additional olive remains from a variety of underwater sites to isotope analysis could enhance our knowledge of the cultivation, trade and consumption of this important species.

5.2.1 Future directions for the stable isotope analysis of waterlogged organics

Future research on ancient olives would benefit from the creation of a comprehensive data set of isotopic values for olive stones of known provenance. By establishing what level of variability in isotopic values can be expected from one olive tree, one orchard, and one entire geographic area, further inferences regarding the geographic origin of the numerous olive stones recovered from ancient shipwrecks may be possible. In addition, the inclusion of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ data would allow for greater accuracy in determining growth conditions and therefore the geographic origin of the remains.

Utilising protocols developed for the ^{14}C dating of waterlogged wood, which isolate the cellulose of plant material, may provide more consistent results. These protocols would potentially exclude the remaining heterogeneous constituents of olive stone endocarp including lignin and remnant lipids. Experimental waterlogging by seawater of modern olive stone samples may increase our understanding of the effect the marine environment may have on isotopic composition. The $\delta^{13}\text{C}$ of dissolved inorganic carbon in seawater collected from northern latitudes can range from 0.30‰ to 1.5‰ depending on the depth (Humphreys et al., 2015, p. 133). Therefore, establishing the $\delta^{13}\text{C}$ of dissolved inorganic carbon at the shipwreck site may prove useful in understanding if saturation by seawater has affected the $\delta^{13}\text{C}$ of organic artefacts.

Waterlogged organic remains from ancient shipwrecks may provide the perfect material to test future strategies for the stable isotope analysis of organics from the marine environment. As more and more submerged prehistoric settlement sites emerge, the possibility that waterlogged organic items from the very inception of agriculture can be uncovered. This material is likely to be scarce, fragmentary, and irreplaceable. Ancient shipwreck organics like the olive stones

examined here can often occur in their thousands and are eminently dateable based on the material artefacts in the ship's cargo, and the construction style of the ship's hull. If stable isotope methods that can be applied to waterlogged organic remains from prehistoric settlement sites are to be honed, we hope that this preliminary study, and future studies of shipwreck organics, can be used for this purpose. While simultaneously providing insights into the growth, trade, and use of agricultural products in later periods, it is more than likely that studies such as the one presented here will form the precursor to much larger studies of waterlogged organics of ever-greater antiquity.

5.3 Organic residue analysis of shipwreck ceramics - *anticipation*

Anticipation of a successful outcome for the organic residue analysis of shipwreck amphorae was based on the many years of successful research into the recovery of molecular evidence for past vessel contents through GC-MS, and for this reason organic residue analysis was selected as a complementary approach to the more novel ancient DNA analysis. Yet the detection of wine and fruit 'biomarkers' in seafloor sediments may muddy the waters, and further research is needed into how these compounds in the natural marine environment effect our results.

There are numerous instances in which preserved macro-remains found within shipwreck amphorae differ from their assumed contents. An example of this is the discovery of thousands of whole olive stones in 'wine' amphorae from the Mazotos shipwreck, discussed above. However, the preservation of macro-remains in shipwreck amphorae remains the exception rather than the rule. Most often, shipwreck amphorae appear empty when discovered on the seafloor, making it necessary to search for molecular evidence for their past contents in order to establish what these ships were transporting on their last voyage.

The well-established methods of organic residue analysis used to investigate ceramic artefacts recovered from terrestrial sites (Charters et al., 1993; Condamin et al., 1976; Evershed, 1993; Evershed et al., 2008; Heron et al., 1991; Knights et al., 1983; Kuksis et al., 1978; Thornton

et al., 1970), may need further examination in their application to objects recovered submerged archaeological sites due to unique aspects of the marine deposition environment. Understanding the composition of seafloor sediment that accumulates around ancient shipwreck sites, and the molecules it contains, is vital.

