REVIEW

Ancient DNA Research in Maritime and Underwater Archaeology: Pitfalls, Promise, and Future Directions

Lisa Briggs

The rapid progression of DNA technology allows for the application of recently developed techniques to an ever-growing body of archaeological and environmental material recovered from submerged archaeological sites. As NGS and DNA Capture replace PCR as the predominant method used to characterise DNA present within an archaeological sample, it is necessary to consider how this effects the possibilities for future research, but also gives cause to reconsider the findings of previously published work from PCR experiments on archaeological material from submerged sites. In this review, the pitfalls, promise, and future directions of ancient DNA research on archaeological material from submerged sites are discussed. Here, it is argued that a common stumbling block in past research has been a lack of characterisation of the deposition environment, and that this has obscured our understanding of DNA identified at submerged sites. Unique aspects of the marine environment may present further complications in our attempts to authenticate ancient DNA. Overcoming these challenges will significantly enhance our ability to confidently assign an archaeological origin to DNA isolated in artefacts, organic remains, and sediments from submerged archaeological sites.

Keywords: aDNA; underwater archaeology; submerged landscapes; shipwrecks

1. Introduction

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 (Bass, 1970; Blackman, 1973; Frost, 1963; Muckelroy, 1978; Throckmorton, 1965). In recent years, however, 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), with ancient DNA research firmly among them. While submerged landscapes offer particular promise for the study of prehistoric humans, all manner of underwater archaeological sites are likely to yield artefacts and organic remains of great use in ancient DNA studies.

Researchers in various avenues of archaeological science have long made use of artefacts recovered from submerged sites, including C14 dating of shipwreck wood (Ralph, 1967) and organic residue analysis of ancient shipwreck ceramics (Formenti et al., 1978). Ancient DNA research is no exception, with one of the earliest successful extractions of DNA from archaeological bone involving a pig bone from the Mary Rose shipwreck (Hagelberg et al., 1989; Hagelberg and Clegg, 1991). This paper offers a review of the application of ancient DNA research to materials recovered from coastal and maritime archaeological sites, and discusses the pitfalls encountered in previously published studies, the promise that DNA analysis may hold for the field of underwater archaeology, including what future directions are likely to help answer long-standing questions about the human past. While artefacts recovered from fluvial and lacustrine environments come under the remit of underwater archaeology, this paper seeks to address issues that are specific to the marine, or saltwater, environment. Some key studies of DNA recovered from lacustrine environments are touched upon, but do not form the focus of this paper, which seeks to review and understand the study of ancient DNA recovered from marine archaeological sites.

Here it is argued that characterisation of the underwater deposition environment is vital to understanding and interpreting the DNA identified. The marine environment is uniquely dynamic and varies considerably throughout the world’s oceans. Temperature, salinity, and sedimentation rate are merely the tip of the proverbial iceberg when it comes to the myriad variables that may affect the preservation of DNA in marine environments. The high ionic environment of seawater, however, appears to be a common factor throughout marine environments, and as low ionic environments accelerate depurination of DNA (Lindahl and Nyberg, 1972), high ionic environments may slow this process. This may have implications for our ability to authenticate DNA recovered as having
an ancient origin. In addition, the potential for modern contaminant DNA to settle on artefacts exposed on the seafloor, or by leaching or vertical migration of DNA throughout submerged strata must be further addressed. Vertical migration of DNA through terrestrial strata has been demonstrated (Haile et al., 2007), yet to what extent this can be applied to underwater contexts remains unclear. Assessing and overcoming these challenges will allow us to more confidently interpret the nature, origin, and archaeological meaning of DNA identified on coastal and maritime archaeological sites.

