Manuscript Details

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Title	Ancient DNA from marine sediments: precautions and considerations for seafloor coring, sample handling and data generation
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Abstract

The study of ancient DNA (aDNA) from sediments (sedaDNA) offers great potential for paleoclimate interpretation, and has recently been applied as a tool to characterise past marine life and environments from deep ocean sediments over geological timescales. Using sedaDNA, palaeo-communities have been detected, including prokaryotes and eukaryotes that do not fossilise, thereby revolutionising the scope of marine micropalaeontological research. However, many studies to date have not reported on the measures taken to prove the authenticity of sedaDNA-derived data from which conclusions are drawn. aDNA is highly fragmented and degraded and extremely sensitive to contamination by non-target environmental DNA. Contamination risks are particularly high on research vessels, drilling ships and platforms, where logistics and facilities do not yet allow for sterile sediment coring, and due consideration needs to be given to sample processing and analysis following aDNA guidelines. This review clarifies the use of aDNA terminology, discusses common pitfalls and highlights the urgency behind adopting new standards for marine sedaDNA research, with a focus on sampling optimisation to facilitate the incorporation of routine sedaDNA research into International Ocean Discovery Program (IODP) operations. Currently available installations aboard drilling ships and platforms are reviewed, improvements suggested, analytical approaches detailed, and the controls and documentation necessary to support the authenticity of aDNA retrieved from deep-sea sediment cores is outlined. Beyond practical considerations, concepts relevant to the study of past marine biodiversity based on aDNA, and the applicability of the new guidelines to the study of other contamination-susceptible environments (permafrost and outer space) are discussed.

Keywords	ancient DNA; marine sediments; deep biosphere; phytoplankton; contamination; seafloor; IODP; biomarkers; Mars

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Suggested reviewers	Fumio Inagaki, Mikkel Winther Pedersen, Chris McKay, Jill Banfield

Submission Files Included in this PDF

File Name [File Type]

Coverletter_LA_et_al_Marine_aDNA_review_R1.pdf [Cover Letter]

Response_to_reviewers_R1.docx [Response to Reviewers]

Armbrecht-et-al_aDNA_Review_R1_highlights.docx [Revised Manuscript with Changes Marked]

Abstract_R1.docx [Abstract]

Armbrecht-et-al_aDNA_Review_R1.docx [Manuscript File]

Fig.1.pdf [Figure]

Fig.2_IODP_CoringSystems.pdf [Figure]

Submission Files Not Included in this PDF

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Table1.xlsx [Table]

Table2.xlsx [Table]

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Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
No data was used for the research described in the article

THE UNIVERSITY of ADELAIDE

Prof. Ian Candy
Mr Timothy J. Horscroft
Editorial Office
Earth-Science Reviews

30.05.2019

Dear Prof. Candy and Mr Horscroft,

We herewith re-submit our invited review manuscript entitled "*Ancient DNA from marine sediments: precautions and considerations for seafloor coring, sample handling and data generation*" to Earth-Science Reviews (EARTH_2018_550).

The manuscript has been revised according to the reviewer's suggestions, and all changes are detailed in the "Response to Reviewers" document.

Our review now includes a 250-word abstract, 9,146 words of main text (including Acknowledgements and Funding Sources), two figures, two tables and 142 references. I declare that all co-authors have agreed to the submission.

Yours sincerely,

L. Armbrecht

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Response to Reviewers - EARTH_2018_550

Reviewer 1 - Comments	Author's response
The paper by Ambrecht et al. "Ancient DNA from marine sediments: precautions and considerations for seafloor coring, sample handling and data generation" aims at presenting the review of state-of-the-art practices in ancient DNA studies of marine sediments, as well as providing general guidelines for sampling and lab protocols, which should be applied in future, in particular in IODP missions.	
In general, the topic of ancient DNA is of the highest interest. However, in the present form, it may be more suitable for a journal of narrower scope. The manuscript would benefit a lot if it would be focused not only on technical aspects of coring, sampling etc. but also if it would provide a review of aDNA applications in various marine environments, time ranges, taxon groups etc. There are many recent studies (many of them included in the reference list) showing the potential of ancient DNA in the progress of science. Although several valuable reviews have been published during the last several years (e.g. Torti et al. 2015, Rawlence et al. 2014, Pedersen et al. 2015), there are still many issues, which could be covered – including the specific character of the marine environment. DNA-related problems revealed by the recent studies incorporate the limits of applications, the challenges, not only related to sampling and contamination, but also to bioinformatics, identification of taxonomic units, qualitative vs quantitative approaches etc.	We disagree with the reviewer's comment. This review forms the base of any future study in the emerging discipline of marine sedimentary ancient DNA, which is interdisciplinary in its core, and thus highly applicable to earth, marine, geo- and climate scientists. As Reviewer 2 also acknowledges, commonly used modern marine genomics techniques have been mis-applied to this new field, demonstrating the urgency to raise awareness amongst earth and marine scientists that appropriate ancient DNA techniques must be used if the aim is to acquire authentic ancient DNA; therefore, this manuscript is ideally suited to ESR and its readership. Our focus is on contamination and best-practise techniques, and we specifically point out in this review that many studies to date fail to provide adequate records of negative controls. Therefore, a review of currently reported taxonomic groups and age estimates is impossible and we do not provide further details than already given. However, we agree with the reviewer that more information can be provided on bioinformatics, identification of taxonomic units and approaches, and have expanded our data-analysis section accordingly.
Moreover, some of the chapters related to planetary exploration, although intriguing, are so far away from the main topic of the paper that they should be removed or shortened.	We have shortened this section in the revised version. However, we would prefer to retain this section and not entirely remove it, as in our opinion the marine aDNA research guidelines are relevant to other low biomass environments such as permafrost and other planets.
l. 38 – 39 "The study of ancient DNA … has recently been applied as a tool to characterize past and modern life in deep ocean sediments". First of all, the study of ancient DNA does not help in the characterization of modern life (we use modern DNA for that). Second, ancient DNA is studied and used for palaeoclimate etc. not only in oceanic sediments (see for instance numerous studies on ancient DNA in lake sediments).	We agree with the reviewer and have removed "modern life" from this sentence. We agree with the reviewer, however, this review focuses on the marine environment, which is introduced in this first sentence.
l. 89, l. 92-93 and elsewhere in the manuscript - "plankton" – here, and also later on in the manuscript, the authors focus only on plankton or deep biosphere. How about benthic organisms, which are also of importance and are used as indicatory species (e.g. foraminifera)? The aDNA of active swimmers (fish) may	We removed plankton in this context, and only refer to planktonic organisms where previous studies have focused on those in particular.

also be preserved and should not be neglected completely.	
l.128-132 – the authors refer to a paper by Kirkpatrick et al 2016, reporting retrieval of 1.4 million years old DNA and underlying that the "origin of DNA must be carefully considered". Later on in the text (l. 139-141), the authors list "to date, the oldest authenticated aDNA records" and do not take into account the one by Kirkpatrick et al (2016). It seems that the authors do not find the finding by Kirkpatrick et al. (2016) to be confirmed. I would expect much more detail information about this case – what was found in original paper and what was wrong in the opinion of the authors and why. Such a case is very interesting and important in terms of making progress in science, as the scientific community may learn from potential failures. Giving reasons for not recognizing the results obtained by Kirkaptrick et al. may give also a chance for them to address the constructive critics in the future correspondence or papers.	We acknowledge that Kirkpatrick et al. have taken utmost care that samples are not contaminated, certain precautions were not taken. For example, while sampling was undertaken immediately following core retrieval on the catwalk of the IODP research ship *RV Joides Resolution*, there is no mention of core liner decontamination before cutting, thus potential contamination of the inner core during cutting cannot be excluded. The authors also describe that PFTs (a chemical tracer) were run and below detection limit, however, it is unclear whether these low PFT concentrations were only measured at the centre of the core or also on the periphery (the latter would be a sign of unsuccessful tracer delivery to the core). All laboratory work was conducted in laminar flow hoods, these create air movement and are not as suitable as special ultra-low background DNA (ancient DNA) facilities; it is also not mentioned whether previous work on marine organisms has been performed in this hood. PCR was used to amplify the 16S V4 and V5 gene regions (each >100bp, thus surprising as aDNA is typically <100bp), then subtracting all but chloroplast derived sequences. We provide detailed information in this review on the biases of PCR and its unsuitability to study aDNA. We acknowledge that the decrease in cpDNA with depth measured by Kirkpatrick et al. is a good indicator for a realistic result, however, the possibility remains that the cpDNA signal might be derived from contaminating seawater DNA. The major diatom taxa detected, *Thalassiosira* and *Chaetoceros*, are indeed important contributors to the fossil record, but also highly represented in the water column. Better indicators for ancient DNA authenticity are, for example, DNA fragment size and degradation.
l.157 "2.1" is missing	Corrected.
l. 160 "where the DNA was not initially preserved for later analysis" – I am not sure what the authors mean. The same expression is used also in table 1. How could be DNA initially preserved for being analysed as ancient DNA?	We changed this to: 'aDNA research involves the biomolecular study of non-modern genetic material preserved in a broad range of biological samples'.
l. 170 – 182 definitions. The authors try to make an order in a number of similar terms used in literature. I find it very useful. However, after reading this paragraph, I find some confusing statements. First of all, the authors contrast aDNA and PalEnDNA (l. 171-172), while they seem to overlap, as also stated by the authors later on (l. 179-180). I suggest presenting the ranges of application of particular terms in form of figure (see for instance somehow similar figure in Torti et al. 2015). I also think that it is not necessary to add a new term 'marine aDNA'. This term is well covered by the existing term 'sedimentary aDNA'. If the authors find it necessary to define a new term then they should provide a precise definition. Does this term refer only to DNA of marine organisms? The DNA pool may	Both Reviewer 1 and 2 commented on the terminology and definitions of PalEnDNA, aDNA, sedaDNA and marine aDNA. Reviewer 2 suggests PalEnDNA to be superfluous, while Reviewer 1 criticises the use of 'marine aDNA' as both marine and freshwater environments can be influenced by freshwater and marine DNA sources, respectively. We agree that in regions characterised by brackish waters our term marine aDNA might indeed be too narrow, as such, we have adjusted our terminology and use an extension of the existing term sedaDNA ('marine sedaDNA') for ancient DNA from marine environments, on which this review focuses. We consider our table of definitions appropriate and do not see the need to display the definitions in a figure, neither did Reviewer 2.

contain also terrigenous DNA delivered with rivers etc (see for instance Torti et al. 2015 and references therein). On the other hand, some processes, e.g. tsunami, may deliver and deposit marine sediments containing DNA of marine organisms on land (e.g. Szczucinski et al 2016). Is the analysis of marine sediments so different from lake sediments to create a specific term? Please note that for instance in case of well-studied Black Sea, some of its older sediments were formed in lake conditions, not in marine. So, shall we use two separate terms in that case?	
l. 186 – chapter 2.21 – this chapter should be in my opinion much better illustrated (table/figure). It is one of the chapters, which potentially may attract attention also of non-aDNA specialists. Particularly interesting may be to show the limits. The authors have mentioned (l. 201-202), that in well-oxygenated deep-sea sediments aDNA was also preserved. However, it was also preserved in much less suitable settings as for instance coastal marshes (tsunami deposits mentioned above).	We have rewritten this paragraph to integrate the reviewers' comment on the retrieval of aDNA from oxygenated sediments, and outlining the limits of marine sedaDNA research with regard to environmental characteristics and age retrieval. However, we have not added a table or figure as neither would not add any information, and solely be a repetition of the text in the manuscript.
l. 195 – 'extremely small grain size … offer a high adsorption surface' – I do not think that it is extremely small grain size that matters, it is the high surface area (ratio of surface to volume).	We agree and modified this sentence.
l. 259 – 'geological' – actually it is a biomechanical process	We replaced 'geological' with 'biomechanical'.
l.280 – chapter 2.26 – it is the next chapter worth to be extended. For instance issue of comparison of various records (micropaleontological and DNA). The problem of quantification of aDNA record. The mentioned results from the Black Sea could be represented by a combined figure showing an example of the application of various proxies.	We expanded this section according to the reviewer's suggestion. However, we are unsure what type of figure the reviewer is requesting here - a timeline of events in the Black Sea, or a hybrid of figure of already published Black Sea results? In either case, we believe that re-illustrating Black Sea results/data exceeds the scope of this review, and have therefore decided against adding another figure in this context. (Instead we focused on refining Figure 1 (sedaDNA workflow) and adding Figure 2 (coring systems)).
l. 314 – chapter 3.1 - I wonder if the specific chapter only about IODP is really necessary.	We shortened this chapter considerably, keeping only information on available coring platforms, which provides important context for the following descriptions of drilling strategies suitable for deep seafloor aDNA recovery.
l. 324 – table 2 is not necessary. It is much easier to include these three points in the text.	We removed this table.
l. 334- 348 – provide at least the project title.	We shortened this section and project titles are no longer applicable.
l. 350 – chapter 3.2 on drilling strategies. Various details of coring systems are discussed. However, the chapter may be difficult to follow for not specialists – consider representing the coring systems and the differences between them on a figure. The authors claim that the paper is to be used by researchers working also in permafrost and other planets – make it accessible for them.	We have included a new figure showing the differences in coring systems.

l. 444 -447 - It is not clear why the authors expect the freezing to affect DNA leaching.	We added an explanation in the text.
l. 546 - 'quantitative' - it is not clear what do the authors mean. A number of sequences?	We removed ‘quantitative’ in this context.
l. 615 - chapter 5. This chapter is poorly linked with the main topic of the paper and in fact, could be shortened to a single paragraph. This paper introduces marine aDNA guidelines (which are not yet established), and in this chapter an extensive description of its potential applications are discussed for non-marine settings. In particular for planetary exploration - very attractive topic, however in situation, when we are not sure if there is any life on other planets, not to mention if it is DNA-based, I do not find useful to discuss if suggested coring techniques, contaminant treatments for marine settings etc. may be useful in planetary exploration on Mars and other planets and moons. In fact, the chapter 5.1 reveals more on applications of experience in studies of aDNA in permafrost for marine settings (630-635) than vice versa.	We have shortened this section in the revised version. However, we would prefer to retain this section and not entirely remove it, as in our opinion the marine aDNA research guidelines are relevant to other low biomass environments such as permafrost and other planets.
The manuscript could be also enriched in figures (there is only a single figure, so far). A good picture is worth a thousand words.	We added a figure showing coring systems.
The references need to be rechecked throughout – many cited references are not in the reference list (over 20!) and vice versa. I have listed below some of the references cited in the text and not included in the reference list but it needs to be rechecked once more. Also, the alphabetic order in the references is not followed, in particular in cases of the same first author. The references cited in the text and missing in the references: l. 84. Ambrecht et al. 2018 l.86 Loucaides et al. 2011 l. 89 Castaneda et al. 2011 (do you mean Castaneda and Schouten?) l. 134 Coolen et al. 2011 l.189 Boere et al. 1008 (should be 2009?) l. 191 (should be Lyon?) l.241 Levy-Booth et al. 2007 l. 291 Calvert et al. 1987; Hay 1988 l.292 Major et al. 2006 l.303 Lyra et al 2013 l. 338 Frueh-Green – be consistent in writing the name with the reference list l. 512 Brotherton et al. 2012 (should be 2013?) l. 547 Klappenback et al 2001 l. 622 Bossenkol et al 2012 l.625 Gittel et al. 2014 l. 631 Neghandhi et al 2016 l. 640 McConnell et al 2007 l.661 Grotzinger et al 2012 l. 1179 (Table 1) - van Everdingen 1998, Fry et al. 2003	We corrected the reference list.

l.1186 (Table 3) - Haile et al. 2012, Leite et al. 2014, Bidle et al 2007	
Reviewer 2 - Comments	**Author's response**
I think this is a nice and timely review in the field of ancient DNA (aDNA) research in marine sediments. Although I am not particularly expert in marine and freshwater sediments I have good knowledge of aDNA studies in terrestrial sediments and the two aDNA research fields suffer of similar contamination problems. As also the authors suggest, many aDNA studies recently have not succeeded in reporting exact measures taken to prove authenticity of results, particularly studies dealing with environmental DNA (eDNA) extracted from sediments and studies to investigate ancient microbiome communities. It is true that modern bacteria and other microorganism are present nearly in every part of our environments, from open research fields to modern clean laboratories. It is also true that in many cases common and standard molecular laboratories have been used in such studies for extracting DNA from ancient sediments and prepare samples for sequencing and that procedures for subsampling from cores have not been documented and reported carefully. This review is therefore very welcome and hopefully will encourage researchers dealing with aDNA data from marine environments to take all necessary precautions during sediment coring, sample handling and data generation.	
I have only some minor comments that hopefully the authors will take into consideration before publication.	
In general I agree with most of the suggestions provided by the authors and with most of their statements. However, I would give more importance to contamination that often occur in the laboratories during DNA extraction and PCR/library preparations rather than in the field during coring when is really hard to avoid it. Contamination is never possible to reduce to zero and will unfortunately always occurs. However it is possible to minimize and to monitor it during all steps. Therefore, rather than insisting on the importance of performing coring in sterilized conditions, which is indeed crucial but very hard to do especially on ships, I would stress much more the importance of avoiding contamination during subsampling and during analyses in the laboratories, as here it is indeed possible to work efficiently to minimize it. No matter how clean we work on the ship and during coring it is very likely that contamination will occur from the modern environment during sampling. What is crucial therefore is to clean samples as much as possible prior to analyses and especially during subsampling to remove the outer part of the samples using sterilized tools, wearing lab mask, lab coats, gloves etc. In order to sample the internal uncontaminated part of the core it is therefore preferable to use larger rather than smaller corers in order to get as much material as possible. A second	We refined section 3.5 "Marine aDNA sample processing and analysis" according to the reviewer's suggestion.

