

Manuscript Details

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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

File Name [File Type]

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,

Dr Linda Armbrecht

| Reviewer 1 - Comments | Author's response |
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| <p>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.</p> | |
| <p>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.</p> | <p>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.</p> <p>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.</p> <p>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.</p> |
| <p>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.</p> | <p>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.</p> |
| <p>I. 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).</p> | <p>We agree with the reviewer and have removed "modern life" from this sentence.</p> <p>We agree with the reviewer, however, this review focuses on the marine environment, which is introduced in this first sentence.</p> |
| <p>I. 89, I. 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</p> | <p>We removed plankton in this context, and only refer to planktonic organisms where previous studies have focused on those in particular.</p> |

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| <p>also be preserved and should not be neglected completely.</p> | |
| <p>I.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 (I. 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 Kirkpatrick et al. may give also a chance for them to address the constructive critics in the future correspondence or papers.</p> | <p>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 <i>RV Joides Resolution</i>, 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, <i>Thalassiosira</i> and <i>Chaetoceros</i>, 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.</p> |
| <p>I.157 “2.1” is missing</p> | <p>Corrected.</p> |
| <p>I. 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?</p> | <p>We changed this to: ‘aDNA research involves the biomolecular study of non-modern genetic material preserved in a broad range of biological samples’.</p> |
| <p>I. 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 (I. 171-172), while they seem to overlap, as also stated by the authors later on (I. 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</p> | <p>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.</p> |

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| <p>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?</p> | |
| <p>I. 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 (I. 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).</p> | <p>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.</p> |
| <p>I. 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).</p> | <p>We agree and modified this sentence.</p> |
| <p>I. 259 – 'geological' – actually it is a biomechanical process</p> | <p>We replaced 'geological' with 'biomechanical'.</p> |
| <p>I.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.</p> | <p>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)).</p> |
| <p>I. 314 – chapter 3.1 - I wonder if the specific chapter only about IODP is really necessary.</p> | <p>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.</p> |
| <p>I. 324 – table 2 is not necessary. It is much easier to include these three points in the text.</p> | <p>We removed this table.</p> |
| <p>I. 334- 348 – provide at least the project title.</p> | <p>We shortened this section and project titles are no longer applicable.</p> |
| <p>I. 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.</p> | <p>We have included a new figure showing the differences in coring systems.</p> |

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| 178 179 | I. 444 -447 - It is not clear why the authors expect the freezing to affect DNA leaching. | We added an explanation in the text. |
| 180 181 | I. 546 - 'quantitative' - it is not clear what do the authors mean. A number of sequences? | We removed 'quantitative' in this context. |
| 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 | I. 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. |
| 199 200 201 | 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. |
| 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 | <p>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.</p> <p>The references cited in the text and missing in the references:</p> <p>I. 84. Ambrecht et al. 2018 I.86 Loucaides et al. 2011 I. 89 Castaneda et al. 2011 (do you mean Castaneda and Schouten?) I. 134 Coolen et al. 2011 I.189 Boere et al. 1008 (should be 2009?) I. 191 (should be Lyon?) I.241 Levy-Booth et al. 2007 I. 291 Calvert et al. 1987; Hay 1988 I.292 Major et al. 2006 I.303 Lyra et al 2013 I. 338 Frueh-Green - be consistent in writing the name with the reference list I. 512 Brotherton et al. 2012 (should be 2013?) I. 547 Klappenback et al 2001 I. 622 Bossenkol et al 2012 I.625 Gittel et al. 2014 I. 631 Neghandhi et al 2016 I. 640 McConnell et al 2007 I.661 Grotzinger et al 2012 I. 1179 (Table 1) - van Everdingen 1998, Fry et al. 2003</p> | We corrected the reference list. |