After the research by Heron et al. (1991) that demonstrated there is little migration between lipids in the soil (i.e. environmental samples) and ceramic artefacts, analysis of corresponding soil samples when analysing ceramics from terrestrial contexts by organic residue analysis has declined during the last few decades. However, to what extent the findings of Heron et al. (1991) applies to archaeological ceramics recovered from underwater contexts remains unclear. In this research, we have shown that several molecules thought to be biomarkers of wine (syringic acid, vanillin, succinic acid, et cetera) were more prevalent in every sample of seafloor sediment than in the shipwreck amphorae thought to have contained wine.

This does not rule out the possibility that these vessels contained wine in the past. Tartaric acid, recognised as the primary biomarker of wine (Pecci et al., 2013; Woodworth et al., 2015) is difficult to detect. Its solubility in water may make it even more difficult to detect in underwater deposition environments. Of the 21 amphorae analysed by organic residue analysis in this study, none yielded indisputable molecular evidence for a past content of wine. However, the majority of the shipwreck amphorae included in this study thought to have contained wine revealed molecular evidence of conifer products.

Conifer products are more likely to survive marine contexts than wine residues due to their hydrophobic nature. In addition, it appears that marine animals are not interested in consuming the visible lining of amphorae, even when these amphorae are entirely exposed on the seafloor. While a conifer lining is not inconsistent with a past content of wine, a lined amphora could have contained other products. Evidence from Pompeii where fish bones were found still clinging to the lining of amphorae in a garum shop have shown that fish sauce amphorae were also lined with *Pinaceae* products (Pecci et al., 2018, p. 494). As such, we can neither confirm the rightful application of the traditional approach to amphorae interpretation,

which assumed the majority of the vessels analysed in this research contained wine, nor deny it based on the molecular evidence found here.

On the other hand, plant oils have been successfully detected in three of the shipwreck amphorae analysed, including two Samian amphorae from the Kyrenia shipwreck thought to have contained wine. This result further strengthens our argument that it is necessary to find molecular evidence for past contents rather than exclusively employing the traditional approach. Evidence for oily products appears to be far easier to detect, due to the lower solubility, and hydrophobic nature of these compounds. Further research, and the testing of new extraction methods, may be necessary to enhance our ability to detect evidence for wine residues, such as tartaric acid, in ancient shipwreck amphorae, but this work has shown that a traditional solvent extraction method is sufficient for the detection of plant oils.

Small acids considered to be biomarkers of wine or fruit were detected in every sample of seafloor sediment analysed in this study, which included samples from both inside and outside of the shipwreck areas of the Mazotos and Babuljaš shipwrecks and one sample collected from inside a Canaanite jar on the Uluburun shipwreck. These ‘biomarkers’ include syringic acid, vanillic acid, succinic acid, and vanillin among others. This result calls into question our ability to use these compounds as effective biomarkers for past human dietary practices. In addition, triterpenes derived from the *Pistacia* resin were detected in the sediment sample collected from a Canaanite jar (KW1553) on the Uluburun shipwreck. This is a clear indication that *Pistacia* biomarkers had successfully migrated from the vessel contents to the sediment at the toe of the vessel. From these findings, we suggest excavators retain sediment recovered from the toe of shipwreck jars and amphorae for analysis, as it is likely that some molecular evidence for the past vessel contents could be found in this seemingly intrusive sediment, that from a visual inspection at first appeared to be composed of sand and bits of shell.

5.3.1 Future directions for the organic residue analysis of shipwreck amphorae

As has been shown in this research, tartaric acid is difficult to detect in archaeological ceramics, likely even more so in ceramics from underwater contexts. The relative ease in detecting lipids, due to their greater resilience in marine environments, could potentially lead to a bias in our interpretation of what shipwreck amphorae contained. That plant oils and similarly oily substances are simply more likely to survive in marine contexts must be acknowledged, in order to avoid such skewing of our view of amphorae contents. Similarly, the propensity for conifer product linings to survive in amphorae has clearly dominated the results of our study, and whether the presence of conifer should indicate a content of wine or not, must be explored further.