1.1. Background of ancient DNA research

Deoxyribonucleic acid (DNA) is the molecule that contains genetic information for a given organism and is present in the cells of all living organisms. Humans, animals, plants, bacteria, and fungi 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 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 found to be false (Austin et al., 1997; Hebsgaard et al., 2005; Palmer et al., 2011; 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, 2000), 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 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, p. 388; Orlando et al., 2015). Next Generation Sequencing (NGS) allows for a far greater number of aDNA molecules present in a sample to be sequenced, without the need for specially designed primers (Poinar et al., 2006). 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; Mardis, 2008, p. 389).

These methods seek to create greater ‘coverage’ across a genome of interest, and ‘depth’ of coverage across as many areas of the genome as possible. Coverage refers to the number of base pairs in a given sample that align to the reference genome, while depth of coverage is the number of times that a specific aligned base pair is represented. The low amount of endogenous DNA in archaeological material has presented problems in aDNA studies, as this can result in low coverage and depth of coverage. ‘Shotgun’ sequencing by NGS can provide a useful screening process by which the DNA of various organisms present in a sample can be identified, which can be followed by DNA ‘capture’ of a chosen organism of interest. DNA capture involves the molecular ‘fishing’ for a targeted genomic sequence and has had fruitful applications in aDNA research (Briggs et al., 2009; Knapp and Hofreiter, 2010). Many studies of environmental and sedimentary DNA use amplicon metagenomics, rather than shotgun metagenomics. Fundamentally, these two methods differ in that amplicon metagenomics sequences the rRNA or ribosomal DNA of organisms while shotgun metagenomics sequences the full genomes of organisms. Amplicon metagenomics targets the highly variable regions of the conserved 16S rRNA gene in bacteria or archaea or the 18S for eukaryotes, which can show what organisms are present, while shotgun metagenomics sequences full genomes allowing for information on both the structure of the organisms present in a community, and their functions within the larger structure (Kozich et al., 2013; Yarza et al., 2014).

A further methodological advancement that must be noted is an understanding and appreciation of the damage patterns that are characteristic of ancient DNA. DNA from long-deceased organisms is highly fragmented, and at the terminal ends of these fragments cytosine residues are prone to deaminate into uracil residues (March, 1977), which can be misread during DNA sequencing as being thymine (Briggs et al., 2007, p. 14616). By assessing the extent of cytosine deamination at the terminal ends of DNA fragments it may be possible to distinguish damaged (and likely ancient) DNA from undamaged (likely
modern) DNA. Software programs such as ‘mapDamage’ allow for the fast and efficient assessment of patterns of nucleotide misincorporation (Jónsson et al., 2013), which can help us to determine if DNA recovered from an archaeological specimen is endogenous or is derived from modern contamination.

These methodological developments, from cloning DNA fragments inside vectors of *Escherichia coli* bacteria, to PCR, to NGS, to amplicon metagenomics, has 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, 2006; Krause et al., 2010; Matisoo-Smith, 2015; Melzzer, 2015; Meyer et al., 2012; Pääbo et al., 2004; Pedersen et al., 2016; Reich, 2018; Slon et al., 2017), animal domestication (Eriksson et al., 2008; Larson et al., 2010, 2007; Meiri et al., 2013; Pires et al., 2019) and plant domestication and the accompanying spread of agriculture (Allaby et al., 2015; Palmer et al., 2011; Salamini et al., 2002; Shomura et al., 2008; 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, shotgun and amplicon 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.

2. Pitfalls and Promise

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 also necessary to explore limitations and pitfalls that may be encountered in this work. In this section, the potential and limits of aDNA research in coastal and maritime archaeology is explored.

2.1. Faunal and human skeletal remains

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 in addition to several human bones from terrestrial sites (Hagelberg and Clegg, 1991). 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 Jack Russell (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 inhabited modern buildings preclude their excavation. DNA analysis of faunal or human skeletal remains from submerged settlement areas 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).