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| I.1186 (Table 3) - Haile et al. 2012, Leite et al. 2014, Bidle et al 2007 | |
| Reviewer 2 - Comments | Author's response |
| <p>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.</p> | |
| <p>I have only some minor comments that hopefully the authors will take into consideration before publication.</p> | |
| <p>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</p> | <p>We refined section 3.5 "Marine aDNA sample processing and analysis" according to the reviewer's suggestion.</p> |

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| <p>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.</p> | |
| <p>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 from remains that are ca 700 kyr (Orlando et al. 2013).</p> | <p>We agree with the reviewer and do not cite this paper.</p> |
| <p>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.</p> | <p>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).</p> |
| <p>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'.</p> | <p>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."</p> |
| <p>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.</p> | <p>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.</p> |
| <p>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.</p> | <p>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).")</p> |
| <p>Line 191, I am a bit uncertain whether Lindhal paper suggests that hydrostatic pressure contribute to DNA preservation. Are the authors sure of this statement?</p> | <p>We removed this statement.</p> |
| <p>Line 201: well-oxygenated is misspelled.</p> | <p>Corrected.</p> |
| <p>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.</p> | <p>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</p> |

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| | 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. |

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3 **Highlights corresponding to changes indicated in 'Response to Reviewers'**
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9 **Title:**

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11 *Ancient DNA from marine sediments: precautions and considerations for seafloor coring,*
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13 *sample handling and data generation*
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108 44 **Abstract**

109
110 45 The study of ancient DNA (aDNA) from sediments (*sedaDNA*) offers great potential for
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112 46 paleoclimate interpretation, and has recently been applied as a tool to characterise past
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114 47 marine life and environments from deep ocean sediments over geological timescales. Using

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121 48 *sed*aDNA, palaeo-communities have been detected, including prokaryotes and eukaryotes
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123 49 that do not fossilise, thereby revolutionising the scope of marine micropalaeontological
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125 50 research. However, many studies to date have not reported on the measures taken to prove
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127 51 the authenticity of *sed*aDNA-derived data from which conclusions are drawn. aDNA is highly
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129 52 fragmented and degraded and extremely sensitive to contamination by non-target
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131 53 environmental DNA. Contamination risks are particularly high on research vessels, drilling
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133 54 ships and platforms, where logistics and facilities do not yet allow for sterile sediment coring,
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135 55 and due consideration needs to be given to sample processing and analysis following aDNA
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137 56 guidelines. This review clarifies the use of aDNA terminology, discusses common pitfalls and
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139 57 highlights the urgency behind adopting new standards for marine *sed*aDNA research, with a
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141 58 focus on sampling optimisation to facilitate the incorporation of routine *sed*aDNA research into
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143 59 International Ocean Discovery Program (IODP) operations. Currently available installations
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145 60 aboard drilling ships and platforms are reviewed, improvements suggested, analytical
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147 61 approaches detailed, and the controls and documentation necessary to support the
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149 62 authenticity of aDNA retrieved from deep-sea sediment cores is outlined. Beyond practical
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151 63 considerations, concepts relevant to the study of past marine biodiversity based on aDNA,
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153 64 and the applicability of the new guidelines to the study of other contamination-susceptible
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155 65 environments (permafrost and outer space) are discussed.

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159 67 **Keywords:** ancient DNA; marine sediments; deep biosphere; phytoplankton; contamination;
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161 68 seafloor; IODP; biomarkers; Mars

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167 70 **Abbreviations:** aDNA, ancient DNA; APC, Advanced Piston Corer; HLAPC, Half-Length
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169 71 Advanced Piston Corer; IODP, International Ocean Discovery Program; mbsf, metres below
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171 72 seafloor; MSP, Mission Specific Platforms; NGS, Next generation Sequencing; PCR,

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180 73 polymerase chain reaction; PFT, perfluorocarbon tracer; PMCH, perfluoromethylcyclohexane;
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182 74 PFMD, perfluoromethyldecalin; *seda*DNA, sedimentary ancient DNA
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186 75 1 Introduction