Advances in organic residue analysis continue rapidly, with new methods such as super-critical fluids offering great promise for archaeological material given their non-destructive nature (Deviese et al., 2018). Any methods that can be developed that would avoid the destruction of irreplaceable artefacts should rightly be explored in order to avoid the unnecessary loss of archaeological material. Proteomics shows great promise as a method for determining past vessel contents (Dallongeville et al., 2011), and as these methods continue to be honed it is likely that the recovery and analysis of proteins will occupy a central place in the amphorae analysis of the future. In addition, methods and protocols that may be more effective in detecting water soluble materials like the biomarkers of wine will greatly enhance our ability to effectively test shipwreck amphorae.

As ancient shipwreck sites can offer direct evidence for long-distance trade in the past, every opportunity should be taken to find increasingly effective ways of characterising the goods these ships carried. That organic molecules can so effectively bind to seafloor sediments may represent both potential future applications, and potential pitfalls of this research. Comprehensive sampling of sediments outside the ancient shipwreck area could easily mitigate these pitfalls, in order to establish what biomarkers are naturally present on these underwater archaeological sites, and what biomarkers may indeed be derived from past shipwreck cargo items.

A detailed study similar to the one conducted by Heron et al. (1991) that explored the extent of lipid migration between ceramics in terrestrial deposition contexts and the soil that surrounds them is necessary for marine contexts. The deliberate saturation of modern ceramic material with a known quantity and concentration of a given compound, such as syringic acid for example, with subsequent burial in seafloor sediment on a controlled site, with regular monitoring of the levels of the compound found in the sediment, or remaining within the ceramic matrix, could offer valuable insight into what extent these small acids which are considered to be biomarkers of wine and fruit can migrate in the marine environment.

5.4 DNA analysis of shipwreck ceramics, cargo items, and environmental samples – *deceptive expectations*

It would appear that deceptive expectations have been set for the successful recovery of DNA derived from past vessel contents in shipwreck amphorae. When Hansson and Foley (2008) and Foley et al. (2012) reported the successful recovery of ancient DNA from shipwreck amphorae through both destructive (2008) and non-destructive methods (2012), a new age of ceramic vessel studies appeared to be beckoning on the horizon (Foley et al., 2012; Hansson and Foley, 2008). The results reported in the 2012 paper appeared to suggest that archaeological scientists, armed only with sterilised cotton swab and some lysis buffer, could non-destructively test any ceramic artefact in the world. One imagined a world where every vessel in the British Museum, the Metropolitan Museum of Art, or the Museum of Egyptian Antiquities in Cairo would now be available for analysis. The enduring argument made by curators and conservators against molecular analysis of their artefacts is based on the destruction it would cause the vessel; but if the work of Foley et al. (2012) presented realistic and replicable results, the arguments against molecular analysis would evaporate as quickly as the smudge of lysis buffer on the surface of an ancient ceramic vessel. Since publication, these two papers have been cited 47 times (*Web of Science Database*, 2019).

Challenging the findings of Hansson and Foley (2008) and Foley et al. (2012), our research has shown that terrestrial plant DNA can be recovered from both artefacts and environmental samples. In our research, five of the 23 vessels analysed contained identifiable contents including olive stones, resin, and pomegranate seeds. These vessels were analysed by either destructive extractions or non-destructive swabbing. No DNA evidence for the known contents of these vessels has been detected by the shotgun metagenomics method used here. Evidence for the species associated with these contents, however, has been detected in numerous environmental samples including swabs of shelving unit dust, seafloor sediment, and seawater. The swabs of shelving-unit dust in particular yielded a broad range of DNA derived from species of interest, indicating the potential for contamination of artefacts post-excavation. While it may still be possible to recover DNA derived from past vessels from shipwreck amphorae, this can only be confidently asserted if we exclude modern environmental contamination as a possible, and far more likely source, of the DNA recovered.