At the inundated early farming site of Atlit Yam, Israel, Hershkovitz et al. (2008) claimed to have isolated and amplified tuberculosis (TB) DNA from two 9000-year-old skeletons. The human remains bore lesions that were consistent with TB, and cattle bones at the site appeared to support the theory that early cases of TB were contracted as a result of animal husbandry (Hershkovitz et al., 2008, p. 3). While no demonstrable amplicons of human nuclear microsatellite DNA were isolated from the skeletal remains, the authors remained confident that the conditions on the underwater site were conducive to ancient DNA preservation (Hershkovitz et al., 2008, p. 4).

Wilbur et al. (2009) questioned these findings and cautioned that destructive analysis in biomolecular archaeology should only proceed when a relevant scientific question remains unanswered. Without additional information about the TB strains involved, attempts to identify ever older cases of TB in humans may not have great scientific value, 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).

In the response paper by Donoghue et al. (2009), the authors defended the destructive analysis of human remains in the interest of investigating the health of an early farming community (Donoghue et al., 2009, p. 2799), rather than attempting to detect the earliest known case of TB in humans. Donoghue et al. (2009) did not address the point that it was now well-established that 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, 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
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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. Full mycobacterial genome sequences and tests 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 (Marchant, 2016). DNA analysis of this individual is ongoing (Schroeder, in preparation), and, if successful, may help illuminate aspects of mobility, seafaring, and the lives of sailors in the Classical Mediterranean. News reports claimed that this would be a feat never attempted before – DNA analysis on someone who has been under the sea for 2,000 years (Merchant, 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.

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 and sediment samples from the submerged settlements and shipwreck sites, 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 therefore would not relate to anthropogenic activities.

### 2.2. Plant remains

The study of archaeobotanical remains can offer insights into both ecological and cultural aspects of the human past (Bogaard and Outram, 2013). However, the majority of research has relied on terrestrial charred plant remains and phytoliths (Fiorentino et al., 2015; Harvey and Fuller, 2005; Hillman, 1981; Jones, 1996, 1984; Lightfoot and Stevens, 2012; Stirling et al., 2017; van Der Veen, 2007). Studies of both terrestrial and underwater sites. Failure to collect soil and sediment samples from the submerged settlements and shipwreck sites, 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 therefore would not relate to anthropogenic activities.

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 B.C.E. 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, 1987, 1986; 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 phyllographic identifiers. Results indicated that while plant chloroplast DNA was difficult to recover because PCR inhibitors in the waterlogged wood presented challenges, new pre-treatments before extraction were helpful, and the authors managed to obtain oak DNA from one of the 10 archaeological samples (Speirs et al., 2009). 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.

The combination of initial screening with NGS followed by DNA capture has been applied to waterlogged grape pips at sites spanning the Iron Age (circa 5th century BCE) to the Medieval period (circa 1200 CE), and has successfully yielded sufficient amounts of endogenous DNA to determine the genetic relationship of these ancient grapes to modern cultivars (Ramos-Madrigal et al., 2019; Wales et al., 2016). These findings offer important information pertaining to the inception and spread of viniculture throughout western Europe. In the latter study, 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). Olive stone samples were collected from the 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. More work on the ancient DNA analysis of waterlogged botanical remains is needed. For example, the largest individual deposit of Bronze Age olives was found inside a Canaanite jar on the Uluburun shipwreck, Turkey, with over 2000 olive stones, preserved in one vessel (Haldane, 1993). Genetic analysis of these olive stones by NGS is underway (Briggs et al. in preparation) and results like these should provide information about DNA preservation through analyses of DNA fragment sizes and damage profiles that can be compared with results from other types of sites. This will allow us to understand better how olive trees and other agricultural products were grown, propagated, and traded around the Mediterranean in prehistory.

2.3. DNA from ancient ceramic vessels

Ancient DNA studies have generally sought to extract DNA molecules for previously living organisms including humans, animals and plants. However, in 2008 and 2012 attempts were made to extract DNA from the ceramic matrix of shipwreck amphorae that had lain on the seafloor for over 2000 years (Foley et al., 2012; Hansson and Foley, 2008). 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, 2008, p. 910). It is therefore theoretically possible that DNA from past vessel contents can bind to the interior walls of amphorae and other ceramic transport containers.