important step to minimize and monitor contamination is the use of negative controls during DNA extraction and PCR/library preparation. Both types of controls, as also mentioned by the authors, should always be processed in parallel with sediment samples from PCR, to DNA sequence and to bioinformatic analyses. It is not enough to measure the DNA amount and even if this is zero, controls must be analyzed all the way along all steps.	
There is one paper on which I have strong doubts about authenticity (Inagaki et al. 2005). I don't think it is possible that the authors have extracted and analyzed DNA from a continental core 108 million years old. Such results are very likely created by contaminants and therefore not authentic and should therefore not be used to support any statement in this paper. This is especially true since the authors say correctly that at the moment the oldest authenticated DNA sequences comes fro remains that are ca 700 kyr (Orlando et al. 2013).	We agree with the reviewer and do not cite this paper.
Please notice that on line 225 the reference is not correct (Inagaki et al. 2015 should be 2005), therefore I would not call this as a 'recent' study.	There are two different publications, Inagaki et al. 2005 and Inagaki et al. 2015. The latter study reports on slow-growing live microbes in 2.5km deep ocean sediments, and is cited in our manuscript. There is no reference to Inagaki et al., 2005 (reporting on 100 Mio. years old ancient microbes).
Line 160. Maybe I miss something here but I don't understand the meaning of the sentence: 'where the DNA was not initially preserved for later analyses'.	We have changed this sentence to: "aDNA research involves the biomolecular study of non-modern genetic material preserved in a broad range of biological samples."
Line 170. The term PalEnDNA is in my opinion superfluous. In literature we have already several established acronyms (aDNA for ancient DNA, eDNA for environmental DNA, sedaDNA or sedDNA for sedimentary ancient DNA, see Pedersen et al. 2015, Ficetola et al. 2015, Parducci et al. 2017). My suggestion is to use only: aDNA, eDNA, sedaDNA and marine aDNA.	We agree with the reviewer that the term PalEnDNA is somewhat superfluous, and have re-written this paragraph to give this term less emphasis. However, as it has been used in the literature to describe ancient DNA from a variety of environmental samples, we decided to retain a brief explanation of this term in the text and Table 1. Additionally, in response to this comment and the comment made by reviewer 1 regarding the terminology, we now use the term 'marine sedaDNA' throughout the text.
Line 178. Some of these references are not correctly cited. Giguet Covex, Pansu and Alsos papers are about lake sediments and investigate mainly plants, but also animals growing around lakes and therefore in terrestrial environments.	We adjusted this sentence. ("Modern sequencing technologies and bioinformatic tools ease the analysis of these complex environmental aDNA samples and of the biological responses to human or climate change, with investigations having focussed on terrestrial settings (Jørgensen et al., 2012; Giguet-Covex et al., 2014; Willerslev et al., 2014; Alsos et al., 2015; Pansu et al., 2015).")
Line 191, I am a bit uncertain wether Lindhal paper suggests that hydrostatic pressure contribute to DNA preservation. Are the authors sure of this statement?	We removed this statement.
Line 201: well-oxygenated is misspelled.	Corrected.
Line 266: There is no leaching in lake sediments (Parducci et al. 2017 New Phyt). There should not be either leaching occurring in marine sediments in my opinion.	We agree with the reviewer and have mentioned this in the text (section 2.2.5). However, in terrestrial non-frozen sediments leaching has been found to be a factor (Haile et al., 2007), and as no studies exist that

	investigate potential leaching in marine environments, its possibility cannot be excluded to date.
Lines 296-298 I don't agree with this statement especially because based on Inagaki et al. 2005 on which I have doubts.	We have changed this sentence, however, as mentioned above we only refer to Inagaki et al. (2015), only (not Inagaki et al., 2005).
Line 358: I don't know what a drill-ship is exactly. Do the authors mean from a stable platform like MSP or from a ship? Maybe this can be explained for non-experts.	We have modified this sentence, to clarify that we are referring to a ship that is capable of performing drilling operations.
Lines 486-490: I am not sure about the statement that samples from the top part of the cores should be subsampled and processed in a non-aDNA laboratory. In these samples DNA even if more abundant is always fragmented and damaged and therefore ancient; contamination risk remains therefore high. If the authors means instead that this increase the risk for 'cross-contamination among samples then I only partially agree since cross-contamination must be always avoided regardless of the amount of DNA present in the ancient samples.	We removed this statement.
Lines 498-501: this depends also on the approach used: metabarcoding or shotgun sequencing. Using the latter in combination with capture technique may increase ability of detecting rare samples/species, particularly if these are present in the reference database.	We added this information to the text.
Lines 533-534: I think it is very good that this review brings up this problem, which is indeed serious. I would strength even more the importance of using strict aDNA methodologies and facilities in this field.	We added a sentence of the end of this paragraph to stress again the importance of strictly using aDNA facilities and methodologies as suggested by the reviewer.
In chapter 4.2 I would add one point here on the importance of negative controls and that these must be always processed and sequenced (and result shown) along with sediment samples.	We welcome this suggestion by the reviewer and have added this point to the 'future priorities' list.
Figure 1 is a too simplified and lacks important details. I suggest the authors to provide more details and improve the figure as well as the legend as this is an important figure for this review.	We updated this figure and added some more details on controls to be taken.

Highlights corresponding to changes indicated in 'Response to Reviewers'

Title:

Ancient DNA from marine sediments: precautions and considerations for seafloor coring, sample handling and data generation

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Abstract

The study of ancient DNA (aDNA) from sediments (*seda*DNA) offers great potential for paleoclimate interpretation, and has recently been applied as a tool to characterise past marine life and environments from deep ocean sediments over geological timescales. Using

*seda*DNA, palaeo-communities have been detected, including prokaryotes and eukaryotes that do not fossilise, thereby revolutionising the scope of marine micropalaeontological research. However, many studies to date have not reported on the measures taken to prove the authenticity of *seda*DNA-derived data from which conclusions are drawn. aDNA is highly fragmented and degraded and extremely sensitive to contamination by non-target environmental DNA. Contamination risks are particularly high on research vessels, drilling ships and platforms, where logistics and facilities do not yet allow for sterile sediment coring, and due consideration needs to be given to sample processing and analysis following aDNA guidelines. This review clarifies the use of aDNA terminology, discusses common pitfalls and highlights the urgency behind adopting new standards for marine *seda*DNA research, with a focus on sampling optimisation to facilitate the incorporation of routine *seda*DNA research into International Ocean Discovery Program (IODP) operations. Currently available installations aboard drilling ships and platforms are reviewed, improvements suggested, analytical approaches detailed, and the controls and documentation necessary to support the authenticity of aDNA retrieved from deep-sea sediment cores is outlined. Beyond practical considerations, concepts relevant to the study of past marine biodiversity based on aDNA, and the applicability of the new guidelines to the study of other contamination-susceptible environments (permafrost and outer space) are discussed.

Keywords: ancient DNA; marine sediments; deep biosphere; phytoplankton; contamination; seafloor; IODP; biomarkers; Mars

Abbreviations: aDNA, ancient DNA; APC, Advanced Piston Corer; HLAPC, Half-Length Advanced Piston Corer; IODP, International Ocean Discovery Program; mbsf, metres below seafloor; MSP, Mission Specific Platforms; NGS, Next generation Sequencing; PCR,

polymerase chain reaction; PFT, perfluorocarbon tracer; PMCH, perfluoromethylcyclohexane; PFMD, perfluoromethyldecalin; *seda*DNA, sedimentary ancient DNA

1 Introduction

Past marine environments have generally been investigated using a suite of methodological approaches and interdisciplinary research fields, such as geology, organic and inorganic geochemistry, paleoceanography and micropaleontology. Discoveries in all of these disciplines have contributed greatly to our understanding of the climatic history of Earth and the evolution and responses of its inhabitants. However, to date, it has not been possible to achieve a detailed picture of all living organisms that have occupied global oceans in the past, restricting estimates of past environmental conditions and climate. The techniques that have traditionally been applied to reconstruct marine palaeo-communities are limited, such as microscopy to investigate the microfossil record (e.g., Winter et al., 2010; Armbrecht et al., 2018). Due to dissolution and degradation of phytoplankton and microzooplankton while sinking to the seafloor post-mortem, only the most robust skeletons and shells are preserved within a complex geological record (Loucaides et al., 2011). Often, these microfossils are broken, altered by chemical processes and unrecognizable. In the absence of well-preserved diagnostic morphological features, lipid biomarkers can provide supplementary information on biological sources in sediment records (Volkman et al., 1998; Coolen et al., 2004; Sinninghe Damste et al., 2004; Brocks et al., 2011), however, the majority of plankton members do not possess highly diagnostic biomarkers.

New marine metagenomic approaches have allowed the routine characterisation of the diversity of both living hard- and soft-bodied plankton communities in the water column and sub-seafloor. Large-scale “omics” studies, such as the Tara Oceans project (a global sampling program to characterise pro- and eukaryotes of the surface ocean), have shed a new light on our understanding of modern (present day) marine ecosystems and diversity (de Vargas et al., 2015; Sunagawa et al., 2015; Carradec et al., 2018). The deep sea and sub-seafloor have

also been targeted with high-resolution metagenomic surveys revealing new insights into the abundance and composition of organisms existing in these largely unexplored environments (e.g., Zinger et al., 2011; Bienhold et al., 2016; Inagaki et al., 2015; Morono and Inagaki, 2016; Orsi et al., 2017, respectively). Such comprehensive studies on living marine communities are continually improving genome reference databases for the hundreds of thousands of pro- and eukaryotic organisms present in the marine environment (Sunagawa et al., 2015; Klemetsen et al., 2017). As a consequence, modern marine metagenomics has not only inspired marine palaeo-research, but also created a means of identifying ancient taxa from marine sediments over geological timescales.

In the last decade, marine palaeo-research has been reinvigorated by genomic techniques that enable the analysis of ancient DNA (aDNA) molecules from long-dead organisms. Past prokaryotic and eukaryotic plankton communities have been reconstructed using aDNA sequencing approaches (e.g., Coolen and Overmann, 1998; 2007; Coolen et al., 2004; 2008; 2013; Bissett et al., 2005; D'Andrea et al., 2006; Boere et al., 2009; Lejzerowicz et al., 2013; Hou et al., 2014; Randlett et al., 2014; More et al., 2018). These studies have confirmed that phyto- and zooplankton are good targets for aDNA-based studies, while also being particularly relevant for ecosystem-climate reconstructions. It is reasonable to assume that obligate photosynthetic plankton (phytoplankton) and/or zooplankton do not survive and reproduce after burial in deep sediments, and represent uncommon lab contaminants (e.g., Lejzerowicz et al., 2013; Hou et al., 2014; More et al., 2018). aDNA analysis has shown that even after their voyage through the water column plankton-derived particles that had settled on the seafloor still reflect the global biogeographic patterns of living species (Morard et al., 2017). Notably, the reconstruction of past marine communities using aDNA is possible using just a few grams of sediment, facilitating sediment sample collection, transport and storage for the purpose of aDNA analyses.

The marine aDNA archive extends back to the Pleistocene, as shown by studies of genomic, 18S rRNA gene markers targeting various eukaryotic groups. For example, aDNA has been

recovered from various eukaryotic plankton taxa in 43,000-year-old Arabian Sea sediments (More et al., 2018). Taxon-specific approaches targeting small, degraded DNA fragments allowed the retrieval of foraminiferal aDNA from ~800-year-old fjord sediments (Pawlowska et al., 2014) and ~30,000-year-old deep-sea sediments with the additional benefit of enabling the detection of rare taxa (Lejzerowicz et al., 2013). However, if a targeted approach is used, the origin and fate of the DNA in question must be carefully considered, especially for very old claims, such as the retrieval of 1.4 million years old DNA from chloroplasts (Kirkpatrick et al., 2016), which are subject to kleptoplasty (sequestration and maintenance of chloroplasts; Bernhard and Bowser, 1999). While Kirkpatrick et al. (2016) used thorough contamination control, the finding of >1 million years old DNA remains to be replicated using adapted control measures (e.g., sediment core decontamination and metagenomic sequencing, as outlined in this review). Most studies to date have involved well-dated sediment records and used a cross-validation through paired analysis of aDNA and diagnostic lipid biomarkers as well as geochemical proxies (e.g., Coolen et al., 2006; 2009). Yet, the absence of modern contaminants in analysed samples was not always verified through sequencing analysis of negative sampling and/or extraction controls, which is crucial for the interpretation of aDNA data even if DNA values measured following amplification (by polymerase chain reaction; PCR) are zero (as DNA may be present but simply be below detection limit). To date, the oldest authenticated aDNA records are from ~400,000-year-old cave sediments (Willerslev et al., 2003) and ~700,000-year-old permafrost mammal bones (Orlando et al., 2013).

Despite technologies now being available to rapidly extract and sequence aDNA from marine sediments, and the enormous potential of aDNA research to improve palaeo-oceanographic, -ecosystem and -climate models, marine *seda*DNA studies remain scarce. This is mainly due to the difficulties and high costs associated with deep-sea aDNA material, for which rarity and hence value justify the deployment of state-of-the-art practices. We review current problems and pitfalls incurred in ship-board sediment sampling, laboratory processing and computational analysis. We suggest solutions to improve sediment coring and sampling

strategies so that aDNA research can become a well-established staple in marine biogeosciences. The focus is on sampling protocols within the framework of the International Ocean Discovery Program (IODP) “Biosphere Frontiers” theme, which is dedicated to understanding sub-seafloor communities. Our guidelines for deep-ocean *seda*DNA isolation are applicable to any low-biomass and setting, including permafrost regions or planet Mars.

2 Definitions and pre-sampling considerations

2.1 Ancient DNA (aDNA), sedimentary ancient DNA (sed aDNA), and palaeo-environmental DNA (PalEnDNA)

aDNA research involves the biomolecular study of non-modern genetic material preserved in a broad range of biological samples (Shapiro und Hofreiter, 2012; Table 1). When an organism dies, mechanisms that ensure DNA repair in the cell are no longer active, leaving the DNA to degrade over time (Allentoft et al., 2012). Eventually, DNA from dead specimens becomes ancient. aDNA is highly fragmented to an average length of less than 100 base pairs (bp), for example, an average length of 48 bp has been determined in the oldest microbial genome assembled to date - from a 48,000-year-old Neandertal (Weyrich et al., 2017). aDNA is affected by post-mortem oxidative and deamination damage, such as thymine enrichment at the end of DNA sequences (Briggs et al., 2007; Ginolhac et al., 2011). Both fragmentation and damage patterns can be used to authenticate aDNA, and damage can even be used to predict its age in certain scenarios (Kistler et al., 2017).

aDNA research mainly focuses on organismal DNA extracted from some tissue remnants of a wide range of single specimen (e.g., tooth, bone, hair, eggshell, feather). In contrast, environmental DNA (eDNA) focuses on disseminated genetic material found in environmental samples such as soil, sediment, water and ice (Taberlet et al., 2012a). Such samples contain complex mixtures of DNA from taxonomically diverse organisms (e.g., bacteria, archaea, plants, animals). In addition to aDNA and eDNA, the term sedimentary aDNA (*seda*DNA) has been coined to describe aDNA that is exclusively recovered from sediments (Willerslev et al.,

2003; Jørgensen et al., 2012). The term fossil DNA has also been used in pioneer studies where sedimentary plankton DNA and lipid biomarkers (i.e., "chemical fossils") derived from the same historical source organisms were analysed in parallel to validate the ancient DNA results (e.g., Coolen and Overmann, 1998; 2007; Coolen et al., 2004). To a lesser degree, 'palaeo-environmental DNA' (PalEnDNA) has also been used to describe disseminated genetic material in a broad range of ancient environmental samples including sediments as well as soil, paleosols, coprolites, water and ice (Rawlence et al., 2014). Modern sequencing technologies and bioinformatic tools ease the analysis of these complex environmental aDNA samples and of the biological responses to human or climate change, with investigations having focussed on terrestrial settings (Jørgensen et al., 2012; Giguet-Covex et al., 2014; Willerslev et al., 2014; Alsos et al., 2015; Pansu et al., 2015). In this review, we use the term 'marine *seda*DNA', which specifically refers to aDNA recovered from ocean sediments. A detailed list of terms frequently used in aDNA research and their definitions is given in Table 1.

2.2 Authenticity of marine aDNA

2.2.1 Environments favourable for marine aDNA preservation

Organic-rich sediments deposited in the deep, cold ocean under stratified and anoxic conditions present several favourable characteristics for the preservation of aDNA (e.g., Coolen and Overmann, 1998; 2007; Coolen et al., 2004; 2013; Boere et al., 2011). Oxidative and deamination damage is reduced in the absence of oxygen (Lindahl, 1993). The absence of irradiation (Lyon et al. 2010), the generally low temperatures (Willerslev et al., 2004), and the high concentration of borate (Furukawa et al., 2013) further contribute to DNA preservation. Additionally, the typically high mud content of deep-sea sediment offers a particularly well-suited matrix for the preservation and accumulation of DNA (Torti et al., 2015). The high surface:volume ratio of extremely small clay minerals in clay-rich sediments offer a high adsorption surface onto which DNA molecules can bind and remain sheltered from the

activity of nucleases (Dell'Anno et al., 2002; Corinaldesi et al., 2008, 2011, 2014, 2018). However, although the above listed properties have been reported to positively impact on DNA preservation, locations with other characteristics that seem less ideal might still be suitable for aDNA research. For example, well-oxygenated Atlantic deep-sea sediments and sand-rich coastal paleo-tsunami deposits have been used to extract and characterise aDNA from foraminifera (Lejzerowicz et al., 2013; Szczuciński et al., 2016, respectively). In conclusion, the preservation of aDNA in marine settings appears to be variable depending on regional environmental characteristics with less favourable to favourable conditions retaining aDNA between a few thousand to, at least, a few ten thousand years. More research is needed to estimate how far back in time authentic marine *seda*DNA can be detected, which could be achieved, for example, by investigating sediment records from various deep seafloor locations over geological timescales.