This research has shown that ‘species of interest’ such as olive tree, grapevine, or pine DNA were found more frequently in the environmental samples of shelving-unit dust, seafloor sediment, and seawater, than in the destructive DNA analysis of vessels of known content. This irrefutably demonstrates the necessity to test the deposition environment in order to establish a baseline of what molecules and DNA are naturally present, before assigning any archaeological meaning to the discovery of the DNA from these species in artefacts.

Notably, we have demonstrated that *Ovis* DNA has been detected in several environmental samples. This calls into question the assertion by Weyrich et al. (2017) that the detection of *Ovis* DNA in Neanderthal dental calculus is evidence for their consumption of sheep (Weyrich et al., 2017). One immediately wonders to what extent was *Ovis* DNA simply detected in the soils and sediments collected from the cave in which these Neanderthal remains were found? Haile et al. (2007) had highlighted this issue a decade earlier when *Ovis* DNA was detected in layers containing extinct Moa bird remains (Haile et al., 2007). Admittedly, the mineralised nature of dental calculus means it would be less susceptible to contamination from exogenous

sources, yet the fact remains that the sediments surrounding the Neanderthal remains should also have been scanned for *Ovis* DNA.

Without first establishing this critical aspect of the deposition environment, it is not possible to assign archaeological meaning to the detection of *Ovis* DNA. This same issue arose when it was claimed that genuinely ancient tuberculosis DNA was detected in Neolithic skeletons recovered from inundated settlement sites (Hershkovitz et al., 2008), and it was pointed out that the tuberculosis mycobacterium is notorious for its survival in soils and sediments so in order to confirm this DNA as deriving from the skeletal remains it was necessary to eliminate its presence in the inundated soils surrounding the human remains (Wilbur et al., 2009).

The resin samples from the Uluburun shipwreck analysed here showed the most promising result, yielding the highest percentage of *Pistacia* DNA of all the sample types. As a type of plant exudate, rather than a physical part of the plant such as a grape seed or olive stone, it remains unclear how much endogenous DNA can be expected to be preserved within this material. Studies that have attempted to recover endogenous DNA from modern wine and olive oil encounter numerous difficulties from nucleases and polysaccharides present in the material inhibiting the recovery of grapevine or olive DNA. Elements of the production process such as fermentation in the case of wine, or heating in the case of resinous plant material, compound the issue of endogenous DNA recovery. Future research on the extent to which endogenous plant DNA is preserved within other cargo items such as grape seeds, olive stones, pomegranate seeds, et cetera, may help to elucidate to what extent ancient DNA analysis can illuminate aspects of ancient trade. Skeletal remains, such as the butchered beef bones found inside amphorae on the Tektaş Burnu shipwreck (Carlson, 2003), and pork shoulders found on the shipwrecks near Pisa (Bruni, 2000), seem far more likely to yield endogenous DNA than processed plant products like pine tar, wine, or even a relatively unprocessed product like olive oil.

Comparison between results from organic residue analysis through solvent extraction and alkaline fusion suggest that these more traditional methods of ceramic analysis may continue

to be more effective at illuminating past vessel contents of ancient shipwreck amphorae than the shotgun metagenomic methods used here. Previous studies that have touted DNA methods as a new way to determine the past contents of ceramic vessels found on ancient shipwrecks did not include analysis of the deposition environment, assess what DNA may be present in museum storage conditions where artefacts are housed post-excavation, or appraise the damage patterns of the DNA fragments identified to determine an ancient origin (Hansson and Foley, 2008, Foley et al., 2012). The identification of terrestrial plant DNA in the seafloor sediments, seawater, and museum storage conditions analysed here suggest that the results from these previous studies may have been due to environmental contamination, rather than constituting genuinely ancient DNA derived from past vessel contents.