Analysis of several shipwreck amphorae assumed to have contained wine was undertaken through destructive analysis (Hansson and Foley, 2008), and by non-destructive swabbing (Foley et al., 2012). Hansson and Foley assert that the conditions on shipwrecks could contribute to the preservation of ancient DNA, as protection from UV light and a lack of contact with subsequent terrestrial plant species would both limit the degradation of the DNA and protect the ceramic matrix from exogenous sources of plant DNA (Hansson and Foley, 2008, p. 1171; 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, 393). The PCR 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. Some grape DNA was detected in the amphorae assumed to have contained wine, but the authors also detected olive DNA, as well as DNA from several families of terrestrial plants including Juglandaceae (walnut family), Zingiberaceae (ginger family), Lamiaceae (family of herbs including thyme, oregano and sage), and Fabaceae (legume family) (Foley et al., 2012).

It is worth noting that swabs or 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 (Charters et al., 1993), as a way to discriminate between endogenous and exogenous sources of residue. 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 success claimed by Foley et al. (2012) for their non-destructive testing of ceramics to determine past vessel contents appeared to offer an undeniable benefit to archaeologists, ceramic specialists, and conservators.

The 2012 study tested both the non-destructive swabbing method, and the destructive method of scraping the interior that the authors claim had been successful in their 2008 study. Through non-destructive analysis by swabbing with a lysis buffer, the authors report ‘43 DNA hits from 27 swabbed samples’ and ‘16 DNA hits from 18 scraped samples’ (Foley et al., 2012, p. 394). Ancient DNA sequences from pine, olive, ginger, juniper, and oregano, among other species of plants, were detected. 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 found on terrestrial sites 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 the type of DNA in this archaeological material was speculative, a
confirmation bias may have been at play in these studies. The authors had decided before analysis what DNA they expected to find. Second, methods for sequencing all the aDNA in a sample and authenticating it have since been greatly enhanced (Briggs et al., 2007; Jónsson et al., 2013), and it is now common to do shotgun sequencing to identify the source(s) of the DNA present in the sample and then capture specific taxa to obtain full genomes with greater coverage, as well as 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 all ancient DNA studies of materials 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 of DNA in archaeological samples from these contexts.

Understanding the coevolutionary relationships amongst humans, plants, and animals can provide insights into how and why modern humans and modern civilisations developed as they did. Ancient DNA research on faunal and botanical remains from submerged sites and shipwrecks has a long history and continuing this research will provide a fruitful future direction for zooarchaeological and archaeobotanical investigations. However, our ability to confidently assign an ancient origin to DNA detected on submerged settlement sites in artefacts from ancient shipwrecks is dependent on the exhaustive exclusion of modern contaminant DNA.

2.4. SedadaDNA from submerged sites

As NGS and DNA capture supplplant PCR as the predominant method for characterising the complex assemblages of DNA present in environmental samples and microbial communities such as found in the microbiome (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 genetic material present within a sample typically using shotgun sequencing (Zepeda Mendoza et al., 2015, p. 745), or amplicon sequencing (Yarza et al., 2014; Ziesemer et al., 2015) and recent applications to archaeological material have focused on environmental DNA (eDNA) isolated from soils and sediments. Crucially, metagenomics characterises all the genetic material from all organisms in a given environment, rather than sequencing the DNA from one preselected organism.

SedadaDNA analysis has been recently applied to sediments and soils recovered from submerged landscape sites and inland lake environments (Niemeyer et al., 2017; Sjögren et al., 2017; Smith et al., 2015). 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). While these studies looked at freshwater, rather than marine environments, the results from these studies would appear to urge caution in our interpretation of sedaDNA from marine sites: as the marine environment is far more dynamic than lacustrine environments, it is even less likely that sedaDNA from marine sites would represent the entire complement of DNA from past environments.