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188 76 Past marine environments have generally been investigated using a suite of methodological
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190 77 approaches and interdisciplinary research fields, such as geology, organic and inorganic
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192 78 geochemistry, paleoceanography and micropaleontology. Discoveries in all of these
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194 79 disciplines have contributed greatly to our understanding of the climatic history of Earth and
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196 80 the evolution and responses of its inhabitants. However, to date, it has not been possible to
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198 81 achieve a detailed picture of all living organisms that have occupied global oceans in the past,
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200 82 restricting estimates of past environmental conditions and climate. The techniques that have
201
202 83 traditionally been applied to reconstruct marine palaeo-communities are limited, such as
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204 84 microscopy to investigate the microfossil record (e.g., Winter et al., 2010; Armbrecht et al.,
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206 85 2018). Due to dissolution and degradation of phytoplankton and microzooplankton while
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208 86 sinking to the seafloor post-mortem, only the most robust skeletons and shells are preserved
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210 87 within a complex geological record (Loucaides et al., 2011). Often, these microfossils are
211
212 88 broken, altered by chemical processes and unrecognizable. In the absence of well-preserved
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214 89 diagnostic morphological features, lipid biomarkers can provide supplementary information on
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216 90 biological sources in sediment records (Volkman et al., 1998; Coolen et al., 2004; Sinninghe
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218 91 Damste et al., 2004; Brocks et al., 2011), however, the majority of plankton members do not
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220 92 possess highly diagnostic biomarkers.

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222 93 New marine metagenomic approaches have allowed the routine characterisation of the
223
224 94 diversity of both living hard- and soft-bodied plankton communities in the water column and
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226 95 sub-seafloor. Large-scale “omics” studies, such as the Tara Oceans project (a global sampling
227
228 96 program to characterise pro- and eukaryotes of the surface ocean), have shed a new light on
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230 97 our understanding of modern (present day) marine ecosystems and diversity (de Vargas et
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232 98 al., 2015; Sunagawa et al., 2015; Carradec et al., 2018). The deep sea and sub-seafloor have
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99 also been targeted with high-resolution metagenomic surveys revealing new insights into the
100 abundance and composition of organisms existing in these largely unexplored environments
101 (e.g., Zinger et al., 2011; Bienhold et al., 2016; Inagaki et al., 2015; Morono and Inagaki, 2016;
102 Orsi et al., 2017, respectively). Such comprehensive studies on living marine communities are
103 continually improving genome reference databases for the hundreds of thousands of pro- and
104 eukaryotic organisms present in the marine environment (Sunagawa et al., 2015; Klemetsen
105 et al., 2017). As a consequence, modern marine metagenomics has not only inspired marine
106 palaeo-research, but also created a means of identifying ancient taxa from marine sediments
107 over geological timescales.

108 In the last decade, marine palaeo-research has been reinvigorated by genomic techniques
109 that enable the analysis of ancient DNA (aDNA) molecules from long-dead organisms. Past
110 prokaryotic and eukaryotic plankton communities have been reconstructed using aDNA
111 sequencing approaches (e.g., Coolen and Overmann, 1998; 2007; Coolen et al., 2004; 2008;
112 2013; Bissett et al., 2005; D'Andrea et al., 2006; Boere et al., 2009; Lejzerowicz et al., 2013;
113 Hou et al., 2014; Randlett et al., 2014; More et al., 2018). These studies have confirmed that
114 phyto- and zooplankton are good targets for aDNA-based studies, while also being particularly
115 relevant for ecosystem-climate reconstructions. It is reasonable to assume that obligate
116 photosynthetic plankton (phytoplankton) and/or zooplankton do not survive and reproduce
117 after burial in deep sediments, and represent uncommon lab contaminants (e.g., Lejzerowicz
118 et al., 2013; Hou et al., 2014; More et al., 2018). aDNA analysis has shown that even after
119 their voyage through the water column plankton-derived particles that had settled on the
120 seafloor still reflect the global biogeographic patterns of living species (Morard et al., 2017).
121 Notably, the reconstruction of past marine communities using aDNA is possible using just a
122 few grams of sediment, facilitating sediment sample collection, transport and storage for the
123 purpose of aDNA analyses.