5.4.1 Future directions for ancient DNA analysis of shipwreck artefacts

A clear limitation of all metagenomic research is that we rely on databases of genomes that have previously been sequenced or studied in part. The curation of such databases must be examined further, to ensure that all genomes available for comparison with our samples have been sequenced properly and transcribed with no errors. In addition, only a small fraction of the species on our planet, be they bacterial, fungal, plant or animal, have yet to be sequenced and included in any database. This creates a profound bias in any DNA study, as species that are of interest to the medical or agricultural industries will be over-represented, (e.g. humans, wheat) and species with little economic value will either never be sequenced, or only represented in a limited fashion. It is entirely possible, if not probable, that species of plants and animals used in the ancient world have not been studied through DNA analysis, and therefore would have no representation in our databases. As DNA sequencing technology continues to become less expensive and therefore more accessible, this problem may abate.

The most promising class of artefact for the recovery of ancient DNA will always be the macro-remains associated with the species itself. This study has shown the only class of artefact to yield what may be endogenous DNA is the resin from the Uluburun shipwreck, which showed

a higher percentage of eukaryotic reads for *Pistacia* than the swabs of ceramics, shelving-unit dust, or the destructive extraction of seafloor sediment.

Similar techniques could be applied to the macro-remains recovered from shipwreck amphorae, such as the butchered beef bones found in amphorae on the Tektaš Burnu shipwreck (Carlson, 2003), the butchered pork shoulders found on shipwrecks at Pisa (Bruni, 2000), olive stones from various wrecks, grape pips, pomegranate, et cetera. The likelihood for endogenous DNA to survive within the cellular material of these species is far greater than for the recovery of DNA derived from these items from inside the ceramic matrix of shipwreck amphorae. These types of remains may offer interesting insight into animal and plant domestication, and the movement of these goods in antiquity.

That DNA from a variety of domesticated species of plants and animals including sheep, dogs, cats, and wheat was found to be present in environmental samples further illustrates the necessity to test the deposition environment, or storage environment, alongside all samples in order to exclude such external contamination from the results.

5.5 Conclusion

5.5.1 Ancient trade and shipwreck evidence

In 2010 a paper was published by Christopher Monroe that attempted, for the first time, to quantify the exact value of the cargo lost on the Uluburun shipwreck (Monroe, 2010). By carefully tallying up the copper ingots, the tin ingots, the scraps of gold and, importantly, the 1 tonne of resin transported in 153 Canaanite jars, a value of around 12,000 Ugaritic silver shekels was determined (Monroe, 2010, p. 28). This was a vast sum in the Late Bronze Age, and the equivalent amount traded for grain could have fed a small city for a year (Monroe 2010, p. 28). This paper, 'Sunk Costs at Late Bronze Age Uluburun' is relevant to the research presented here for several reasons. First, it deals with the Uluburun shipwreck whose artefacts are also analysed here. Second, it attempts to reconstruct aspects of ancient maritime trade based on shipwreck evidence, as this research also seeks to do. Third, the work of Monroe is dependent on an empirical knowledge of what the ceramic transport vessels on the Uluburun contained. As stated by Monroe 'After the 11 tons of copper and tin aboard the Uluburun ship, the load of resin-filled jars was the bulkiest and heaviest, and one might suspect it was among the more valuable cargoes' (Monroe, 2010, p. 24).

Monroe was able to use the macro-remains discovered inside the Canaanite jars as empirical evidence for what they contained. This greatly enhanced his ability to confidently quantify a minimum value for Uluburun cargo. Even in the case of the Canaanite jars from the Uluburun questions have been posed as to the true nature of the contents, with some asserting that the chunks of amber-coloured resin were the true cargo (Mills and White, 1989; Stern et al., 2008), while others claim it was a combined cargo of resin and resinated wine (McGovern and Hall, 2016; McGovern and Michel, 1996). Had molecular evidence for wine been found, Monroe would have had to retally all his careful estimations of the value of the Uluburun cargo.

Here we recall Trimalchio, our fictional freedman from Petronius *Satyricon* discussed in the Introduction of this thesis. The cargo of the ships he describes to his dinner guests was first composed only of wine, but on the subsequent voyage he hedged his bets by including beans, bacon, Capuan perfumes, and slaves, alongside the ever-popular trade item of wine. If one were to attempt to quantify the total value of these cargoes, we would see a considerable difference between the total value of the former and the latter. In order to understand the extent, importance, and value of ancient maritime trade, we must know what was being traded, and in what quantities.