2.2.2 Marine *seda*DNA degradation and fragment length

18S rRNA gene fragments of past dinoflagellates, diatoms, and haptophytes as long as 500 bp in length have been amplified and sequenced (e.g., Coolen et al., 2004), after DNA was isolated from sediments exhibiting characteristics favourable for aDNA preservation (Section 2.2.1). Up to 20% of genomic DNA from haptophyte algae has been reported to still be of high molecular weight after 2,700 years of deposition in Black Sea sediments, and the ratio between 500 bp-long haptophyte 18S rDNA fragments and the concentration of haptophyte-diagnostic long-chain alkenones did not vary substantially for at least 7,500 years after deposition, indicative that both types of biomolecules from the same plankton source were equally well preserved (Coolen et al., 2006). This contradicts the generalised view that aDNA is characterised by fragment lengths of <100bp. Nevertheless, studies that report the recovery of exceedingly long aDNA fragments should be viewed with scepticism especially in the absence of sampling and extraction controls, where there is no indication on whether the data might reflect modern signals. However, to date, no data are available on average aDNA

fragment length for deep-sea sediments, which could be obtained from metagenomic shotgun sequencing. Gaining insights into the latter should be the focus of future research as this information will ultimately help to choose the most suitable and efficient aDNA extraction and sequencing library preparation techniques for degraded *seda*DNA (see Section 3.5).

2.2.3 Contamination sources by modern DNA

Key to the viability of marine *seda*DNA studies is the capability to differentiate between true ancient signals (representative that lived at a particular time-period in the past) and modern contamination (introduced through the sampling and analysis process, or naturally by the environment). Microorganisms and their DNA coat nearly every part of this planet (Weyrich et al., 2015) and a recent study has shown that slow-growing microbes even occur in marine sediments up to 2.5 km deep (Inagaki et al., 2015). The DNA of active deep-biosphere organisms is likely to blur the aDNA signal, as would be the case for microorganisms introduced to ancient sediment samples through the drilling process (see Section 3.2). Moreover, microbial DNA is widely present in laboratory environments and reagents, including in those labelled DNA-free (Salter et al., 2014). If PCR is applied to amplify aDNA, the DNA from modern microorganisms may amplify preferentially over damaged, fragmented aDNA and obscure the true aDNA signals within the sample (Willerslev and Cooper, 2005). Therefore, utmost care must be taken to control and account for contaminants and background DNA throughout the whole process of collecting, processing and sequencing aDNA, e.g., by including negative controls in every step of the analysis process (Fig. 1).

2.2.4 Intracellular vs. extracellular DNA

One approach to separating ancient from modern DNA in sediments has been to differentiate between intracellular and extracellular DNA. Intracellular DNA is defined as DNA contained within living cells, structurally intact dead cells and intact resting stages (e.g., bacterial spores,

or other cyst-forming plankton). Extracellular DNA is defined as DNA that has been released from cells and preserved for substantial periods of time through mineral and/or microfossil adsorption or within clay aggregates (Levy-Booth et al., 2007). Extracellular DNA represent an archive of taxa that were autochthonous at the time of deposition (Cornaldesi et al., 2008; 2011). DNA extraction methods have been developed to target either of these DNA fractions (Corinaldesi et al., 2005; Taberlet et al., 2012b; Alawi et al., 2014). However, it is difficult to prove at what time in the past the organism died, and its DNA became extracellular. Furthermore, the extra- and intracellular DNA pool may not always be clearly distinguishable as genetic material present in the environment might have been taken up by competent bacteria (Demanèche et al., 2001; Dell'Anno et al., 2004) and even by eukaryotes (Overballe-Petersen and Willerslev, 2014). It is also important to note that if only the extracellular pool was to be studied, the paleontological value of dormant yet ancient DNA (e.g., from cysts deposited far back in time) will be lost. Due to these issues, extraction techniques targeting only the extracellular portion are currently not recommended for marine *seda*DNA studies. Alternatively, bioinformatics approaches that can clearly identify ancient signals (Ginolhac et al., 2011; Kistler et al., 2017) are preferred options for authenticating aDNA sequences (Jónsson et al., 2013).

2.2.5 Vertical DNA movement in marine sediment cores

Three major processes are associated with the vertical movement of DNA in sediment cores: DNA leaching, bioturbation and migration. Bioturbation is a biomechanical process that results in the multidirectional re-organisation of sediments primarily in the upper 10 cm of the sub-seafloor (Boudreau, 1998). DNA leaching is a passive process describing the downward movement of DNA across sediment layers (Haile et al., 2007), without a lowermost boundary. The mixing of sediment layers, and consequently of modern and ancient DNA, can lead to misinterpretations of genomic data. Experimental trials to assess DNA leaching through terrestrial sediments exist (Ceccherini et al., 2007; Poté et al., 2007), with initial results

indicating that the extent of leaching depends on the taxonomic source (Haile et al., 2007). In Previous studies from lake sediments have shown that leaching is not a factor (Parducci et al., 2017), and in seafloor sediments DNA it seems to play a minor role as aDNA and lipid biomarkers derived from the same microbial source were found to co-exist or to be both below detection limit in marine sediments just centimetres apart (Boere et al., 2009; Coolen et al., 2006; 2009; 2013). In the latter studies it therefore appears that the pore size of the laminated sediments was too small for intracellular DNA to migrate, and that all extracellular plankton DNA was adsorbed to the mineral matrices. Recent studies showing *upwards* vertical pore fluid movement also demonstrate the potential for vertical migration of relict or intact DNA within sediments (Torres et al., 2015), and should likewise be considered. Vertical migration of relict or intact DNA is expected to be especially a concern in sediments with micron scale pore sizes and/or a low clay content and a poor capacity to adsorb extracellular DNA. Future experimental research is required to quantify DNA leaching and/or migration through marine sediments, acknowledging the challenge of replicating a complex environmental system varying widely in hydrodynamics and sediment type.

2.2.6 Cross validation of marine aDNA and palaeo-environmental proxies

In addition to using proper contamination controls, downcore changes in past plankton compositions inferred from marine *seda*DNA can be validated through a complementary analysis of independent biological (e.g., microfossils, lipid biomarkers) and geochemical proxies (indicative of the prevailing paleoenvironmental conditions) (Boere et al., 2009; Coolen et al., 2004; 2006; 2013; Hou et al., 2014; More et al., 2018). The most detailed comparison between past ecosystem changes using marine *seda*DNA and the paleo-depositional environment to date has been performed on Holocene sediments from the permanently anoxic and sulfidic Black Sea (Coolen, 2011; Coolen et al., 2006; 2009; 2013; Giosan et al., 2012; Manske et al., 2008). The anoxic and laminated sediments of this semi-isolated sea are devoid of bioturbation and form high-resolution archives of climate-driven hydrological and

environmental changes (Calvert et al., 1987; Hay, 1988). Episodes of postglacial sea-level rise ~9,000 years ago (Major et al., 2006) and sea surface salinity increase ~5,200 years ago (Giosan et al., 2012) have been associated based on *seda*DNA with freshwater to brackish/marine planktonic community transitions (Coolen et al., 2013). For example, the gradual increase in sea surface salinity coincided with the arrival of marine copepods (*Calanus euxinus*), which could only be identified through *seda*DNA analysis (Coolen et al., 2013) as these important zooplankton members generally do not leave other diagnostic remains in the fossil record besides difficult to distinguish resting eggs (Marcus et al., 1996).

Vice versa, paleoenvironmental conditions inferred from more traditional geochemical and micropaleontological proxies have been verified from parallel *seda*DNA analysis. By way of example, Black Sea sediments deposited since the last 2,500 years contain coccoliths from the calcified marine haptophyte *Emiliania huxleyi* whereas haptophyte-derived diagnostic long chain alkenones in the absence of coccoliths were abundant in up to 7,500-year-old sediments (Hay et al., 1991; Coolen et al., 2009). Paired analysis of long-chain alkenones and *seda*DNA analysis (18S rRNA) revealed that that the first haptophytes that colonized the Black Sea ~7,500 years ago were initially a mixture of *E. huxleyi* and a highly diverse suite of previously overlooked non-calcified haptophytes related to alkenone-producing brackish *Isochrysis* species. *E. huxleyi* remained the only alkenone producer after 5,200 years BP when salinity reached modern day levels (Coolen et al., 2009). It was concluded that while calcite dissolution prevented the preservation of *E. huxleyi* coccoliths in sediments older than 2,500 years ago, their molecular fossils (DNA fragments and long-chain alkenones) survived much longer and showed that in reality this marine haptophyte entered the Black Sea already shortly after the marine reconnection which occurred ~9,000 years ago (Coolen et al., 2009; 2013). Even more detailed analyses of *E. huxleyi* (targeting 250-bp-long mitochondrial cytochrome oxidase subunit I; mtCOI) indicate a series of transitions from possibly low-salinity to high-salinity adapted strains of *E. huxleyi* in the Black Sea (7.5 – 5.2 ka BP), to a different suite of strains during the most marine stage (5.2 – 2.5 ka BP), returning to low salinity strains after 2.5 ka BP. The latter transition coincides with the onset of the cold and wet Subatlantic climate

(Coolen, 2011) when the Black Sea experienced re-freshening from 32 to 18 ppt (Van der Meer et al., 2011; Giosan et al., 2012; Coolen et al., 2013). The analysis of similar length preserved sequences of viral major capsid protein (mcp) genes revealed a continuous co-existence of *E. huxleyi* and coccolithoviruses in the Black Sea since the last 7,000 years and that the same *E. huxleyi* strains, which occurred shortly after the marine reconnection returned with the same viral strains after the re-freshening during the Subatlantic climate thousands of years later (Coolen, 2011). More recently, detailed sedimentary 18S rDNA profiling targeting the shorter (130 bp) V9 region revealed that long-term expansion of past oxygen minimum zones (OMZ) created isolated habitats for unicellular eukaryotes (protists) capable of sustaining oxygen depletion either by adapting a parasitic life cycle (e.g., apicomplexans) or by establishing mutualistic connections with others (e.g., radiolarians and mixotrophic dinoflagellates). These examples show that *sed* aDNA can be used to identify biological sources of lipid biomarkers, to verify the reliability of paleoenvironmental information inferred from more traditional proxies, and to reconstruct past ecosystems at multiple trophic levels.

The reconstruction of subseafloor prokaryote communities is more complicated since the DNA may be derived from living intact cells in the sediment (see Section 2.2.4). However, 16S rRNA gene profiling from total (intracellular and extracellular) sedimentary DNA has revealed useful insights into sub-seafloor microbial indicators of the palaeo-depositional environment. For example, microbiomes in 20 million years-old coalbeds underlying 2 km of marine sediments were shown to resemble forest soil communities (Inagaki et al., 2015). Variations in bacterial communities found in Baltic Sea sediments have been linked to palaeo-salinity changes (Lyra et al., 2013). Orsi et al. (2017) showed that the genomic potential for denitrification correlated with past proxies for oxygen minimum zone strength in up to 43 ka-old Arabian Sea sediments. The presence of fermentation pathways and their correlation with the depth distribution of the same denitrifier groups, however, suggests that these microbes were possibly alive upon burial, but low postdepositional selection criteria may explain why they nevertheless formed a long-term genomic archive of past environmental conditions

spanning the last glacial-interglacial cycle (Orsi et al., 2017). Further studies are required to determine as to how far the persistence of this phenomenon extends with increased depth in the biosphere. Nevertheless, these examples show that the complementary analysis of marine *sedaDNA*-inferred past plankton composition and biological and geochemical proxies is a powerful tool to reconstruct palaeo-environments.

3 aDNA research in the International Ocean Discovery Program (IODP) framework

3.1 IODP infrastructure

IODP is the global community's longest marine geoscience program, operating for 51 years. Its scientific strategy has been to answer globally-significant research questions about the Earth's structure, and the processes that have, and continue to, shape our planet and its climatic history. More recently, additional focus has been cast on biological evolution and limits, particularly in the sub-seafloor environment, under the new Biosphere Frontiers theme (Bickle et al., 2011). This theme has been inspired by the rapidly evolving knowledge and technical capabilities across the multiple merging fields of molecular biology, microbiology, organic and inorganic geochemistry, and micropalaeontology and includes scope for the integration of marine *sedaDNA* research. IODP is currently serviced through three platforms, the United States of America's research vessel *JOIDES Resolution*, Japan's *Chikyu* and by the European consortium's Mission Specific Platforms (MSP). In recent years, the laboratories and storage facilities on the ships were modified, or purpose built, to ensure addressing Deep Biosphere questions was possible. As a result, the latest IODP decadal plan considered options to enable access to uncontaminated samples, their processing and preservation on-board. The latter has led to new coring technologies such as the Half-Length Advanced Piston Corer (HLAPC) allowing a coring depth extension of the conventionally used Advanced Piston Corer (APC), and the use of chemical contamination tracers such as perfluorocarbon tracers (PFTs) (see Sections 3.2 and 3.3, respectively). Particularly useful to aDNA studies may be

the development of remotely controlled instruments allowing sediment sampling at ambient pressure (MeBo; Pape et al., 2017) and a rock-drilling device (RD2; Früh-Green et al., 2015). Notable achievements under the new Deep Biosphere theme include the finding of millions of years old active microbial community from coal beds buried at 2.5 km below the seafloor (Inagaki et al., 2015), and the preservation of an imprint of the Chicxulub impact catastrophe (Cockell et al., 2017). A lot remains to be understood before this theme and its challenges are satisfactorily addressed and it is clear that scientists engaging in Biosphere Frontiers will push methodological, technological and multidisciplinary studies.

3.2 Coring strategies suitable for marine *seda*DNA retrieval

Ideally, marine *seda*DNA sampling involves multiple spatial replicates to ensure that the biodiversity captured is representative of a particular site and time period. However, the ability to collect multiple deep ocean sediment cores to characterise palaeo-plankton is hindered by high costs and logistical issues associated with drilling operations. Thorough planning and collaboration to maximise the use of expensive expeditions and precious deep ocean sediment core material are indispensable in marine *seda*DNA research. To date, several coring strategies exist that differ in machinery as well as sub-seafloor depth that can be reached, and their application is largely dependent on which drilling platform is used (ship or MSP), and what type of sediment is to be cored/drilled (soft sediment or hard rock). This review concentrates on describing piston coring strategies, which are generally better suited to retrieve sediments for aDNA analysis due to relatively low contamination risks. Rotary core barrel systems are required to drill some sedimentary and most igneous rocks, and as they operate with drill-heads and drilling fluids (e.g., seawater) the risk of contamination is dramatically increased (see Section 3.3, Fig. 2).

Piston coring, referred to as Advanced Piston Coring (APC) or Hydraulic Piston Coring System (HPCS), is used to sample unconsolidated or poorly consolidated (i.e., softer) marine

sediments. Briefly, these instruments are pushed into the sediment while a piston inside the core pipe creates a vacuum so that the collected sediment remains in the pipe during retrieval. Sediments obtained by piston coring preserve laminated sediments well, are associated with a relatively low risk of environmental contamination and the preferred method to obtain sediments for aDNA analysis (Lever et al., 2006; Smith et al., 2000; More et al., 2018; Fig. 2). Using the piston coring approach, a successive recovery of marine sediments has been achieved to a depth of ~490 m below seafloor (mbsf) (Tada et al., 2013). If only a few metres long (soft) sediment cores are required, gravity-based coring systems, such as a Kasten-, or a Multicorer provide a good alternative (Coolen et al., 2004; 2009). Progress has also been made towards modifying piston coring instruments so that contamination-free sampling is possible, at least for short (<4 m) sediments (Feek et al., 2011). For example, the 'Mk II sampler' uses an air and water-tight piston coring system with a pointed aluminium head, preventing contamination of the sampled sediment from smearing or water infiltration (Feek et al., 2011). However, to date this corer has only been used in shallow waters, thus it remains to be tested whether use of such an instrument would be feasible during coring operations in deeper waters and which modifications may be required.

3.3 Contamination tracing during coring

Deep ocean coring requires the lowering of coring instruments through hundreds to thousands of metres of seawater before the seafloor is reached, hence exposes the instruments to contamination by modern DNA (Fig. 1). This unavoidable issue has called for the development of methods for environmental DNA contaminant tracing during coring operations. One approach has been to compare biological material found in the contaminating source material (e.g., seawater, drilling fluid) to that of sub-seafloor communities, and to exclude all signals occurring in either from the final analyses (e.g., Expedition 330 Scientists, 2012; Cox et al., 2018). This approach can be implemented for either piston coring or rotary core barrel drilling, provided other sampling constraints associated with these coring systems can be

accommodated. However, this procedure does not account for potential “false negative” DNA signals that might indeed occur in both ancient sediments *and* modern contaminating material. However, in some cases, the microbial community structure of modern contamination (e.g., drilling “mud”) can be resolved, particularly if functional genes are being targeted in sediment samples (Cox et al., 2018).