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 geoaechaeologist, 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 8030 and 7980 years B.P. and represents a period in the British Isles for which there is little terrestrial archaeological (Smith et al., 2015, p. 999).

While interesting archaeological artefacts like corded fibre, burnt hazelnut shells and worked flint were found (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 6000 B.P., meaning that the Bouldner Cliff discovery pushed back the earliest example of wheat in this region by 2000 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, 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 as ancient in origin greatly enhances the credibility of novel DNA discoveries, the results from the Smith et al. (2015) study have proven to be somewhat controversial, and some would argue that more work is needed in order to securely establish the presence of wheat in the British isles 2000 years earlier than previously suspected.

As metagenomics data generated by NGS ostensibly 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). On the other hand, sedaDNA from sea-ice environments has detected taxa not represented in the fossil record (De Schepper et al., 2019), indicating that where the fossil record is incomplete, sedaDNA may be able to provide additional insights into what organisms inhabited a given environment in the past. These tandem studies that assess how well NGS data can detect nearby species which have been independently confirmed by palynological, fossil, 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. In addition, 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 such shifts in climate. 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.

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., 2009; 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 have been submerged due to rising sea levels. 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 processes that resulted in the peopling of the Americas. Potentially of great significance for the coastal and maritime archaeology of prehistoric sites are emerging theories that the peopling of the Americas involved coastal exploration by early communities, rather than exclusively relying on migration over land (Pedersen et al., 2016). 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.

A recent review of marine sedaDNA research has offered several suggestions on how best to obtain cores, avoid contamination after core collection, and prevent false positives during bioinformatics data analysis (Armbrecht et al., 2019). Suggestions include piston coring as being the most effective coring method for avoiding contamination, collection of seawater samples to assess the contaminant DNA likely present, and rigorous laboratory protocols to avoid introducing modern DNA during the extraction process (Armbrecht et al., 2019, p. 10).

As with skeletal remains, plant remains, and ceramics, the potential for the successful recovery of well-preserved sedaDNA from marine environments will depend on a number of factors including temperature, salinity, depth, sedimentation rate and proximity to land masses. The warm and fluctuating temperatures of shallow seas may not prove to be an ideal environment for the preservation of DNA. The consistently cold temperatures of seawater in Northern latitudes is likely to create a far more favourable conditions for the preservation of sedaDNA.

### 3. Discussion

#### 3.1. DNA and the marine environment

As a whole, the growing corpus of ancient DNA research conducted on material from submerged sites provides a fresh look at past environments, plant and animal domestication, migration events, and connectivity among past societies. Yet a chasm remains between our understanding of what DNA can be identified, and our ability to confidently assign an archaeological meaning to this data. This issue appears to be more prevalent in cases where the DNA identified is not said to derive from the organic material itself, i.e. human DNA from skeletal remains, or grape DNA from grape seeds. Two such case studies are examined above. The first study involved the detection of TB DNA in Neolithic human skeletons from the submerged site of Atlit Yam (Hershkovitz et al., 2008), and the second involved the detection of DNA derived from the past contents of ancient shipwreck amphorae (Foley et al., 2012; Hansson and Foley, 2008).

The most common pitfall of past research has been a failure to properly characterise the DNA present in the underwater deposition environment. For the Hershkovitz et al. (2008) study, it remains unclear whether or not the
TB detected in the skeletal remains could simply have been present in submerged soil layers present at the inundated Neolithic settlement site. For the Hansson and Foley (2008) and Foley et al. (2012) studies of ancient shipwreck amphorae, the DNA detected was claimed to derive from the past contents of the amphorae analysed. However, all the taxa detected are still cultivated in the Mediterranean today: olive, thyme, oregano, and legumes are not particularly exotic in Mediterranean contexts, and it remains a distinct possibility that the DNA reported to be from past vessel contents in these studies was, in fact, simply present in the either the underwater deposition environment, or in the museum storage areas where the recovered amphorae have been housed. It must be highlighted that both these studies utilised a PCR amplification approach. PCR is an immensely powerful tool for amplifying DNA, therefore if a tiny fragment of olive, oregano, or TB DNA was present in the environment, the primers designed to amplify these specific taxa may amplify this DNA to the extent that it appears to derive from the artefact analysed.