This issue, and the work of Monroe (2010), illustrates why it is vital that we understand what cargo items were being traded on the ships we find at the bottom of the Mediterranean. Complex quantifications of cargo costs (Monroe 2010), attempts to gauge patterns in shipping strategies (Horden and Purcell, 2000; Wilson, 2011), and even Bayesian analysis Roman trade (Rubio-Campillo et al., 2017), all rely on an accurate assessment of what shipwreck transport vessels contained. Research such as ours, which seeks molecular evidence for amphorae contents, can serve as the foundation on which these economic estimations are made. If complex quantifications and financial estimations of ancient trade are built from the ‘best-guess’ scenario that is the traditional approach to amphorae interpretation, these laboriously constructed economic models will topple as if they were constructed on quicksand.

By challenging the traditional approach to vessel interpretation, and using shipwreck evidence to do so, this research may be opening Pandora’s proverbial pithos; if the tidy assumptions regarding the nature of shipwreck cargoes are challenged, it could disrupt attempts to quantify trade in the ancient world. Yet this is how scientific research must progress, by seeking empirical evidence at the expense of convenient assumptions.

5.5.2 Scientific methods for understanding shipwreck cargoes

The techniques and methods used in bioarchaeology to characterise material from the ancient world continue to progress. As new and innovative methods are developed, we must assess how effective their application to archaeological material will be. Here, we have analysed cargo items from ancient shipwrecks by three discrete methods: stable isotope analysis, organic residue analysis, and shotgun metagenomic DNA analysis. Our goal has been to address issues surrounding the traditional approach to amphora interpretation, in which vessel contents are assumed based on the shape of a ceramic vessel, with no molecular evidence presented to confirm what these vessels contained in antiquity.

Stable isotope analysis shows great promise for waterlogged organic material from shipwrecks. Human, plant, and faunal remains have been found on ancient shipwrecks, and the future application of stable isotope analysis to this material requires that we continue to investigate what affect waterlogging by seawater may have on the isotopic ratios of the archaeological material. The results presented here indicate that there is not a statistically significant difference between the range of values found for the stable isotopes of carbon in ancient waterlogged olive stones and that found for modern olive stones grown in one island of the Eastern Mediterranean. This is a promising result as it suggests that waterlogged archaeological material may indeed be suitable for stable isotope analysis.

Organic residue analysis has long been applied to ancient shipwreck ceramics, but the biomarkers of wine have proven difficult to detect. In order to address assumptions regarding vessel content, 21 amphorae have been analysed, 19 of which were thought to have contained wine. Over 50% showed evidence for conifer products, which may indicate a past content of wine or other products like fish sauce, while two of the so-called 'wine' amphorae were found to contain plant oils, further eroding our confidence in the traditional approach. Vitally, several small acids thought to be biomarkers of wine, including syringic acid, succinic acid, vanillin and vanillic acid, were detected in every sample of seafloor sediment analysed in this study. This is a significant discovery as it calls into question the suitability of these molecules as 'biomarkers' of anthropogenic activity, as they are demonstrably present in the natural seafloor composition. To what extent these molecules can migrate between the seafloor and the porous

shipwreck ceramics that are buried in it remains unclear, and further experimental analysis will allow great insight into whether or not this phenomenon occurs.

Shotgun metagenomic DNA analysis of ancient shipwreck artefacts yielded mixed results. The most promising results came from the analysis of actual cargo material, rather than the ceramics that once held such material. The recovery of very small amounts of what may be ancient *Pistacia* DNA from the amber-coloured resin carried on the Uluburun shipwreck suggests that these methods are most successful when applied to the contents of amphorae, rather than the pulverised ceramic matrix of the amphorae themselves.