Another approach has been the introduction of fluorescent microspheres, which are particulate tracers of 0.2 - 1.0 mm in diameter physically mimicking contaminating organisms. The microspheres have been introduced near the coring head, i.e., where the sediment enters the corer and coring pipe, spreading across the outside of the core (inside the pipe) while drilling, simulating particle movement (Expedition 330 Scientists, 2012; Orcutt et al., 2017). Microscopy has been used to quantify the number of microspheres at the periphery and in the centre of the core to assess contamination (Expedition 330 Scientists, 2012; Orcutt et al., 2017). Similar methods using other perfluorocarbon tracers (PFT’s) including perfluoromethylcyclohexane (PMCH) have been developed for the USA drilling vessel *JOIDES Resolution* (Smith et al., 2000) already in the early phases of IODP. Later, PMCH-based contamination tracing has also been applied during riser drilling on the *Chikyu* (Inagaki et al., 2015). During the IODP Expedition 357 (Atlantis Massif Serpentinization and Life), the PMCH tracer delivery system was further developed to fit the seafloor-based drilling systems MeBO (Pape et al., 2017) and RD2 (Früh-Green et al., 2015) (see Section 3.1). PMCH is highly volatile which can lead to false positive measurements in uncontaminated samples, therefore, more recent investigations during IODP expeditions have moved to the use of the heavier chemical tracer perfluoromethyldecalin (PFMD, 512.09 g mol^{-1}) (e.g., Fryer et al., 2018).

3.4 Subsampling after core acquisition

Key to enable interdisciplinary sampling and correlations of independent measurements is a detailed sampling plan, specifying sample types as well as the sequence in which these samples are to be collected. Sampling for aDNA is time-sensitive (to avoid exposure to oxygen, high temperatures and contamination), thus should be conducted immediately after core retrieval on an untreated core-half (i.e., prior to any type of scanning such as by X-Ray). The laboratory in which subsampling for aDNA is carried out should be clean and workbenches and surfaces decontaminated with bleach (considered to be most efficient at removing contaminating DNA) and, if applicable, ethanol (to prevent corrosion of metal after bleach-treatment). Detailed records on whether molecular and amplification techniques (i.e., PCR) have been employed in on-board laboratories and which organisms were targeted should be kept on record within IODP to ensure sampling for aDNA can be spatially separated from these laboratories. While most vessels are not currently equipped for complete DNA decontamination, such records may be invaluable for post-expedition aDNA data analyses.

Two sampling approaches are the most feasible on board IODP ships and MSP's: cutting whole round cores or direct subsampling after core cutting into 1.5 m long sections. The choice of approach needs to be made on a case-by-case basis, and depends on the specific facilities, consumables, chemicals and researcher expertise available during each mission. It is recommended that cutting or subsampling are performed under filtered air, e.g., a portable type of a horizontal laminar flow clean air system as described in Morono and Inagaki (2016). Additionally, subsampling should be conducted from the bottom to the top of the core (ancient to modern), using clean (e.g., bleach and ethanol treated) sampling tools for each sample to avoid any form of cross-contamination. Most commonly, soft sediments acquired by piston coring are used for *seda*DNA analyses, therefore, we focus on subsampling procedures of the latter here, subsequently briefly outlining sampling recommendations for hard rock material.

If the sampling decision is in favour of whole round core samples, the newly acquired core sections are cut into 5 - 50 cm sections (preferably under cold conditions), which should be

packed in sterile bags or wrap and transferred directly into a fridge or freezer. Although quick and providing a large amount of material for later sub-sampling, this approach has the disadvantage that a lot of freezer space is required, and post-expedition transport can be costly due to the high sample volume and weight.

An alternative to whole round core cutting is direct subsampling immediately after core cutting, either directly from the centre of the top or bottom of each unsplit core section (usually 1.5 m long), or after or splitting the core sections into two halves. In any case the core liner should be cleaned with bleach to remove potential contamination from seawater, and core cutters and splitting-wires, usually metal and sensitive to bleach should be cleaned with ethanol. If sampling from uncut sections, surface material (~0.5 cm) should be removed with bleach and ethanol-treated scrapers before sampling, which is most easily done with sterile cut-tip syringes, placed into sterile plastic bags and stored frozen.

If sampling is undertaken on split core halves, simultaneous visual sedimentological assessments are possible that enable more targeted sampling at specific depths of interest. Using DNA-clean tools, the top 0.5 cm of the core surface should be scraped off perpendicular to the core pipe using sterile scrapers (from bottom to top of the core). Alternatively, the core half to be sampled can be covered with plastic wrap, followed by powdered dry ice, which will result in the top 0.5 cm to become solid frozen. After 5 min, the frozen outer sediment layer can be lifted at one edge with a sterile scalpel creating a contaminant-free surface, from which subsamples can be taken (Coolen and Overmann, 1998). Then, subsampling should be undertaken using sterile (e.g., gamma-irradiated) plastic syringes or centrifuge tubes (e.g., capacity of ~15 mL). Cut-tip syringes have the advantage that more sediment can be collected as no pressure builds up when pushing the syringe into the sediment (the filled syringe should be placed into a sterile plastic bag immediately, e.g., Whirl-Pak®). Alternatively, sterile centrifuge tubes can be used as is to collect ‘plunge-samples’, usually providing ~1 - 3 cc of sediment material. The outside of the ‘mini-cores’ should be cleaned with bleach and placed

into sterile plastic bags to avoid cross-contamination between samples. For subsamples, storage at -20 °C or -80 °C is recommended as freezing has been shown to facilitate phytoplankton cell-lysis during DNA extractions (Armbrecht et al., *in prep.*). Sub-samples can also be collected by transferring a small amount of sediment into a sterile microcentrifuge tube using clean metal or disposable spatulas (particular care needs to be taken to avoid cross-contamination when using the same sampling tool for different samples). The latter approach may be a good solution when only a few small samples are required, e.g., to supplement other scientific questions of an ongoing expedition. For replication purposes it is recommended that duplicate samples are taken at each depth.

If the material is hard rock or similar, subsamples are most easily collected from whole round or split cores. The same decontamination procedures as outlined above should be considered throughout the subsampling procedure (i.e., decontamination of work-surfaces and sampling tools with bleach and ethanol, sampling under cold conditions and filtered or low air-flow, packing of samples into sterile bags before storage). A de-contaminated metal cutter or a hammer and chisel are best used to remove the outer layer of the exposed sediment, at least at those depths where subsampling is anticipated.

3.5 Marine aDNA sample processing and analysis

Marine aDNA samples should be processed in a specialised aDNA laboratory to prevent contamination with modern DNA. Such a laboratory is generally characterised by creating a low-DNA environment, with a clear separation of no-DNA (e.g., buffer preparation) and DNA-containing (e.g., DNA extraction) workflows, regular and thorough sterilisation procedures, positive air pressure, and protective clothing of the analyst (lab coat/suit, gloves, facemask, visor). Details on optimised laboratory set-up, techniques and workflows have been reviewed before (Cooper and Poinar, 2000; Pedersen et al., 2015). The introduction of aDNA samples

into such facilities is relatively straight-forward, as the outer packaging and surface of the sample can be easily sterilised (e.g., using bleach and/or UV).

As on-board subsampling, DNA extractions should be carried out from the most ancient to most recent samples, to prevent modern DNA indivertibly being carried to ancient samples. The amount of sediment used in DNA extractions should capture a representative picture of the biota present in a sample. Despite suggestions that bulk DNA extractions from up to 10 g of material can improve detection of taxa and better represent the diversity of the area of interest (e.g., Taberlet et al., 2012b; Coolen et al., 2013), using such large volumes of sediment is often not practical and can be quite costly in this field where typically many samples are processed. Instead, numerous studies have used replicate extractions of a smaller sample size (e.g., 0.25 g; Table 2) to increase the likelihood of yielding aDNA from rare taxa, as well as successive DNA extractions from a single 0.25 g sediment sample (e.g., Willerslev et al., 2003). Post-extraction, the use of RNA-probe based enrichment approaches coupled with shotgun sequencing, a common technique in aDNA research, may furthermore drastically improve the detection of rare taxa (Horn et al., 2012).

While it would be ideal to find one extraction method that will yield the best quality data and enable standardisation across ancient marine sediment studies, the type of sediment or target organisms may require some adjustments of standard protocols (Hermans et al., 2018). Extraction methods can bias the diversity observed due to differential resilience of taxa to the cell-lysis method (Zhou et al., 1996; Young et al., 2015) and DNA binding capacities of different soil and sediment types (Lorenz and Wackernagel, 1994; Miller et al., 1999). As a result, the aDNA extraction efficiency can be poor and the detection of an aDNA signal lost. To date, a variety of commercial kits have been successfully used to isolate aDNA from sediments (Table 3). To further increase the yield of very low amounts of highly fragmented aDNA several studies have been utilising extraction protocols that include a liquid-silica DNA binding step (e.g., Brotherton et al., 2013 and Weyrich et al., 2017 for non-sediment samples) or ethylenediaminetetraacetic acid (EDTA) cell-lysis step (Slon et al., 2017; utilising cave-

sediment samples). Other studies have replaced the Bead Solution in the DNeasy extraction kits (Qiagen; Table 2) by 1M sodium phosphate pH 9 - 10 and 15 vol% ethanol to efficiently release clay-adsorbed DNA, and to prevent DNA released from intact cells from adsorbing to clay minerals during the extraction (Direito et al., 2012; Orsi et al., 2017; More et al., 2018). The latter is especially important when working with low organic, high carbonate rocks and sediments (Direito et al., 2012).

Two points are particularly important to prevent contamination during extractions. Firstly, as with the samples themselves, it is crucial that all tools and reagents undergo rigorous sterilisation procedures before utilisation, such as by bleach and UV treatment of any packing material before entering ancient DNA facilities. Secondly, blank controls should be included for every step of the laboratory process, i.e., extraction/library preparation blank controls, sequencing and bioinformatic analysis controls (Ficetola et al., 2016). Controlling and monitoring contamination is particularly important when analysing bacterial diversity due to their presence in all laboratory environments and reagents (Weyrich et al., 2015). Optimally, extraction blanks are included in a 1:5 ratio (Willerslev and Cooper, 2005), with a bare minimum of one control with each set of extractions. Aside from bioinformatically removing any organisms determined in such extraction blanks from the investigated sample material, the contaminants should be tracked within a laboratory, and contaminant lists published alongside the data for reasons of data transparency and authenticity.

Post-extraction, many marine aDNA studies have employed methods that are routinely used for modern marine DNA analysis. Although modern DNA work is not exempt from precautions, there are several issues with aDNA work: (i) as outlined in Sections 2.1. and 2.2. aDNA is highly fragmented and degraded and any small amount of modern DNA present in the sample (from reagents, labs or living cells that were present in the sediment sample) will amplify over the aDNA; (ii) sampling and extraction controls are often not included in the sequencing sample; (iii) PCRs are often inhibited due to the co-extraction of humic substances, pigments and heavy metals along with DNA (Webster et al., 2003 and references therein), requiring

adequate removal of these impurities (e.g., Coolen et al., 2009); (iv) successful PCRs are prone to bias due to random amplification in reactions that contain very low amounts of DNA template, thus PCR drift (stochastic variation in the first PCR cycles) can occur (Wagner et al., 1994; Polz and Cavanaugh, 1998; Webster et al., 2003). More importantly, the number of, e.g., bacterial 16S and eukaryotic 18S rRNA operons can greatly vary between per genome and per cell and can cause a biased representation of the past community structure (e.g., Klappenbach et al., 2001). The above biases can be reduced and the detection limit lowered when PCR approaches selectively, amplifying particular groups of organisms indicative of environmental changes, are paired with independent geochemical proxies (e.g., Coolen et al., 2004; 2006; 2009). However, we strongly advocate for the use of strict aDNA methodologies and facilities in order to achieve the generation of authentic marine *sed*aDNA data, following the guidelines in this review.

Shotgun metagenomics are currently widely accepted and the least biased method to analyse the broad diversity of ancient environmental samples (e.g., Slon et al., 2017). Although only a small portion of the generated sequence data might be attributable to the ancient organism in question (Morard et al., 2017), next generation sequencing (NGS) generates large quantities of data that enable meaningful statistics, with the additional benefit of preserving the relative proportion of detected taxa. To analyse aDNA sequence data, robust bioinformatic pipelines (e.g., Paleomix, Schubert et al., 2014) have been developed and are available for the application to marine *sed*aDNA, integrating damage detection algorithms (e.g., Ginolhac et al. 2011; Kistler et al., 2017) that enable the distinction between ancient and modern signals. Determining the extent of cytosine residues deamination (C to T and G to A, Weyrich et al. 2017) should also be considered to assess authenticity of aDNA sequences, especially when the data was generated from mixed communities, such as from marine *sed*aDNA. It is furthermore crucial to carefully screen sequencing data for any low-complexity reads, which may get incorrectly assigned to taxa during alignments against genetic databases, as well as ensuring that taxonomic assignments in the database of choice are correct. Bioinformatic

pipelines removing such misidentification-derived errors do not currently exist and should be the focus of future research, as well as the comparison of shotgun and amplicon marine *sed*aDNA data to accurately determine biases and analysis strategies best suited to this new discipline.

4 Future marine aDNA sampling considerations

4.1 Equipment and installations required aboard IODP platforms

In addition to the recent upgrades and investments IODP has made to enable sediment sampling suitable for Biosphere Frontiers theme (Section 3.1) we suggest the following items to facilitate contamination-free sediment sampling and the tracing of contaminants.

(i) Laboratories in which sampling for aDNA is undertaken should be carefully chosen to minimise contamination. Rapid transport of the core from the deck to the lab, thorough decontamination measures (see Section 3.4), and easy access to fridges or freezers are crucial. While a positively air-pressured lab (standard for aDNA laboratories) may not be feasible, air-flow can be reduced by keeping all doors shut and fans off during aDNA sampling. Contamination by human DNA from analysts can be greatly reduced by wearing adequate protective clothing (gloves, facemask, freshly laundered/disposable lab coat/overall). A detailed record or any molecular work undertaken in ship-board labs should be maintained by IODP, and under no circumstances should aDNA sampling be conducted in labs used previously to run PCRs (see Section 3.4). Alternatively, the equipment of a shipping container exclusively dedicated to aDNA sampling could be a good solution to spatially separate aDNA sampling aboard drilling-platforms and installation could be as required during expeditions that involve aDNA sampling.

(ii) DNA is likely to behave quite different from chemical tracers and microspheres currently used to track contamination. With constantly advancing technologies in the field of synthetic

biology, the possibility arises to develop 'non-biological DNA' with known sequences. Such non-viable DNA tags are already used in the oil industry, where a different tag is introduced into oil pipes monthly to monitor when and where leaks occur (Forecast Technology Ltd). Using such tags during seafloor coring operations instead of chemical tracers should enable a precise assessment of contamination by environmental DNA, where bioinformatics pipelines could be adjusted to detect and quantify the amount of tags present in the final sequencing data.

4.2 Ground-truthing marine aDNA research and data

To ground-truth marine aDNA studies and to ensure the generation of authentic aDNA data we suggest future research in this field to focus on the following aspects:

(i) The establishment of a public record of common contaminants. This can be achieved, for example, through an inter-lab comparison focused on analysing the same samples and integrating extraction blanks to trace contaminants associated with particular coring equipment, ship- and land- based laboratories.

(ii) Investigation of factors that might considerably bias marine *seda*DNA data. This might include information on sediment-type and environmental condition dependent aDNA preservation, taxon-specific DNA degradation rates, average aDNA fragment length, and shotgun and amplification-based aDNA data comparisons.

(iii) Ongoing enrichment of genetic reference databases for modern marine plankton, to enable taxonomic assignment of the hundreds of thousands of ancient sequences expected to be found in marine sediments.

(iv) The inclusion of negative controls during extractions, library preparations and in sequencing runs, and the publication of the results in the context of independent multiproxy biological and environmental metadata obtained from the same sediment interval.

(iv) Once (i) - (iv) are addressed, the development of a dedicated aDNA coring proposal is encouraged, in which sediment cores are collected using the above outlined, best-suited coring strategies, sampling and analysis procedures. During such an expedition, basic questions such as optimal on-board contamination tracing techniques, feasible work-flows, spatial replication required to achieve representative community data, and age to which marine *seda*DNA can be determined should be addressed. Such baseline data is missing to date and remains the most important step towards the generation of authentic aDNA data from marine sediments.

5 Application of marine *seda*DNA research guidelines to other contamination susceptible environments

5.1 Permafrost

Permafrost molecular biological studies provide the opportunity to study living organisms that have successfully adapted to extremely cold environments and comprise an analogous cryogenic environment to that found on other planets, such as Mars (Amato et al., 2010). Molecular investigations have focussed on humans (Rasmussen et al., 2010), plants (Willerslev et al., 2003), megafauna (Boessenkool et al. 2012), fungi (Bellemain et al., 2013) and microbes (Willerslev et al., 2004). Permafrost top layers are characterised by a more abundant and diverse microbial community compared to the deeper soil (Gittel et al. 2014). To overcome the hurdle of distinguishing between the modern and ancient DNA signal, metatranscriptomics have been applied to identify the active community only (e.g., Coolen and Orsi, 2015). Despite the challenges in experimental approaches, such as rapid community shifts after thawing even at nearly ambient conditions (Negandhi et al. 2016), studies of permafrost environments have advanced our understanding of feedback loops associated with the response of extremophiles to warming, ultimately informing modelling studies including marine palaeo-environments.

Sampling for ice and permafrost in polar regions is challenging in terms of logistics and minimising contamination risks for both the sample and the sampled environment. For example, permafrost soil samples are, like marine sediment cores, retrieved through drilling, which can introduce microbial contaminants to the deeper permafrost soil layers as the drill head and liquid pass through the top active soil layer (Bang-Andreasen et al., 2017). Additionally, the cryosphere has been accumulating industrial chemicals and metals since the 1850's (McConnell et al., 2007), so that the present-day microbial community is now capable of degrading industrial contaminants, thereby representing an anthropogenically-adapted rather than an original pristine community (Hauptmann et al., 2017). With both these newly adapted anthropogenic and drilling fluid communities containing characteristics for heavy metal degradation, distinguishing indigenous ice core or permafrost communities from drilling fluid communities will become more difficult in the future (Miteva et al., 2014). Therefore, the described guidelines in this review for distinguishing ancient from modern and contaminant signals, as well as the need for aseptic sampling procedures, are highly applicable to permafrost environments and, more generally, the cryosphere.