124 The marine aDNA archive extends back to the Pleistocene, as shown by studies of genomic,
125 18S rRNA gene markers targeting various eukaryotic groups. For example, aDNA has been

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126 recovered from various eukaryotic plankton taxa in 43,000-year-old Arabian Sea sediments
127 (More et al., 2018). Taxon-specific approaches targeting small, degraded DNA fragments
128 allowed the retrieval of foraminiferal aDNA from ~800-year-old fjord sediments (Pawlowska et
129 al., 2014) and ~30,000-year-old deep-sea sediments with the additional benefit of enabling
130 the detection of rare taxa (Lejzerowicz et al., 2013). However, if a targeted approach is used,
131 the origin and fate of the DNA in question must be carefully considered, especially for very old
132 claims, such as the retrieval of 1.4 million years old DNA from chloroplasts (Kirkpatrick et al.,
133 2016), which are subject to kleptoplasty (sequestration and maintenance of chloroplasts;
134 Bernhard and Bowser, 1999). While Kirkpatrick et al. (2016) used thorough contamination
135 control, the finding of >1 million years old DNA remains to be replicated using adapted control
136 measures (e.g., sediment core decontamination and metagenomic sequencing, as outlined in
137 this review). Most studies to date have involved well-dated sediment records and used a cross-
138 validation through paired analysis of aDNA and diagnostic lipid biomarkers as well as
139 geochemical proxies (e.g., Coolen et al., 2006; 2009). Yet, the absence of modern
140 contaminants in analysed samples was not always verified through sequencing analysis of
141 negative sampling and/or extraction controls, which is crucial for the interpretation of aDNA
142 data even if DNA values measured following amplification (by polymerase chain reaction;
143 PCR) are zero (as DNA may be present but simply be below detection limit). To date, the
144 oldest authenticated aDNA records are from ~400,000-year-old cave sediments (Willerslev et
145 al., 2003) and ~700,000-year-old permafrost mammal bones (Orlando et al., 2013).

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

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357 154 strategies so that aDNA research can become a well-established staple in marine
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359 155 biogeosciences. The focus is on sampling protocols within the framework of the International
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361 156 Ocean Discovery Program (IODP) “Biosphere Frontiers” theme, which is dedicated to
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363 157 understanding sub-seafloor communities. Our guidelines for deep-ocean *sedaDNA* isolation
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365 158 are applicable to any low-biomass and setting, including permafrost regions or planet Mars.
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369 159 **2 Definitions and pre-sampling considerations**

370 371 160 2.1 Ancient DNA (aDNA), sedimentary ancient DNA (sed aDNA), and palaeo-environmental 372 373 161 DNA (PalEnDNA)