A second pitfall identified in this review is the failure to authenticate DNA recovered from marine archaeological sites as ancient in origin by analysing the damage patterns that may, or may not be, present in isolated DNA. It has long been established that the cytosine residues at the terminal ends of ancient DNA fragments are likely to deaminate, and that this can assist in the authentication of DNA fragments as having an archaeological origin (Briggs et al., 2007; Green et al., 2009; Jónsson et al., 2013). Neither the PCR experiments of Hershkovitz et al. (2008) or Hansson and Foley (2008) and Foley et al. (2012) were able to show that the DNA they identified was ancient in origin. In the case of the Smith et al. (2015) study that identified wheat DNA in Mesolithic archaeological layers, it is certainly possible that Mesolithic communities at the periphery of advancing agricultural trade routes had access to new food items via long networks of exchange that were not yet cultivated on site (Larson, 2015). Despite authentication by phylogenetic intersection analysis (Smith et al., 2015), these results have been questioned as the number of reads identified were too few to authenticate by analysis of the damage patterns of cytosine deamination (Weiß et al., 2015).

However, this second pitfall may be more complex than it initially appears. We still lack a complete understanding of the intricate relationship between the marine environment and the preservation of ancient DNA. The relative temperature, salinity, dissolved oxygen, and high ionic content of the seawater at a given marine archaeological site will doubtless contribute to the rate and extent of DNA damage. These factors could either accelerate, slow, or virtually halt cytosine deamination and nucleotide misincorporation. Permafrost environments on terrestrial sites have been shown to provide for exceptional DNA preservation (Poinar et al., 2006), and analysis of sea ice contexts suggest that preservation in very cold marine environments is also possible (De Schepper et al., 2019). Warm, shallow seas, on the other hand, that exhibit a frequent seasonal shift in temperature are less likely to create an environment conducive to DNA preservation.

The exclusion of oxygen may also affect the rate of decay of DNA on marine archaeological sites as cytosine deamination involves the addition of an oxygen molecule (March, 1977), and bacterial diagenesis will be significantly slowed in an anoxic environment. The newly discovered ancient shipwrecks of the Black Sea exhibit exceptional preservation of organic material due to the anoxic seawater environment that exists there (Davis et al., 2018; Markey, 2003), and it is possible that these sites will offer outstanding DNA preservation. In addition, early research into DNA depurination showed that there was a clear ‘salt effect’ in samples incubated in high ionic environments; the ‘salt effect’ significantly slowed the process of depurination in these samples (Lindahl and Nyberg, 1972).

Adding further complexity to the issue of DNA in marine strata is the fact that while work has been done on the vertical leaching and migration of DNA through terrestrial strata (Haile et al., 2007), our understanding as to what extent this applies to submerged paleosols is not yet clear. Bioturbation by marine organisms may also shift DNA through layers of marine sediment, while cephalopods have been observed transporting organic artefacts into ceramic transport containers on ancient shipwrecks (Frost, 1963), which can confuse our assessment of ‘vessel contents’ and could likely move around DNA associated with the organisms transported.

Given these unique aspects of the marine environment, which differ significantly between underwater archaeological sites, we must question whether established methods of assessing the characteristic damage patterns of ancient DNA from terrestrial sites can confidently be applied to underwater environments where extremes in temperature and salinity may significantly slow the process of DNA degradation. Further work is needed in this area. Organic remains from shipwrecks, which are eminently dateable, may offer great promise for this area of research. By developing new methods of ancient DNA authentication that can take into account these unique aspects of the marine environment, we can more confidently assess the damage patterns of DNA recovered from underwater archaeological sites.