5.2 Planetary exploration

The methodologies advocated in this review that enable aDNA in marine sediments to be distinguished from modern DNA are also applicable to the search for life on other planets or moons. Astrobiologists are especially interested in the possibility of detection of Life 2.0, where the life has an independent genesis to that on Earth. The search for life beyond Earth has been potentially possible since the 1970s, with the two Viking lander missions to Mars, but there are other possible targets in our solar system, notably some of the moons around Jupiter and Saturn (e.g., Europa, Titan). Space technology has now reached the point where the detection of life, if it exists or existed elsewhere in the solar system, is becoming a realistic possibility in the next 50 years. There have been several rovers that have carried out successful exploration of the surface of Mars, including Curiosity, the Mars Science Laboratory

that in 2018 is mid-way through its predicted mission (Grotzinger et al., 2014). The rover Mars 2020 is being designed at present to test for evidence of life in the near-surface environment. It will drill, collect and cache samples from the Martian surface, which will then be returned to Earth for more detailed analysis (Beaty et al., 2015). Sample return from Mars to Earth is planned for the end of the 2020's (Foust, 2018). Active planning is also ongoing for possible missions to land and analyse materials from the surfaces of moons such as Europa and Titan, by both NASA and the European Space Agency. For example, Europa (a moon of Jupiter) is known to have a global saltwater ocean below its icy crust, as well as a rocky seafloor, so is one of the highest priority targets in the search for present-day life beyond Earth (Hand et al., 2017). A key concern with this solar system exploration is planetary protection, which is governed by the United Nations Outer Space Treaty (United Nations Office for Disarmament Affairs, 2015) and the Committee on Space Research (COSPAR) of the International Committee for Science. There are two important categories of planetary protection. The first is "forward contamination", where Earth-derived microbial life hitches a ride on spacecraft and contaminates parts of a planetary surface being explored. The second is "backward contamination', where life from an explored planet or moon is inadvertently returned to Earth, maybe in a spacecraft or within a rock sample. The relevance to aDNA analytical protocols is in forward contamination (i.e., the risk of contaminating sample material that could lead to data misinterpretations, and/or generally introducing Earth contaminants to other planets; Rummel and Conley, 2017). It should be noted that if indeed there is or was life on other planetary bodies, it may well not be based on a genetic code composed of DNA and RNA. Independently originated Life 2.0 would be highly unlikely to have evolved exactly the same nucleic acid genetic code as life on Earth (e.g., Rummel and Conley, 2017). Indeed, it has been postulated that an alternative biosphere could exist as a "shadow biosphere" on Earth (Davies et al., 2009). If DNA or RNA-based extant life is found on Mars, for example, then it is most likely that it would represent either past natural exchange of rocks between the two planets (panspermia), or anthropogenic forward contamination. Therefore, the procedures used for distinguishing indigenous life in planetary exploration will need broadening to include the

possibility of life with a different genetic code. The protocols developed for aDNA sampling of marine sediments on Earth, including the ability to distinguish from modern DNA, have relevance for the designing of methods to look for past life on Mars or outer solar system moons using molecular biology techniques (Beaty et al., 2015; Hand et al., 2017).

Conclusions

Ancient DNA in marine deep-sea sediments holds the potential to open a new era of marine palaeo-environment and -climate reconstruction. However, anti-contamination measures central to all aDNA research have logistical constraints and are particularly poorly-suited to shipboard sediment sampling and processing. For example, sterile coring equipment and ultra-clean laboratories are usually not available on any type of drilling platform. Current and future IODP drilling vessels are aware of the increasing need for improved and innovative solutions to coring, non-contaminant drill fluids and appropriate laboratories and storage facilities. Such logistical advances should go hand-in-hand with the establishment of new criteria and standards to ensure the acquisition and preservation of sediment cores with minimal environmental contaminants. Complementary genetic and geochemical information currently available to date suggests that, realistically, environmental reconstructions based on marine *seda*DNA from past plankton can be achieved for at least the last glacial-interglacial cycle, and potentially back to ~400,000 years. These guidelines can be applied in other scientific areas to facilitate and optimise research conducted in extremely remote locations, contamination-susceptible environmental samples, and even during the future exploration of other planets.

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Figures:

Figure 1: Schematic showing the key steps involved in acquiring deep marine sediment cores, subsampling, DNA extraction, aDNA preparation for sequencing and data generation. Indicated are sources of potential contamination and reduction in data quality, as well as

recommended precautions to be considered and/or controls to be taken. An impact score (1-3 stars) is given to indicate the severity of potential contamination or the impact that impaired data would have on the results at each step in the process. Schematic graphics are not to scale.

Figure 2: Overview of IODP coring systems. A) Advanced piston coring system (APC), shown before and after stroking; only small volumes of drill fluid can enter the space between the core barrel and collar from above after stroking, greatly reducing the risk of contamination. B) Extended core barrel system (XCB) and C) Rotary core barrel system (RCB); both containing circulation jets at the bottom of the core barrel through which drill-fluid enters and removes coring debris by transporting it upwards within the drill hole to the surface. D) Comparison of rotary and piston cored sediments demonstrating the well-preserved lamination in Piston cored material. Figure adapted from Sun et al. (2018) and IODP (iodp.tamu.edu/tools/index.html).

Table 1: Terms commonly used in marine aDNA research and their definition. aDNA terms are listed hierarchically, all other terms are listed alphabetically.

Table 2: Commonly used DNA extraction kits in aDNA studies to date.

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Abstract

The study of ancient DNA (aDNA) from sediments (*seda*DNA) offers great potential for paleoclimate interpretation, and has recently been applied as a tool to characterise past marine life and environments from deep ocean sediments over geological timescales. Using *seda*DNA, palaeo-communities have been detected, including prokaryotes and eukaryotes that do not fossilise, thereby revolutionising the scope of marine micropalaeontological research. However, many studies to date have not reported on the measures taken to prove the authenticity of *seda*DNA-derived data from which conclusions are drawn. aDNA is highly fragmented and degraded and extremely sensitive to contamination by non-target environmental DNA. Contamination risks are particularly high on research vessels, drilling ships and platforms, where logistics and facilities do not yet allow for sterile sediment coring, and due consideration needs to be given to sample processing and analysis following aDNA guidelines. This review clarifies the use of aDNA terminology, discusses common pitfalls and highlights the urgency behind adopting new standards for marine *seda*DNA research, with a focus on sampling optimisation to facilitate the incorporation of routine *seda*DNA research into International Ocean Discovery Program (IODP) operations. Currently available installations aboard drilling ships and platforms are reviewed, improvements suggested, analytical approaches detailed, and the controls and documentation necessary to support the authenticity of aDNA retrieved from deep-sea sediment cores is outlined. Beyond practical considerations, concepts relevant to the study of past marine biodiversity based on aDNA, and the applicability of the new guidelines to the study of other contamination-susceptible environments (permafrost and outer space) are discussed.

Title:

Ancient DNA from marine sediments: precautions and considerations for seafloor coring, sample handling and data generation

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Abstract

The study of ancient DNA (aDNA) from sediments (*seda*DNA) offers great potential for paleoclimate interpretation, and has recently been applied as a tool to characterise past marine life and environments from deep ocean sediments over geological timescales. Using *seda*DNA, palaeo-communities have been detected, including prokaryotes and eukaryotes that do not fossilise, thereby revolutionising the scope of marine micropalaeontological

research. However, many studies to date have not reported on the measures taken to prove the authenticity of *seda*DNA-derived data from which conclusions are drawn. aDNA is highly fragmented and degraded and extremely sensitive to contamination by non-target environmental DNA. Contamination risks are particularly high on research vessels, drilling ships and platforms, where logistics and facilities do not yet allow for sterile sediment coring, and due consideration needs to be given to sample processing and analysis following aDNA guidelines. This review clarifies the use of aDNA terminology, discusses common pitfalls and highlights the urgency behind adopting new standards for marine *seda*DNA research, with a focus on sampling optimisation to facilitate the incorporation of routine *seda*DNA research into International Ocean Discovery Program (IODP) operations. Currently available installations aboard drilling ships and platforms are reviewed, improvements suggested, analytical approaches detailed, and the controls and documentation necessary to support the authenticity of aDNA retrieved from deep-sea sediment cores is outlined. Beyond practical considerations, concepts relevant to the study of past marine biodiversity based on aDNA, and the applicability of the new guidelines to the study of other contamination-susceptible environments (permafrost and outer space) are discussed.

Keywords: ancient DNA; marine sediments; deep biosphere; phytoplankton; contamination; seafloor; IODP; biomarkers; Mars

Abbreviations: aDNA, ancient DNA; APC, Advanced Piston Corer; HLAPC, Half-Length Advanced Piston Corer; IODP, International Ocean Discovery Program; mbsf, metres below seafloor; MSP, Mission Specific Platforms; NGS, Next generation Sequencing; PCR, polymerase chain reaction; PFT, perfluorocarbon tracer; PMCH, perfluoromethylcyclohexane; PFMD, perfluoromethyldecalin; *seda*DNA, sedimentary ancient DNA

1 Introduction

Past marine environments have generally been investigated using a suite of methodological approaches and interdisciplinary research fields, such as geology, organic and inorganic geochemistry, paleoceanography and micropaleontology. Discoveries in all of these disciplines have contributed greatly to our understanding of the climatic history of Earth and the evolution and responses of its inhabitants. However, to date, it has not been possible to achieve a detailed picture of all living organisms that have occupied global oceans in the past, restricting estimates of past environmental conditions and climate. The techniques that have traditionally been applied to reconstruct marine palaeo-communities are limited, such as microscopy to investigate the microfossil record (e.g., Winter et al., 2010; Armbrecht et al., 2018). Due to dissolution and degradation of phytoplankton and microzooplankton while sinking to the seafloor post-mortem, only the most robust skeletons and shells are preserved within a complex geological record (Loucaides et al., 2011). Often, these microfossils are broken, altered by chemical processes and unrecognizable. In the absence of well-preserved diagnostic morphological features, lipid biomarkers can provide supplementary information on biological sources in sediment records (Volkman et al., 1998; Coolen et al., 2004; Sinninghe Damste et al., 2004; Brocks et al., 2011), however, the majority of plankton members do not possess highly diagnostic biomarkers.

New marine metagenomic approaches have allowed the routine characterisation of the diversity of both living hard- and soft-bodied plankton communities in the water column and sub-seafloor. Large-scale “omics” studies, such as the Tara Oceans project (a global sampling program to characterise pro- and eukaryotes of the surface ocean), have shed a new light on our understanding of modern (present day) marine ecosystems and diversity (de Vargas et al., 2015; Sunagawa et al., 2015; Carradec et al., 2018). The deep sea and sub-seafloor have also been targeted with high-resolution metagenomic surveys revealing new insights into the abundance and composition of organisms existing in these largely unexplored environments (e.g., Zinger et al., 2011; Bienhold et al., 2016; Inagaki et al., 2015; Morono and Inagaki, 2016;

Orsi et al., 2017, respectively). Such comprehensive studies on living marine communities are continually improving genome reference databases for the hundreds of thousands of pro- and eukaryotic organisms present in the marine environment (Sunagawa et al., 2015; Klemetsen et al., 2017). As a consequence, modern marine metagenomics has not only inspired marine palaeo-research, but also created a means of identifying ancient taxa from marine sediments over geological timescales.

In the last decade, marine palaeo-research has been reinvigorated by genomic techniques that enable the analysis of ancient DNA (aDNA) molecules from long-dead organisms. Past prokaryotic and eukaryotic plankton communities have been reconstructed using aDNA sequencing approaches (e.g., Coolen and Overmann, 1998; 2007; Coolen et al., 2004; 2008; 2013; Bissett et al., 2005; D'Andrea et al., 2006; Boere et al., 2009; Lejzerowicz et al., 2013; Hou et al., 2014; Randlett et al., 2014; More et al., 2018). These studies have confirmed that phyto- and zooplankton are good targets for aDNA-based studies, while also being particularly relevant for ecosystem-climate reconstructions. It is reasonable to assume that obligate photosynthetic plankton (phytoplankton) and/or zooplankton do not survive and reproduce after burial in deep sediments, and represent uncommon lab contaminants (e.g., Lejzerowicz et al., 2013; Hou et al., 2014; More et al., 2018). aDNA analysis has shown that even after their voyage through the water column plankton-derived particles that had settled on the seafloor still reflect the global biogeographic patterns of living species (Morard et al., 2017). Notably, the reconstruction of past marine communities using aDNA is possible using just a few grams of sediment, facilitating sediment sample collection, transport and storage for the purpose of aDNA analyses.

The marine aDNA archive extends back to the Pleistocene, as shown by studies of genomic, 18S rRNA gene markers targeting various eukaryotic groups. For example, aDNA has been recovered from various eukaryotic plankton taxa in 43,000-year-old Arabian Sea sediments (More et al., 2018). Taxon-specific approaches targeting small, degraded DNA fragments allowed the retrieval of foraminiferal aDNA from ~800-year-old fjord sediments (Pawlowska et

al., 2014) and ~30,000-year-old deep-sea sediments with the additional benefit of enabling the detection of rare taxa (Lejzerowicz et al., 2013). However, if a targeted approach is used, the origin and fate of the DNA in question must be carefully considered, especially for very old claims, such as the retrieval of 1.4 million years old DNA from chloroplasts (Kirkpatrick et al., 2016), which are subject to kleptoplasty (sequestration and maintenance of chloroplasts; Bernhard and Bowser, 1999). While Kirkpatrick et al. (2016) used thorough contamination control, the finding of >1 million years old DNA remains to be replicated using adapted control measures (e.g., sediment core decontamination and metagenomic sequencing, as outlined in this review). Most studies to date have involved well-dated sediment records and used a cross-validation through paired analysis of aDNA and diagnostic lipid biomarkers as well as geochemical proxies (e.g., Coolen et al., 2006; 2009). Yet, the absence of modern contaminants in analysed samples was not always verified through sequencing analysis of negative sampling and/or extraction controls, which is crucial for the interpretation of aDNA data even if DNA values measured following amplification (by polymerase chain reaction; PCR) are zero (as DNA may be present but simply be below detection limit). To date, the oldest authenticated aDNA records are from ~400,000-year-old cave sediments (Willerslev et al., 2003) and ~700,000-year-old permafrost mammal bones (Orlando et al., 2013).

Despite technologies now being available to rapidly extract and sequence aDNA from marine sediments, and the enormous potential of aDNA research to improve palaeo-oceanographic, -ecosystem and -climate models, marine *seda*DNA studies remain scarce. This is mainly due to the difficulties and high costs associated with deep-sea aDNA material, for which rarity and hence value justify the deployment of state-of-the-art practices. We review current problems and pitfalls incurred in ship-board sediment sampling, laboratory processing and computational analysis. We suggest solutions to improve sediment coring and sampling strategies so that aDNA research can become a well-established staple in marine biogeosciences. The focus is on sampling protocols within the framework of the International Ocean Discovery Program (IODP) “Biosphere Frontiers” theme, which is dedicated to

understanding sub-seafloor communities. Our guidelines for deep-ocean *seda*DNA isolation are applicable to any low-biomass and setting, including permafrost regions or planet Mars.

2 Definitions and pre-sampling considerations

2.1 Ancient DNA (aDNA), sedimentary ancient DNA (sed aDNA), and palaeo-environmental DNA (PalEnDNA)

aDNA research involves the biomolecular study of non-modern genetic material preserved in a broad range of biological samples (Shapiro und Hofreiter, 2012; Table 1). When an organism dies, mechanisms that ensure DNA repair in the cell are no longer active, leaving the DNA to degrade over time (Allentoft et al., 2012). Eventually, DNA from dead specimens becomes ancient. aDNA is highly fragmented to an average length of less than 100 base pairs (bp), for example, an average length of 48 bp has been determined in the oldest microbial genome assembled to date - from a 48,000-year-old Neandertal (Weyrich et al., 2017). aDNA is affected by post-mortem oxidative and deamination damage, such as thymine enrichment at the end of DNA sequences (Briggs et al., 2007; Ginolhac et al., 2011). Both fragmentation and damage patterns can be used to authenticate aDNA, and damage can even be used to predict its age in certain scenarios (Kistler et al., 2017).

aDNA research mainly focuses on organismal DNA extracted from some tissue remnants of a wide range of single specimen (e.g., tooth, bone, hair, eggshell, feather). In contrast, environmental DNA (eDNA) focuses on disseminated genetic material found in environmental samples such as soil, sediment, water and ice (Taberlet et al., 2012a). Such samples contain complex mixtures of DNA from taxonomically diverse organisms (e.g., bacteria, archaea, plants, animals). In addition to aDNA and eDNA, the term sedimentary aDNA (*seda*DNA) has been coined to describe aDNA that is exclusively recovered from sediments (Willerslev et al., 2003; Jørgensen et al., 2012). The term fossil DNA has also been used in pioneer studies where sedimentary plankton DNA and lipid biomarkers (i.e., “chemical fossils”) derived from the same historical source organisms were analysed in parallel to validate the ancient DNA

results (e.g., Coolen and Overmann, 1998; 2007; Coolen et al., 2004). To a lesser degree, 'palaeo-environmental DNA' (PalEnDNA) has also been used to describe disseminated genetic material in a broad range of ancient environmental samples including sediments as well as soil, paleosols, coprolites, water and ice (Rawlence et al., 2014). Modern sequencing technologies and bioinformatic tools ease the analysis of these complex environmental aDNA samples and of the biological responses to human or climate change, with investigations having focussed on terrestrial settings (Jørgensen et al., 2012; Giguet-Covex et al., 2014; Willerslev et al., 2014; Alsos et al., 2015; Pansu et al., 2015). In this review, we use the term 'marine *seda*DNA', which specifically refers to aDNA recovered from ocean sediments. A detailed list of terms frequently used in aDNA research and their definitions is given in Table 1.