In vessels of known contents, including olive stones, resin, and pomegranate, no DNA related to these known contents was detected by the shotgun metagenomic methods used here. Conifer product was detected by organic residue analysis in several of the vessels also subjected to ancient DNA analysis, but no appreciable amounts of *Pinus* DNA was detected in these vessels.

Based on the research presented here, the traditional approach to amphora interpretation maintains its utility as a ‘best guess’ as to what a certain type of amphora contained. The limitation of this approach is that adhering to assumptions based only on what has come before, we are less likely to incorporate new information and archaeological findings into our understanding of amphorae and shipwrecks. When making a declarative statement as to what a ceramic vessel contained in antiquity, the strongest arguments will come from molecular evidence. When molecular evidence is not present, it is necessary to caveat statements about vessel contents with explicit language - ‘we assume this Africana III/Keay 25 amphora contained olive oil, but we have no evidence to say for sure’ or ‘it is likely these Chian amphorae contained wine, but this has not yet been firmly established.’

5.5.3 Ancient shipwreck artefacts and the marine environment

Perhaps the most significant findings presented here relate to the complexity of the molecules and DNA recovered from the marine environment. While the marine environment does not appear to have a seriously deleterious effect on the carbon isotope composition of waterlogged olive stones, the same may not be true for the organic residue analysis and ancient DNA research conducted on shipwreck artefacts.

We have shown that ‘biomarkers’ of wine can be detected in marine sediments collected from natural seafloor sediments a great distance from ancient shipwreck sites. If such molecules are simply present in the deposition environment, we cannot confidently assume that they relate to food preparation actions such as fermentation, wine preparation, or wine consumption. If these molecules are not related to anthropogenic activities in this case, it is possible that other studies which did not test the deposition environment, are simply reporting on molecules that are nearly ubiquitous in the natural environment. This makes a strong argument for the inclusion of environmental samples in further organic residue analysis research of shipwreck artefacts.

DNA related to ‘organisms of interest’ including grapevine, olive, and pine DNA, was detected in several environmental samples of shelving-unit dust, seafloor sediment, and seawater. After alignment with reference genomes and analysis by mapDamage2.0, in every instance this detected DNA was shown to be modern. This further illustrates the caution needed in interpreting what DNA is detected in metagenomic datasets, and why analysing corresponding environmental samples is vital to establishing what DNA may be naturally present in the environment in which these artefacts are buried, or stored, post-excavation.

When applying scientific methods developed for artefacts recovered from archaeological sites on land to shipwreck artefacts, one must consider the ways in which the marine environment differs from that of the terrestrial. Sedimentation rates, bioturbation by cephalopods, proximity to fluvial deposits, salinity, temperature, currents, recreational diving activities, can all effect the molecules detected in shipwreck artefacts. Despite these challenges, shipwreck assemblages continue to be the best source for information pertaining to ancient trade in the Mediterranean.

By carefully calibrating our methods to account for the dynamic nature of the marine environment, there is every reason to have great expectations for a long and fruitful relationship between the investigations into ancient shipwrecks and newly developed methods in archaeological science.

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Supplementary Material

Co-Author Statements

Co-author Statement

January 2019

I hereby declare that I am aware that the work in the paper/manuscript entitled:

DNA from the Wine-Dark Sea: searching for DNA in ceramics, cargo items, and environmental samples from the Uluburun, Cape Gelidonya, Kyrenia, Mazotos, and Babuljaš shipwrecks and the Hellenistic island of Yeronisos

of which I am co-author, will form part of DPhil thesis for the degree of DPhil in Archaeological Science, at the University of Oxford, submitted by DPhil student:

Elizabeth (Lisa) Briggs

who made a major contribution to the work in the research phase and who made a major contribution to the work in the writing phase.

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Name: Cemal Pulak

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Co-author Statement

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I hereby declare that I am aware that the work in the paper/manuscript entitled:

Biomarkers in ceramics and sediments: using residue analysis to explore cargo compositions of the Uluburun, Mazotos, Kyrenia and Babuljaš shipwrecks, and the use of pottery on the Hellenistic island of Yeronisos

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Signature:

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