### 3.2. Future directions

It is vital that we address these challenges, as marine archaeological sites likely contain human remains, artefacts, and botanical remains that can offer direct insights into many of the ‘big questions’ that continue to propel archaeological research. The study of submerged sites can help to answer some outstanding research questions including the search for evidence of the peopling of the Americas, the earliest cultivation of plants and how these taxa co-evolved with their human cultivators, and how international trade and exchange propelled social transformations.

Evidence for human migration events by coastal navigation continues to build (Pedersen et al., 2016), giving us every reason to believe that some of the most important sites for understanding the peopling of the Americas, Australia, and Polynesia are currently underwater. Given the richness of marine resources, and the
propensity for Mesolithic communities to include fish as a major part of their diet (Alexandru Dinu, 2010; Mcquade and O’Donnell, 2007; Moundrea-Agrafioti, 2003; Perri et al., 2018), it is more than likely that a significant number of Mesolithic sites are now underwater due to rising sea levels. This has already been demonstrated at Doggerland (Gaffney et al., 2009, 2007). The discovery, excavation, and analysis of artefacts from such submerged prehistoric sites represents an important future direction for maritime archaeology.

While there is evidence that Neolithic communities did not consume marine resources to the same extent (Richards and Schulting, 2006), people continued to settle on the coast in this period. Many early farming communities are now submerged, and such sites on the Levantine coast have already provided new information about the cultivation of important crops such as olives (Gallili et al., 1997, 1993). Further investigation into the genetic makeup into the organic remains recovered from these submerged coastal sites may allow us greater insight into plant domestication and early agricultural practices.

The discovery of deep-sea shipwrecks far from shore has already disrupted long-held beliefs surrounding ancient navigation methods (Ballard et al., 2002), and it is likely that by a further examination of the genetic makeup of their cargoes we will better understand trade, connectivity, and social transformations in antiquity. The largest deposit of olive stones from the Bronze Age ever excavated was found on a shipwreck (Haldane, 1993), which suggests that many more discoveries of equal or greater importance to our understanding of plant domestication, animal domestication, and networks of exchange will be possible by the further study of ancient shipwrecks and the cargoes they carried. A watery world of ancient DNA discoveries is waiting for us under the sea.

4. Conclusion
As with ancient DNA research in general, which suffered from missteps and false positives in the early phases of the development of the discipline, the successful genetic analysis of archaeological material from coastal and maritime sites has not always been smooth sailing. However, advances in ancient DNA sequencing, amplification, recovery, and capture continue unabated, while simultaneously costs for this analysis continue to fall. This will allow a far greater number of archaeologists and Principal Investigators to utilise this technology in an effort to better understand the sites we study.

In this review, the pitfalls, promise, and future directions of ancient DNA research of coastal and maritime sites have been discussed, with an emphasis on the promise this field continues to hold. Just as insights into human evolution, migrations, agricultural developments, and animal domestication can be arrived at from DNA research of material from terrestrial sites, there is great promise for this research to continue to extend into the watery realm of underwater archaeology. As submerged prehistoric sites have been a key topic of archaeological discourse in the last decade (Sturt et al., 2018), it is this area of research that is predicted to have the greatest focus for ancient DNA research in the coming years. However, as the allure of these sites, shrouded in the greatest antiquity, hold an undeniable appeal, it is not only inundated landscapes that hold important clues to our human past. Ancient shipwrecks, harbours, lake dwellings, and sunken cities are also likely to yield numerous artefacts and environmental samples of great utility to the archaeological scientist in the coming decades. By following increasingly comprehensive methods for authenticating aDNA, and by understanding how DNA breaks down in archaeological material on both terrestrial and underwater sites, we can continue to confidently incorporate aDNA studies into coastal and maritime archaeological research.

Competing Interests
The author has no competing interests to declare.

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