2.2 Authenticity of marine aDNA

2.2.1 Environments favourable for marine aDNA preservation

Organic-rich sediments deposited in the deep, cold ocean under stratified and anoxic conditions present several favourable characteristics for the preservation of aDNA (e.g., Coolen and Overmann, 1998; 2007; Coolen et al., 2004; 2013; Boere et al., 2011). Oxidative and deamination damage is reduced in the absence of oxygen (Lindahl, 1993). The absence of irradiation (Lyon et al. 2010), the generally low temperatures (Willerslev et al., 2004), and the high concentration of borate (Furukawa et al., 2013) further contribute to DNA preservation. Additionally, the typically high mud content of deep-sea sediment offers a particularly well-suited matrix for the preservation and accumulation of DNA (Torti et al., 2015). The high surface:volume ratio of extremely small clay minerals in clay-rich sediments offer a high adsorption surface onto which DNA molecules can bind and remain sheltered from the activity of nucleases (Dell'Anno et al., 2002; Corinaldesi et al., 2008, 2011, 2014, 2018). However, although the above listed properties have been reported to positively impact on DNA preservation, locations with other characteristics that seem less ideal might still be suitable for

aDNA research. For example, well-oxygenated Atlantic deep-sea sediments and sand-rich coastal paleo-tsunami deposits have been used to extract and characterise aDNA from foraminifera (Lejzerowicz et al., 2013; Szczuciński et al., 2016, respectively). In conclusion, the preservation of aDNA in marine settings appears to be variable depending on regional environmental characteristics with less favourable to favourable conditions retaining aDNA between a few thousand to, at least, a few ten thousand years. More research is needed to estimate how far back in time authentic marine *seda*DNA can be detected, which could be achieved, for example, by investigating sediment records from various deep seafloor locations over geological timescales.

2.2.2 Marine *seda*DNA degradation and fragment length

18S rRNA gene fragments of past dinoflagellates, diatoms, and haptophytes as long as 500 bp in length have been amplified and sequenced (e.g., Coolen et al., 2004), after DNA was isolated from sediments exhibiting characteristics favourable for aDNA preservation (Section 2.2.1). Up to 20% of genomic DNA from haptophyte algae has been reported to still be of high molecular weight after 2,700 years of deposition in Black Sea sediments, and the ratio between 500 bp-long haptophyte 18S rDNA fragments and the concentration of haptophyte-diagnostic long-chain alkenones did not vary substantially for at least 7,500 years after deposition, indicative that both types of biomolecules from the same plankton source were equally well preserved (Coolen et al., 2006). This contradicts the generalised view that aDNA is characterised by fragment lengths of <100bp. Nevertheless, studies that report the recovery of exceedingly long aDNA fragments should be viewed with scepticism especially in the absence of sampling and extraction controls, where there is no indication on whether the data might reflect modern signals. However, to date, no data are available on average aDNA fragment length for deep-sea sediments, which could be obtained from metagenomic shotgun sequencing. Gaining insights into the latter should be the focus of future research as this

information will ultimately help to choose the most suitable and efficient aDNA extraction and sequencing library preparation techniques for degraded *seda*DNA (see Section 3.5).

2.2.3 Contamination sources by modern DNA

Key to the viability of marine *seda*DNA studies is the capability to differentiate between true ancient signals (representative that lived at a particular time-period in the past) and modern contamination (introduced through the sampling and analysis process, or naturally by the environment). Microorganisms and their DNA coat nearly every part of this planet (Weyrich et al., 2015) and a recent study has shown that slow-growing microbes even occur in marine sediments up to 2.5 km deep (Inagaki et al., 2015). The DNA of active deep-biosphere organisms is likely to blur the aDNA signal, as would be the case for microorganisms introduced to ancient sediment samples through the drilling process (see Section 3.2). Moreover, microbial DNA is widely present in laboratory environments and reagents, including in those labelled DNA-free (Salter et al., 2014). If PCR is applied to amplify aDNA, the DNA from modern microorganisms may amplify preferentially over damaged, fragmented aDNA and obscure the true aDNA signals within the sample (Willerslev and Cooper, 2005). Therefore, utmost care must be taken to control and account for contaminants and background DNA throughout the whole process of collecting, processing and sequencing aDNA, e.g., by including negative controls in every step of the analysis process (Fig. 1).

2.2.4 Intracellular vs. extracellular DNA

One approach to separating ancient from modern DNA in sediments has been to differentiate between intracellular and extracellular DNA. Intracellular DNA is defined as DNA contained within living cells, structurally intact dead cells and intact resting stages (e.g., bacterial spores, or other cyst-forming plankton). Extracellular DNA is defined as DNA that has been released from cells and preserved for substantial periods of time through mineral and/or microfossil

adsorption or within clay aggregates (Levy-Booth et al., 2007). Extracellular DNA represent an archive of taxa that were autochthonous at the time of deposition (Cornaldesi et al., 2008; 2011). DNA extraction methods have been developed to target either of these DNA fractions (Corinaldesi et al., 2005; Taberlet et al., 2012b; Alawi et al., 2014). However, it is difficult to prove at what time in the past the organism died, and its DNA became extracellular. Furthermore, the extra- and intracellular DNA pool may not always be clearly distinguishable as genetic material present in the environment might have been taken up by competent bacteria (Demanèche et al., 2001; Dell'Anno et al., 2004) and even by eukaryotes (Overballe-Petersen and Willerslev, 2014). It is also important to note that if only the extracellular pool was to be studied, the paleontological value of dormant yet ancient DNA (e.g., from cysts deposited far back in time) will be lost. Due to these issues, extraction techniques targeting only the extracellular portion are currently not recommended for marine *sed*aDNA studies. Alternatively, bioinformatics approaches that can clearly identify ancient signals (Ginolhac et al., 2011; Kistler et al., 2017) are preferred options for authenticating aDNA sequences (Jónsson et al., 2013).

2.2.5 Vertical DNA movement in marine sediment cores

Three major processes are associated with the vertical movement of DNA in sediment cores: DNA leaching, bioturbation and migration. Bioturbation is a biomechanical process that results in the multidirectional re-organisation of sediments primarily in the upper 10 cm of the sub-seafloor (Boudreau, 1998). DNA leaching is a passive process describing the downward movement of DNA across sediment layers (Haile et al., 2007), without a lowermost boundary. The mixing of sediment layers, and consequently of modern and ancient DNA, can lead to misinterpretations of genomic data. Experimental trials to assess DNA leaching through terrestrial sediments exist (Ceccherini et al., 2007; Poté et al., 2007), with initial results indicating that the extent of leaching depends on the taxonomic source (Haile et al., 2007). In Previous studies from lake sediments have shown that leaching is not a factor (Parducci et

al., 2017), and in seafloor sediments DNA it seems to play a minor role as aDNA and lipid biomarkers derived from the same microbial source were found to co-exist or to be both below detection limit in marine sediments just centimetres apart (Boere et al., 2009; Coolen et al., 2006; 2009; 2013). In the latter studies it therefore appears that the pore size of the laminated sediments was too small for intracellular DNA to migrate, and that all extracellular plankton DNA was adsorbed to the mineral matrices. Recent studies showing *upwards* vertical pore fluid movement also demonstrate the potential for vertical migration of relict or intact DNA within sediments (Torres et al., 2015), and should likewise be considered. Vertical migration of relict or intact DNA is expected to be especially a concern in sediments with micron scale pore sizes and/or a low clay content and a poor capacity to adsorb extracellular DNA. Future experimental research is required to quantify DNA leaching and/or migration through marine sediments, acknowledging the challenge of replicating a complex environmental system varying widely in hydrodynamics and sediment type.

2.2.6 Cross validation of marine aDNA and palaeo-environmental proxies

In addition to using proper contamination controls, downcore changes in past plankton compositions inferred from marine *sed*aDNA can be validated through a complementary analysis of independent biological (e.g., microfossils, lipid biomarkers) and geochemical proxies (indicative of the prevailing paleoenvironmental conditions) (Boere et al., 2009; Coolen et al., 2004; 2006; 2013; Hou et al., 2014; More et al., 2018). The most detailed comparison between past ecosystem changes using marine *sed*aDNA and the paleo-depositional environment to date has been performed on Holocene sediments from the permanently anoxic and sulfidic Black Sea (Coolen, 2011; Coolen et al., 2006; 2009; 2013; Giosan et al., 2012; Manske et al., 2008). The anoxic and laminated sediments of this semi-isolated sea are devoid of bioturbation and form high-resolution archives of climate-driven hydrological and environmental changes (Calvert et al., 1987; Hay, 1988). Episodes of postglacial sea-level rise ~9,000 years ago (Major et al., 2006) and sea surface salinity increase ~5,200 years ago

(Giosan et al., 2012) have been associated based on *seda*DNA with freshwater to brackish/marine planktonic community transitions (Coolen et al., 2013). For example, the gradual increase in sea surface salinity coincided with the arrival of marine copepods (*Calanus euxinus*), which could only be identified through *seda*DNA analysis (Coolen et al., 2013) as these important zooplankton members generally do not leave other diagnostic remains in the fossil record besides difficult to distinguish resting eggs (Marcus et al., 1996).

Vice versa, paleoenvironmental conditions inferred from more traditional geochemical and micropaleontological proxies have been verified from parallel *seda*DNA analysis. By way of example, Black Sea sediments deposited since the last 2,500 years contain coccoliths from the calcified marine haptophyte *Emiliania huxleyi* whereas haptophyte-derived diagnostic long chain alkenones in the absence of coccoliths were abundant in up to 7,500-year-old sediments (Hay et al., 1991; Coolen et al., 2009). Paired analysis of long-chain alkenones and *seda*DNA analysis (18S rRNA) revealed that that the first haptophytes that colonized the Black Sea ~7,500 years ago were initially a mixture of *E. huxleyi* and a highly diverse suite of previously overlooked non-calcified haptophytes related to alkenone-producing brackish *Isochrysis* species. *E. huxleyi* remained the only alkenone producer after 5,200 years BP when salinity reached modern day levels (Coolen et al., 2009). It was concluded that while calcite dissolution prevented the preservation of *E. huxleyi* coccoliths in sediments older than 2,500 years ago, their molecular fossils (DNA fragments and long-chain alkenones) survived much longer and showed that in reality this marine haptophyte entered the Black Sea already shortly after the marine reconnection which occurred ~9,000 years ago (Coolen et al., 2009; 2013). Even more detailed analyses of *E. huxleyi* (targeting 250-bp-long mitochondrial cytochrome oxidase subunit I; mtCOI) indicate a series of transitions from possibly low-salinity to high-salinity adapted strains of *E. huxleyi* in the Black Sea (7.5 – 5.2 ka BP), to a different suite of strains during the most marine stage (5.2 – 2.5 ka BP), returning to low salinity strains after 2.5 ka BP. The latter transition coincides with the onset of the cold and wet Subatlantic climate (Coolen, 2011) when the Black Sea experienced re-freshening from 32 to 18 ppt (Van der Meer et al., 2011; Giosan et al., 2012; Coolen et al., 2013). The analysis of similar length

preserved sequences of viral major capsid protein (mcp) genes revealed a continuous co-existence of *E. huxleyi* and coccolithoviruses in the Black Sea since the last 7,000 years and that the same *E. huxleyi* strains, which occurred shortly after the marine reconnection returned with the same viral strains after the re-freshening during the Subatlantic climate thousands of years later (Coolen, 2011). More recently, detailed sedimentary 18S rDNA profiling targeting the shorter (130 bp) V9 region revealed that long-term expansion of past oxygen minimum zones (OMZ) created isolated habitats for unicellular eukaryotes (protists) capable of sustaining oxygen depletion either by adapting a parasitic life cycle (e.g., apicomplexans) or by establishing mutualistic connections with others (e.g., radiolarians and mixotrophic dinoflagellates). These examples show that *sed* aDNA can be used to identify biological sources of lipid biomarkers, to verify the reliability of paleoenvironmental information inferred from more traditional proxies, and to reconstruct past ecosystems at multiple trophic levels.

The reconstruction of subseafloor prokaryote communities is more complicated since the DNA may be derived from living intact cells in the sediment (see Section 2.2.4). However, 16S rRNA gene profiling from total (intracellular and extracellular) sedimentary DNA has revealed useful insights into sub-seafloor microbial indicators of the palaeo-depositional environment. For example, microbiomes in 20 million years-old coalbeds underlying 2 km of marine sediments were shown to resemble forest soil communities (Inagaki et al., 2015). Variations in bacterial communities found in Baltic Sea sediments have been linked to palaeo-salinity changes (Lyra et al., 2013). Orsi et al. (2017) showed that the genomic potential for denitrification correlated with past proxies for oxygen minimum zone strength in up to 43 ka-old Arabian Sea sediments. The presence of fermentation pathways and their correlation with the depth distribution of the same denitrifier groups, however, suggests that these microbes were possibly alive upon burial, but low postdepositional selection criteria may explain why they nevertheless formed a long-term genomic archive of past environmental conditions spanning the last glacial-interglacial cycle (Orsi et al., 2017). Further studies are required to determine as to how far the persistence of this phenomenon extends with increased depth in

the biosphere. Nevertheless, these examples show that the complementary analysis of marine *seda*DNA-inferred past plankton composition and biological and geochemical proxies is a powerful tool to reconstruct palaeo-environments.

3 aDNA research in the International Ocean Discovery Program (IODP) framework

3.1 IODP infrastructure

IODP is the global community's longest marine geoscience program, operating for 51 years. Its scientific strategy has been to answer globally-significant research questions about the Earth's structure, and the processes that have, and continue to, shape our planet and its climatic history. More recently, additional focus has been cast on biological evolution and limits, particularly in the sub-seafloor environment, under the new Biosphere Frontiers theme (Bickle et al., 2011). This theme has been inspired by the rapidly evolving knowledge and technical capabilities across the multiple merging fields of molecular biology, microbiology, organic and inorganic geochemistry, and micropalaeontology and includes scope for the integration of marine *seda*DNA research. IODP is currently serviced through three platforms, the United States of America's research vessel *JOIDES Resolution*, Japan's *Chikyu* and by the European consortium's Mission Specific Platforms (MSP). In recent years, the laboratories and storage facilities on the ships were modified, or purpose built, to ensure addressing Deep Biosphere questions was possible. As a result, the latest IODP decadal plan considered options to enable access to uncontaminated samples, their processing and preservation on-board. The latter has led to new coring technologies such as the Half-Length Advanced Piston Corer (HLAPC) allowing a coring depth extension of the conventionally used Advanced Piston Corer (APC), and the use of chemical contamination tracers such as perfluorocarbon tracers (PFTs) (see Sections 3.2 and 3.3, respectively). Particularly useful to aDNA studies may be the development of remotely controlled instruments allowing sediment sampling at ambient pressure (MeBo; Pape et al., 2017) and a rock-drilling device (RD2; Früh-Green et al., 2015).

Notable achievements under the new Deep Biosphere theme include the finding of millions of years old active microbial community from coal beds buried at 2.5 km below the seafloor (Inagaki et al., 2015), and the preservation of an imprint of the Chicxulub impact catastrophe (Cockell et al., 2017). A lot remains to be understood before this theme and its challenges are satisfactorily addressed and it is clear that scientists engaging in Biosphere Frontiers will push methodological, technological and multidisciplinary studies.

3.2 Coring strategies suitable for marine *seda*DNA retrieval

Ideally, marine *seda*DNA sampling involves multiple spatial replicates to ensure that the biodiversity captured is representative of a particular site and time period. However, the ability to collect multiple deep ocean sediment cores to characterise palaeo-plankton is hindered by high costs and logistical issues associated with drilling operations. Thorough planning and collaboration to maximise the use of expensive expeditions and precious deep ocean sediment core material are indispensable in marine *seda*DNA research. To date, several coring strategies exist that differ in machinery as well as sub-seafloor depth that can be reached, and their application is largely dependent on which drilling platform is used (ship or MSP), and what type of sediment is to be cored/drilled (soft sediment or hard rock). This review concentrates on describing piston coring strategies, which are generally better suited to retrieve sediments for aDNA analysis due to relatively low contamination risks. Rotary core barrel systems are required to drill some sedimentary and most igneous rocks, and as they operate with drill-heads and drilling fluids (e.g., seawater) the risk of contamination is dramatically increased (see Section 3.3, Fig. 2).

Piston coring, referred to as Advanced Piston Coring (APC) or Hydraulic Piston Coring System (HPCS), is used to sample unconsolidated or poorly consolidated (i.e., softer) marine sediments. Briefly, these instruments are pushed into the sediment while a piston inside the core pipe creates a vacuum so that the collected sediment remains in the pipe during retrieval.

Sediments obtained by piston coring preserve laminated sediments well, are associated with a relatively low risk of environmental contamination and the preferred method to obtain sediments for aDNA analysis (Lever et al., 2006; Smith et al., 2000; More et al., 2018; Fig. 2). Using the piston coring approach, a successive recovery of marine sediments has been achieved to a depth of ~490 m below seafloor (mbsf) (Tada et al., 2013). If only a few metres long (soft) sediment cores are required, gravity-based coring systems, such as a Kasten-, or a Multicorer provide a good alternative (Coolen et al., 2004; 2009). Progress has also been made towards modifying piston coring instruments so that contamination-free sampling is possible, at least for short (<4 m) sediments (Feek et al., 2011). For example, the 'Mk II sampler' uses an air and water-tight piston coring system with a pointed aluminium head, preventing contamination of the sampled sediment from smearing or water infiltration (Feek et al., 2011). However, to date this corer has only been used in shallow waters, thus it remains to be tested whether use of such an instrument would be feasible during coring operations in deeper waters and which modifications may be required.

3.3 Contamination tracing during coring

Deep ocean coring requires the lowering of coring instruments through hundreds to thousands of metres of seawater before the seafloor is reached, hence exposes the instruments to contamination by modern DNA (Fig. 1). This unavoidable issue has called for the development of methods for environmental DNA contaminant tracing during coring operations. One approach has been to compare biological material found in the contaminating source material (e.g., seawater, drilling fluid) to that of sub-seafloor communities, and to exclude all signals occurring in either from the final analyses (e.g., Expedition 330 Scientists, 2012; Cox et al., 2018). This approach can be implemented for either piston coring or rotary core barrel drilling, provided other sampling constraints associated with these coring systems can be accommodated. However, this procedure does not account for potential "false negative" DNA signals that might indeed occur in both ancient sediments *and* modern contaminating material.

However, in some cases, the microbial community structure of modern contamination (e.g., drilling "mud") can be resolved, particularly if functional genes are being targeted in sediment samples (Cox et al., 2018).

Another approach has been the introduction of fluorescent microspheres, which are particulate tracers of 0.2 - 1.0 mm in diameter physically mimicking contaminating organisms. The microspheres have been introduced near the coring head, i.e., where the sediment enters the corer and coring pipe, spreading across the outside of the core (inside the pipe) while drilling, simulating particle movement (Expedition 330 Scientists, 2012; Orcutt et al., 2017). Microscopy has been used to quantify the number of microspheres at the periphery and in the centre of the core to assess contamination (Expedition 330 Scientists, 2012; Orcutt et al., 2017). Similar methods using other perfluorocarbon tracers (PFT's) including perfluoromethylcyclohexane (PMCH) have been developed for the USA drilling vessel *JOIDES Resolution* (Smith et al., 2000) already in the early phases of IODP. Later, PMCH-based contamination tracing has also been applied during riser drilling on the *Chikyu* (Inagaki et al., 2015). During the IODP Expedition 357 (Atlantis Massif Serpentinization and Life), the PMCH tracer delivery system was further developed to fit the seafloor-based drilling systems MeBO (Pape et al., 2017) and RD2 (Früh-Green et al., 2015) (see Section 3.1). PMCH is highly volatile which can lead to false positive measurements in uncontaminated samples, therefore, more recent investigations during IODP expeditions have moved to the use of the heavier chemical tracer perfluoromethyldecalin (PFMD, 512.09 g mol^{-1}) (e.g., Fryer et al., 2018).

3.4 Subsampling after core acquisition

Key to enable interdisciplinary sampling and correlations of independent measurements is a detailed sampling plan, specifying sample types as well as the sequence in which these samples are to be collected. Sampling for aDNA is time-sensitive (to avoid exposure to

oxygen, high temperatures and contamination), thus should be conducted immediately after core retrieval on an untreated core-half (i.e., prior to any type of scanning such as by X-Ray). The laboratory in which subsampling for aDNA is carried out should be clean and workbenches and surfaces decontaminated with bleach (considered to be most efficient at removing contaminating DNA) and, if applicable, ethanol (to prevent corrosion of metal after bleach-treatment). Detailed records on whether molecular and amplification techniques (i.e., PCR) have been employed in on-board laboratories and which organisms were targeted should be kept on record within IODP to ensure sampling for aDNA can be spatially separated from these laboratories. While most vessels are not currently equipped for complete DNA decontamination, such records may be invaluable for post-expedition aDNA data analyses.

Two sampling approaches are the most feasible on board IODP ships and MSP's: cutting whole round cores or direct subsampling after core cutting into 1.5 m long sections. The choice of approach needs to be made on a case-by-case basis, and depends on the specific facilities, consumables, chemicals and researcher expertise available during each mission. It is recommended that cutting or subsampling are performed under filtered air, e.g., a portable type of a horizontal laminar flow clean air system as described in Morono and Inagaki (2016). Additionally, subsampling should be conducted from the bottom to the top of the core (ancient to modern), using clean (e.g., bleach and ethanol treated) sampling tools for each sample to avoid any form of cross-contamination. Most commonly, soft sediments acquired by piston coring are used for *seda*DNA analyses, therefore, we focus on subsampling procedures of the latter here, subsequently briefly outlining sampling recommendations for hard rock material.

If the sampling decision is in favour of whole round core samples, the newly acquired core sections are cut into 5 - 50 cm sections (preferably under cold conditions), which should be packed in sterile bags or wrap and transferred directly into a fridge or freezer. Although quick and providing a large amount of material for later sub-sampling, this approach has the

disadvantage that a lot of freezer space is required, and post-expedition transport can be costly due to the high sample volume and weight.

An alternative to whole round core cutting is direct subsampling immediately after core cutting, either directly from the centre of the top or bottom of each unsplit core section (usually 1.5 m long), or after or splitting the core sections into two halves. In any case the core liner should be cleaned with bleach to remove potential contamination from seawater, and core cutters and splitting-wires, usually metal and sensitive to bleach should be cleaned with ethanol. If sampling from uncut sections, surface material (~0.5 cm) should be removed with bleach and ethanol-treated scrapers before sampling, which is most easily done with sterile cut-tip syringes, placed into sterile plastic bags and stored frozen.

If sampling is undertaken on split core halves, simultaneous visual sedimentological assessments are possible that enable more targeted sampling at specific depths of interest. Using DNA-clean tools, the top 0.5 cm of the core surface should be scraped off perpendicular to the core pipe using sterile scrapers (from bottom to top of the core). Alternatively, the core half to be sampled can be covered with plastic wrap, followed by powdered dry ice, which will result in the top 0.5 cm to become solid frozen. After 5 min, the frozen outer sediment layer can be lifted at one edge with a sterile scalpel creating a contaminant-free surface, from which subsamples can be taken (Coolen and Overmann, 1998). Then, subsampling should be undertaken using sterile (e.g., gamma-irradiated) plastic syringes or centrifuge tubes (e.g., capacity of ~15 mL). Cut-tip syringes have the advantage that more sediment can be collected as no pressure builds up when pushing the syringe into the sediment (the filled syringe should be placed into a sterile plastic bag immediately, e.g., Whirl-Pak®). Alternatively, sterile centrifuge tubes can be used as is to collect ‘plunge-samples’, usually providing ~1 - 3 cc of sediment material. The outside of the ‘mini-cores’ should be cleaned with bleach and placed into sterile plastic bags to avoid cross-contamination between samples. For subsamples, storage at -20 °C or -80 °C is recommended as freezing has been shown to facilitate

phytoplankton cell-lysis during DNA extractions (Armbrecht et al., *in prep.*). Sub-samples can also be collected by transferring a small amount of sediment into a sterile microcentrifuge tube using clean metal or disposable spatulas (particular care needs to be taken to avoid cross-contamination when using the same sampling tool for different samples). The latter approach may be a good solution when only a few small samples are required, e.g., to supplement other scientific questions of an ongoing expedition. For replication purposes it is recommended that duplicate samples are taken at each depth.

If the material is hard rock or similar, subsamples are most easily collected from whole round or split cores. The same decontamination procedures as outlined above should be considered throughout the subsampling procedure (i.e., decontamination of work-surfaces and sampling tools with bleach and ethanol, sampling under cold conditions and filtered or low air-flow, packing of samples into sterile bags before storage). A de-contaminated metal cutter or a hammer and chisel are best used to remove the outer layer of the exposed sediment, at least at those depths where subsampling is anticipated.

3.5 Marine aDNA sample processing and analysis

Marine aDNA samples should be processed in a specialised aDNA laboratory to prevent contamination with modern DNA. Such a laboratory is generally characterised by creating a low-DNA environment, with a clear separation of no-DNA (e.g., buffer preparation) and DNA-containing (e.g., DNA extraction) workflows, regular and thorough sterilisation procedures, positive air pressure, and protective clothing of the analyst (lab coat/suit, gloves, facemask, visor). Details on optimised laboratory set-up, techniques and workflows have been reviewed before (Cooper and Poinar, 2000; Pedersen et al., 2015). The introduction of aDNA samples into such facilities is relatively straight-forward, as the outer packaging and surface of the sample can be easily sterilised (e.g., using bleach and/or UV).

As on-board subsampling, DNA extractions should be carried out from the most ancient to most recent samples, to prevent modern DNA indivertibly being carried to ancient samples. The amount of sediment used in DNA extractions should capture a representative picture of the biota present in a sample. Despite suggestions that bulk DNA extractions from up to 10 g of material can improve detection of taxa and better represent the diversity of the area of interest (e.g., Taberlet et al., 2012b; Coolen et al., 2013), using such large volumes of sediment is often not practical and can be quite costly in this field where typically many samples are processed. Instead, numerous studies have used replicate extractions of a smaller sample size (e.g., 0.25 g; Table 2) to increase the likelihood of yielding aDNA from rare taxa, as well as successive DNA extractions from a single 0.25 g sediment sample (e.g., Willerslev et al., 2003). Post-extraction, the use of RNA-probe based enrichment approaches coupled with shotgun sequencing, a common technique in aDNA research, may furthermore drastically improve the detection of rare taxa (Horn et al., 2012).

While it would be ideal to find one extraction method that will yield the best quality data and enable standardisation across ancient marine sediment studies, the type of sediment or target organisms may require some adjustments of standard protocols (Hermans et al., 2018). Extraction methods can bias the diversity observed due to differential resilience of taxa to the cell-lysis method (Zhou et al., 1996; Young et al., 2015) and DNA binding capacities of different soil and sediment types (Lorenz and Wackernagel, 1994; Miller et al., 1999). As a result, the aDNA extraction efficiency can be poor and the detection of an aDNA signal lost. To date, a variety of commercial kits have been successfully used to isolate aDNA from sediments (Table 3). To further increase the yield of very low amounts of highly fragmented aDNA several studies have been utilising extraction protocols that include a liquid-silica DNA binding step (e.g., Brotherton et al., 2013 and Weyrich et al., 2017 for non-sediment samples) or ethylenediaminetetraacetic acid (EDTA) cell-lysis step (Slon et al., 2017; utilising cave-sediment samples). Other studies have replaced the Bead Solution in the DNeasy extraction kits (Qiagen; Table 2) by 1M sodium phosphate pH 9 - 10 and 15 vol% ethanol to efficiently

release clay-adsorbed DNA, and to prevent DNA released from intact cells from adsorbing to clay minerals during the extraction (Direito et al., 2012; Orsi et al., 2017; More et al., 2018). The latter is especially important when working with low organic, high carbonate rocks and sediments (Direito et al., 2012).

Two points are particularly important to prevent contamination during extractions. Firstly, as with the samples themselves, it is crucial that all tools and reagents undergo rigorous sterilisation procedures before utilisation, such as by bleach and UV treatment of any packing material before entering ancient DNA facilities. Secondly, blank controls should be included for every step of the laboratory process, i.e., extraction/library preparation blank controls, sequencing and bioinformatic analysis controls (Ficetola et al., 2016). Controlling and monitoring contamination is particularly important when analysing bacterial diversity due to their presence in all laboratory environments and reagents (Weyrich et al., 2015). Optimally, extraction blanks are included in a 1:5 ratio (Willerslev and Cooper, 2005), with a bare minimum of one control with each set of extractions. Aside from bioinformatically removing any organisms determined in such extraction blanks from the investigated sample material, the contaminants should be tracked within a laboratory, and contaminant lists published alongside the data for reasons of data transparency and authenticity.

Post-extraction, many marine aDNA studies have employed methods that are routinely used for modern marine DNA analysis. Although modern DNA work is not exempt from precautions, there are several issues with aDNA work: (i) as outlined in Sections 2.1. and 2.2. aDNA is highly fragmented and degraded and any small amount of modern DNA present in the sample (from reagents, labs or living cells that were present in the sediment sample) will amplify over the aDNA; (ii) sampling and extraction controls are often not included in the sequencing sample; (iii) PCRs are often inhibited due to the co-extraction of humic substances, pigments and heavy metals along with DNA (Webster et al., 2003 and references therein), requiring adequate removal of these impurities (e.g., Coolen et al., 2009); (iv) successful PCRs are prone to bias due to random amplification in reactions that contain very low amounts of DNA

template, thus PCR drift (stochastic variation in the first PCR cycles) can occur (Wagner et al., 1994; Polz and Cavanaugh, 1998; Webster et al., 2003). More importantly, the number of, e.g., bacterial 16S and eukaryotic 18S rRNA operons can greatly vary between per genome and per cell and can cause a biased representation of the past community structure (e.g., Klappenbach et al., 2001). The above biases can be reduced and the detection limit lowered when PCR approaches selectively, amplifying particular groups of organisms indicative of environmental changes, are paired with independent geochemical proxies (e.g., Coolen et al., 2004; 2006; 2009). However, we strongly advocate for the use of strict aDNA methodologies and facilities in order to achieve the generation of authentic marine *seda*DNA data, following the guidelines in this review.

Shotgun metagenomics are currently widely accepted and the least biased method to analyse the broad diversity of ancient environmental samples (e.g., Slon et al., 2017). Although only a small portion of the generated sequence data might be attributable to the ancient organism in question (Morard et al., 2017), next generation sequencing (NGS) generates large quantities of data that enable meaningful statistics, with the additional benefit of preserving the relative proportion of detected taxa. To analyse aDNA sequence data, robust bioinformatic pipelines (e.g., Paleomix, Schubert et al., 2014) have been developed and are available for the application to marine *seda*DNA, integrating damage detection algorithms (e.g., Ginolhac et al. 2011; Kistler et al., 2017) that enable the distinction between ancient and modern signals. Determining the extent of cytosine residues deamination (C to T and G to A, Weyrich et al. 2017) should also be considered to assess authenticity of aDNA sequences, especially when the data was generated from mixed communities, such as from marine *seda*DNA. It is furthermore crucial to carefully screen sequencing data for any low-complexity reads, which may get incorrectly assigned to taxa during alignments against genetic databases, as well as ensuring that taxonomic assignments in the database of choice are correct. Bioinformatic pipelines removing such misidentification-derived errors do not currently exist and should be the focus of future research, as well as the comparison of shotgun and amplicon marine

*seda*DNA data to accurately determine biases and analysis strategies best suited to this new discipline.

4 Future marine aDNA sampling considerations

4.1 Equipment and installations required aboard IODP platforms

In addition to the recent upgrades and investments IODP has made to enable sediment sampling suitable for Biosphere Frontiers theme (Section 3.1) we suggest the following items to facilitate contamination-free sediment sampling and the tracing of contaminants.

(i) Laboratories in which sampling for aDNA is undertaken should be carefully chosen to minimise contamination. Rapid transport of the core from the deck to the lab, thorough decontamination measures (see Section 3.4), and easy access to fridges or freezers are crucial. While a positively air-pressured lab (standard for aDNA laboratories) may not be feasible, air-flow can be reduced by keeping all doors shut and fans off during aDNA sampling. Contamination by human DNA from analysts can be greatly reduced by wearing adequate protective clothing (gloves, facemask, freshly laundered/disposable lab coat/overall). A detailed record or any molecular work undertaken in ship-board labs should be maintained by IODP, and under no circumstances should aDNA sampling be conducted in labs used previously to run PCRs (see Section 3.4). Alternatively, the equipment of a shipping container exclusively dedicated to aDNA sampling could be a good solution to spatially separate aDNA sampling aboard drilling-platforms and installation could be as required during expeditions that involve aDNA sampling.

(ii) DNA is likely to behave quite different from chemical tracers and microspheres currently used to track contamination. With constantly advancing technologies in the field of synthetic biology, the possibility arises to develop ‘non-biological DNA’ with known sequences. Such non-viable DNA tags are already used in the oil industry, where a different tag is introduced

into oil pipes monthly to monitor when and where leaks occur (Forecast Technology Ltd). Using such tags during seafloor coring operations instead of chemical tracers should enable a precise assessment of contamination by environmental DNA, where bioinformatics pipelines could be adjusted to detect and quantify the amount of tags present in the final sequencing data.

4.2 Ground-truthing marine aDNA research and data

To ground-truth marine aDNA studies and to ensure the generation of authentic aDNA data we suggest future research in this field to focus on the following aspects:

(i) The establishment of a public record of common contaminants. This can be achieved, for example, through an inter-lab comparison focused on analysing the same samples and integrating extraction blanks to trace contaminants associated with particular coring equipment, ship- and land- based laboratories.

(ii) Investigation of factors that might considerably bias marine *seda*DNA data. This might include information on sediment-type and environmental condition dependent aDNA preservation, taxon-specific DNA degradation rates, average aDNA fragment length, and shotgun and amplification-based aDNA data comparisons.

(iii) Ongoing enrichment of genetic reference databases for modern marine plankton, to enable taxonomic assignment of the hundreds of thousands of ancient sequences expected to be found in marine sediments.

(iv) The inclusion of negative controls during extractions, library preparations and in sequencing runs, and the publication of the results in the context of independent multiproxy biological and environmental metadata obtained from the same sediment interval.

(iv) Once (i) - (iv) are addressed, the development of a dedicated aDNA coring proposal is encouraged, in which sediment cores are collected using the above outlined, best-suited

coring strategies, sampling and analysis procedures. During such an expedition, basic questions such as optimal on-board contamination tracing techniques, feasible work-flows, spatial replication required to achieve representative community data, and age to which marine *seda*DNA can be determined should be addressed. Such baseline data is missing to date and remains the most important step towards the generation of authentic aDNA data from marine sediments.

5 Application of marine *seda*DNA research guidelines to other contamination susceptible environments

5.1 Permafrost

Permafrost molecular biological studies provide the opportunity to study living organisms that have successfully adapted to extremely cold environments and comprise an analogous cryogenic environment to that found on other planets, such as Mars (Amato et al., 2010). Molecular investigations have focussed on humans (Rasmussen et al., 2010), plants (Willerslev et al., 2003), megafauna (Boessenkool et al. 2012), fungi (Bellemain et al., 2013) and microbes (Willerslev et al., 2004). Permafrost top layers are characterised by a more abundant and diverse microbial community compared to the deeper soil (Gittel et al. 2014). To overcome the hurdle of distinguishing between the modern and ancient DNA signal, metatranscriptomics have been applied to identify the active community only (e.g., Coolen and Orsi, 2015). Despite the challenges in experimental approaches, such as rapid community shifts after thawing even at nearly ambient conditions (Negandhi et al. 2016), studies of permafrost environments have advanced our understanding of feedback loops associated with the response of extremophiles to warming, ultimately informing modelling studies including marine palaeo-environments.

Sampling for ice and permafrost in polar regions is challenging in terms of logistics and minimising contamination risks for both the sample and the sampled environment. For

example, permafrost soil samples are, like marine sediment cores, retrieved through drilling, which can introduce microbial contaminants to the deeper permafrost soil layers as the drill head and liquid pass through the top active soil layer (Bang-Andreasen et al., 2017). Additionally, the cryosphere has been accumulating industrial chemicals and metals since the 1850's (McConnell et al., 2007), so that the present-day microbial community is now capable of degrading industrial contaminants, thereby representing an anthropogenically-adapted rather than an original pristine community (Hauptmann et al., 2017). With both these newly adapted anthropogenic and drilling fluid communities containing characteristics for heavy metal degradation, distinguishing indigenous ice core or permafrost communities from drilling fluid communities will become more difficult in the future (Miteva et al., 2014). Therefore, the described guidelines in this review for distinguishing ancient from modern and contaminant signals, as well as the need for aseptic sampling procedures, are highly applicable to permafrost environments and, more generally, the cryosphere.

5.2 Planetary exploration

The methodologies advocated in this review that enable aDNA in marine sediments to be distinguished from modern DNA are also applicable to the search for life on other planets or moons. Astrobiologists are especially interested in the possibility of detection of Life 2.0, where the life has an independent genesis to that on Earth. The search for life beyond Earth has been potentially possible since the 1970s, with the two Viking lander missions to Mars, but there are other possible targets in our solar system, notably some of the moons around Jupiter and Saturn (e.g., Europa, Titan). Space technology has now reached the point where the detection of life, if it exists or existed elsewhere in the solar system, is becoming a realistic possibility in the next 50 years. There have been several rovers that have carried out successful exploration of the surface of Mars, including Curiosity, the Mars Science Laboratory that in 2018 is mid-way through its predicted mission (Grotzinger et al., 2014). The rover Mars 2020 is being designed at present to test for evidence of life in the near-surface environment.

It will drill, collect and cache samples from the Martian surface, which will then be returned to Earth for more detailed analysis (Beaty et al., 2015). Sample return from Mars to Earth is planned for the end of the 2020's (Foust, 2018). Active planning is also ongoing for possible missions to land and analyse materials from the surfaces of moons such as Europa and Titan, by both NASA and the European Space Agency. For example, Europa (a moon of Jupiter) is known to have a global saltwater ocean below its icy crust, as well as a rocky seafloor, so is one of the highest priority targets in the search for present-day life beyond Earth (Hand et al., 2017). A key concern with this solar system exploration is planetary protection, which is governed by the United Nations Outer Space Treaty (United Nations Office for Disarmament Affairs, 2015) and the Committee on Space Research (COSPAR) of the International Committee for Science. There are two important categories of planetary protection. The first is "forward contamination", where Earth-derived microbial life hitches a ride on spacecraft and contaminates parts of a planetary surface being explored. The second is "backward contamination', where life from an explored planet or moon is inadvertently returned to Earth, maybe in a spacecraft or within a rock sample. The relevance to aDNA analytical protocols is in forward contamination (i.e., the risk of contaminating sample material that could lead to data misinterpretations, and/or generally introducing Earth contaminants to other planets; Rummel and Conley, 2017). It should be noted that if indeed there is or was life on other planetary bodies, it may well not be based on a genetic code composed of DNA and RNA. Independently originated Life 2.0 would be highly unlikely to have evolved exactly the same nucleic acid genetic code as life on Earth (e.g., Rummel and Conley, 2017). Indeed, it has been postulated that an alternative biosphere could exist as a "shadow biosphere" on Earth (Davies et al., 2009). If DNA or RNA-based extant life is found on Mars, for example, then it is most likely that it would represent either past natural exchange of rocks between the two planets (panspermia), or anthropogenic forward contamination. Therefore, the procedures used for distinguishing indigenous life in planetary exploration will need broadening to include the possibility of life with a different genetic code. The protocols developed for aDNA sampling of marine sediments on Earth, including the ability to distinguish from modern DNA, have

relevance for the designing of methods to look for past life on Mars or outer solar system moons using molecular biology techniques (Beaty et al., 2015; Hand et al., 2017).

Conclusions

Ancient DNA in marine deep-sea sediments holds the potential to open a new era of marine palaeo-environment and -climate reconstruction. However, anti-contamination measures central to all aDNA research have logistical constraints and are particularly poorly-suited to shipboard sediment sampling and processing. For example, sterile coring equipment and ultra-clean laboratories are usually not available on any type of drilling platform. Current and future IODP drilling vessels are aware of the increasing need for improved and innovative solutions to coring, non-contaminant drill fluids and appropriate laboratories and storage facilities. Such logistical advances should go hand-in-hand with the establishment of new criteria and standards to ensure the acquisition and preservation of sediment cores with minimal environmental contaminants. Complementary genetic and geochemical information currently available to date suggests that, realistically, environmental reconstructions based on marine *sedaDNA* from past plankton can be achieved for at least the last glacial-interglacial cycle, and potentially back to ~400,000 years. These guidelines can be applied in other scientific areas to facilitate and optimise research conducted in extremely remote locations, contamination-susceptible environmental samples, and even during the future exploration of other planets.

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Figures:

Figure 1: Schematic showing the key steps involved in acquiring deep marine sediment cores, subsampling, DNA extraction, aDNA preparation for sequencing and data generation. Indicated are sources of potential contamination and reduction in data quality, as well as recommended precautions to be considered and/or controls to be taken. An impact score (1-3 stars) is given to indicate the severity of potential contamination or the impact that impaired

data would have on the results at each step in the process. Schematic graphics are not to scale.

Figure 2: Overview of IODP coring systems. A) Advanced piston coring system (APC), shown before and after stroking; only small volumes of drill fluid can enter the space between the core barrel and collar from above after stroking, greatly reducing the risk of contamination. B) Extended core barrel system (XCB) and C) Rotary core barrel system (RCB); both containing circulation jets at the bottom of the core barrel through which drill-fluid enters and removes coring debris by transporting it upwards within the drill hole to the surface. D) Comparison of rotary and piston cored sediments demonstrating the well-preserved lamination in Piston cored material. Figure adapted from Sun et al. (2018) and IODP (iodp.tamu.edu/tools/index.html).

Table 1: Terms commonly used in marine aDNA research and their definition. aDNA terms are listed hierarchically, all other terms are listed alphabetically.

Table 2: Commonly used DNA extraction kits in aDNA studies to date.

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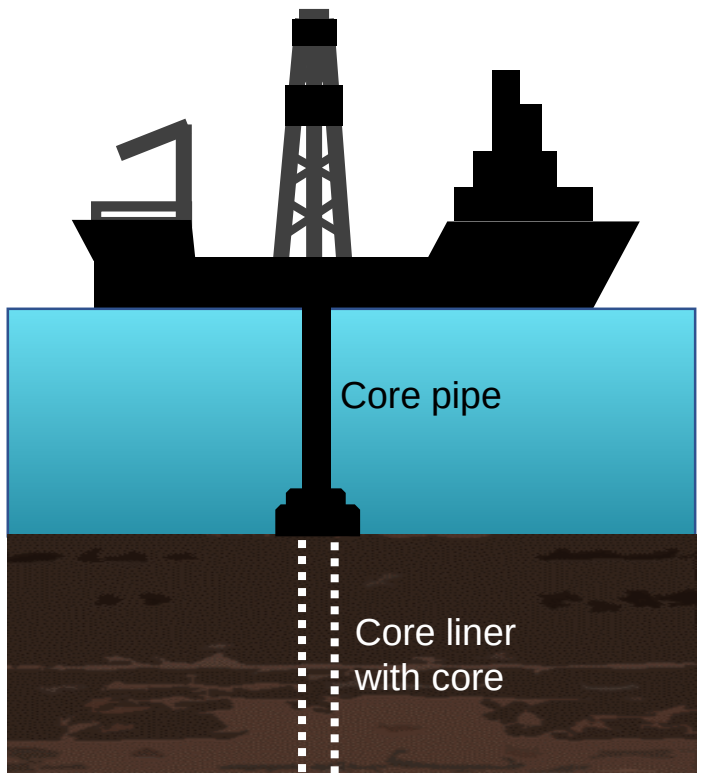
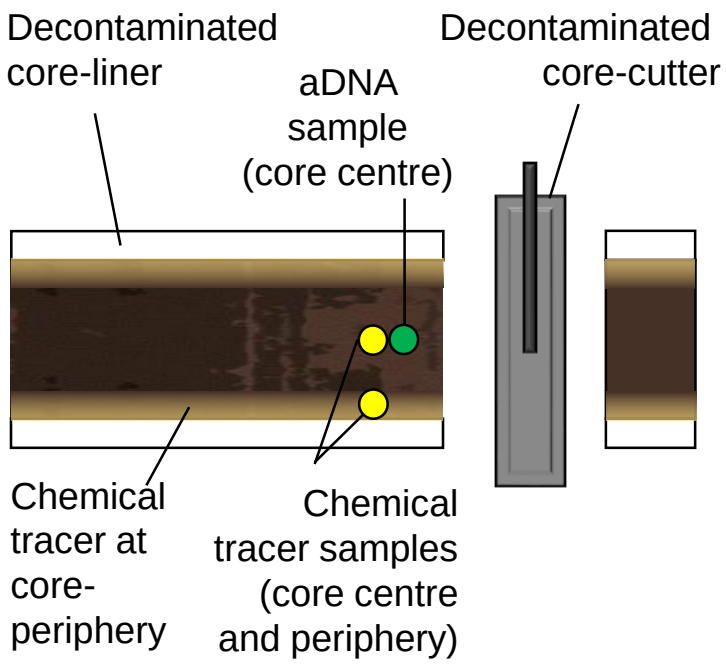
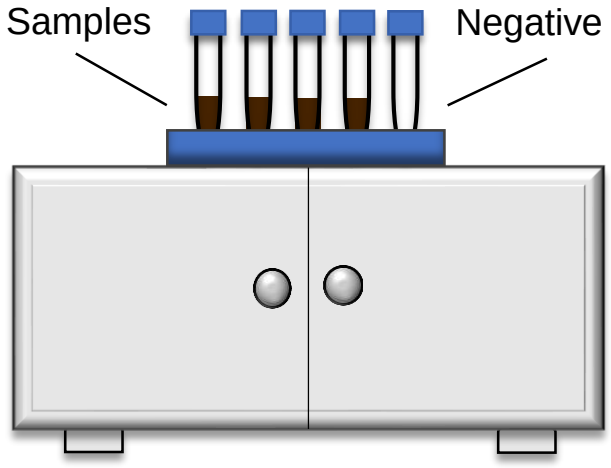
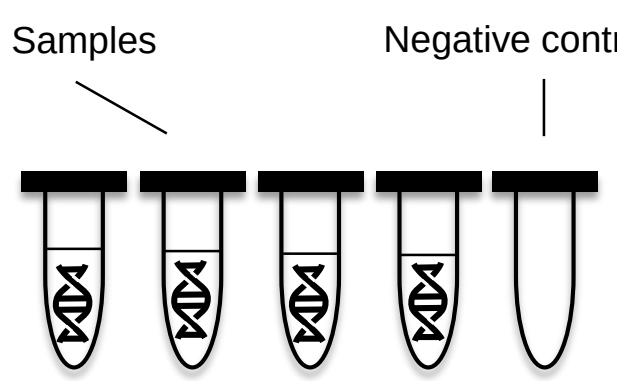
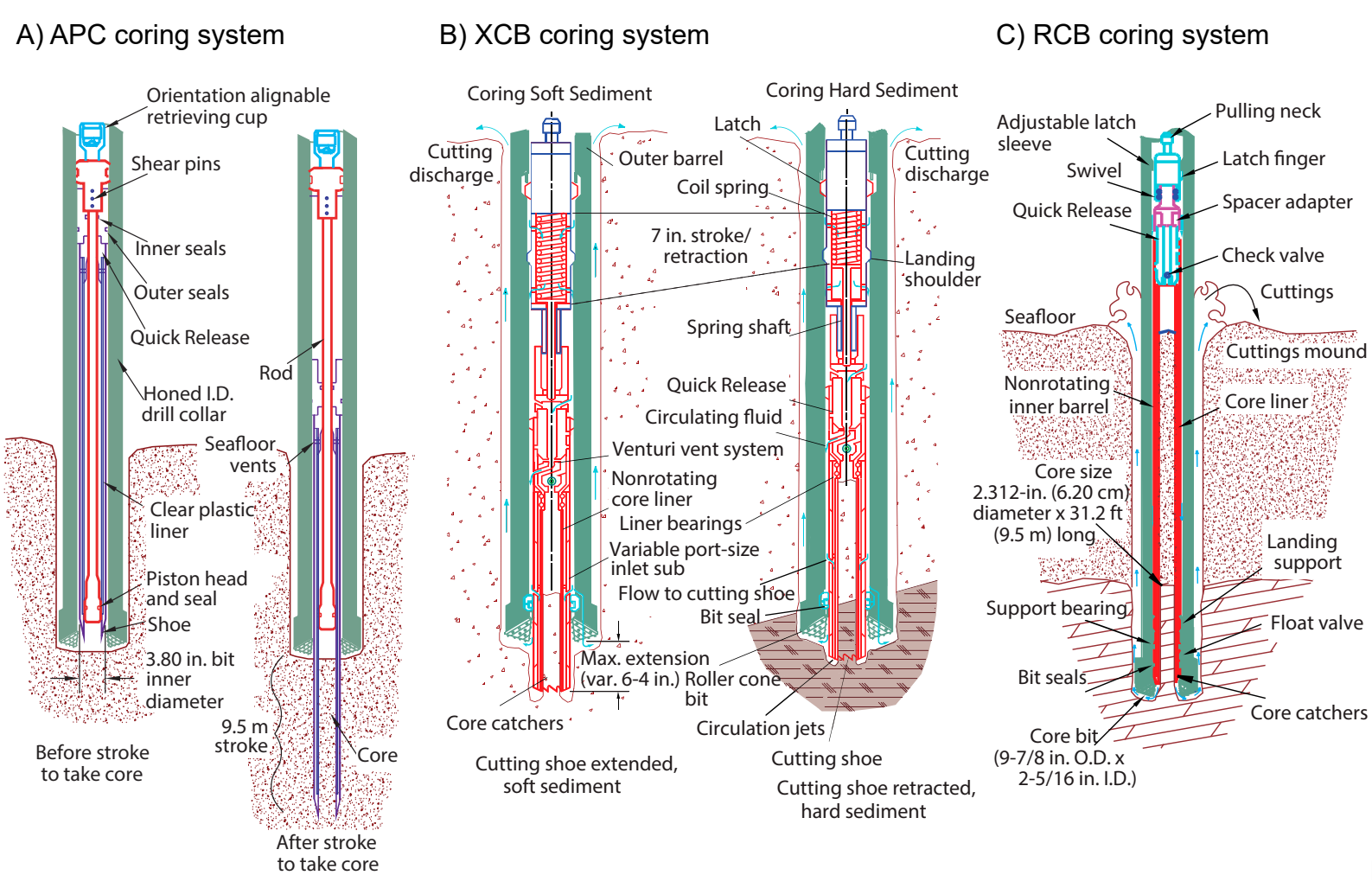
		Impact subject	Impact score
Sediment coring		• Modern DNA in seawater • Contaminant DNA in drilling fluid • Surface sediment DNA pushed downwards during coring *Recommended contamination control measures: seawater control sample, application of chemical tracers*	***
Sediment core subsampling		• Human DNA from analysts • Contaminant DNA present in working area • Cross-contamination *Recommended contamination control measures: reduction of airflow, sampling under cold conditions, decontamination of core-liners, removal of exposed surface sediments, air controls and lab swabs*	**
aDNA extraction		• Contaminant DNA present in reagents • Contaminant DNA present in laboratory • Cross-contamination *Recommended contamination control measures: working on low-DNA environment, personal protective equipment, negative controls*	*
aDNA preparation for sequencing		• Metagenomic library preparation (reagent contaminants) • PCR and amplicon library preparation (PCR bias) *Recommended contamination control measures: working on low-DNA environment, personal protective equipment, negative controls*	* **
aDNA data analysis		• Vigorous data filtering and quality control *Recommended contamination control measures: analysis of negative controls alongside samples*	***



D) Rotary (left) and Piston (right) cored sediment core