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375 162 aDNA research involves the biomolecular study of non-modern genetic material preserved in
376
377 163 a broad range of biological samples (Shapiro und Hofreiter, 2012; Table 1). When an organism
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379 164 dies, mechanisms that ensure DNA repair in the cell are no longer active, leaving the DNA to
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381 165 degrade over time (Allentoft et al., 2012). Eventually, DNA from dead specimens becomes
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383 166 ancient. aDNA is highly fragmented to an average length of less than 100 base pairs (bp), for
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385 167 example, an average length of 48 bp has been determined in the oldest microbial genome
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387 168 assembled to date - from a 48,000-year-old Neandertal (Weyrich et al., 2017). aDNA is
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389 169 affected by post-mortem oxidative and deamination damage, such as thymine enrichment at
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391 170 the end of DNA sequences (Briggs et al., 2007; Ginolhac et al., 2011). Both fragmentation and
392
393 171 damage patterns can be used to authenticate aDNA, and damage can even be used to predict
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395 172 its age in certain scenarios (Kistler et al., 2017).
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398 173 aDNA research mainly focuses on organismal DNA extracted from some tissue remnants of
399
400 174 a wide range of single specimen (e.g., tooth, bone, hair, eggshell, feather). In contrast,
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402 175 environmental DNA (eDNA) focuses on disseminated genetic material found in environmental
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404 176 samples such as soil, sediment, water and ice (Taberlet et al., 2012a). Such samples contain
405
406 177 complex mixtures of DNA from taxonomically diverse organisms (e.g., bacteria, archaea,
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408 178 plants, animals). In addition to aDNA and eDNA, the term sedimentary aDNA (*sedaDNA*) has
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410 179 been coined to describe aDNA that is exclusively recovered from sediments (Willerslev et al.,
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180 2003; Jørgensen et al., 2012). The term fossil DNA has also been used in pioneer studies
181 where sedimentary plankton DNA and lipid biomarkers (i.e., “chemical fossils”) derived from
182 the same historical source organisms were analysed in parallel to validate the ancient DNA
183 results (e.g., Coolen and Overmann, 1998; 2007; Coolen et al., 2004). To a lesser degree,
184 ‘palaeo-environmental DNA’ (PalEnDNA) has also been used to describe disseminated
185 genetic material in a broad range of ancient environmental samples including sediments as
186 well as soil, paleosols, coprolites, water and ice (Rawlence et al., 2014). Modern sequencing
187 technologies and bioinformatic tools ease the analysis of these complex environmental aDNA
188 samples and of the biological responses to human or climate change, with investigations
189 having focussed on terrestrial settings (Jørgensen et al., 2012; Giguet-Covex et al., 2014;
190 Willerslev et al., 2014; Alsos et al., 2015; Pansu et al., 2015). In this review, we use the term
191 ‘marine *seda*aDNA’, which specifically refers to aDNA recovered from ocean sediments. A
192 detailed list of terms frequently used in aDNA research and their definitions is given in Table
193 1.

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195 2.2 Authenticity of marine aDNA

196 2.2.1 Environments favourable for marine aDNA preservation

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

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

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220 2.2.2 Marine *seda*DNA degradation and fragment length

221 18S rRNA gene fragments of past dinoflagellates, diatoms, and haptophytes as long as 500
222 bp in length have been amplified and sequenced (e.g., Coolen et al., 2004), after DNA was
223 isolated from sediments exhibiting characteristics favourable for aDNA preservation (Section
224 2.2.1). Up to 20% of genomic DNA from haptophyte algae has been reported to still be of high
225 molecular weight after 2,700 years of deposition in Black Sea sediments, and the ratio
226 between 500 bp-long haptophyte 18S rDNA fragments and the concentration of haptophyte-
227 diagnostic long-chain alkenones did not vary substantially for at least 7,500 years after
228 deposition, indicative that both types of biomolecules from the same plankton source were
229 equally well preserved (Coolen et al., 2006). This contradicts the generalised view that aDNA
230 is characterised by fragment lengths of <100bp. Nevertheless, studies that report the recovery
231 of exceedingly long aDNA fragments should be viewed with scepticism especially in the
232 absence of sampling and extraction controls, where there is no indication on whether the data
233 might reflect modern signals. However, to date, no data are available on average aDNA

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

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239 2.2.3 Contamination sources by modern DNA

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

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256 2.2.4 Intracellular vs. extracellular DNA

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

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260 or other cyst-forming plankton). Extracellular DNA is defined as DNA that has been released
261 from cells and preserved for substantial periods of time through mineral and/or microfossil
262 adsorption or within clay aggregates (Levy-Booth et al., 2007). Extracellular DNA represent
263 an archive of taxa that were autochthonous at the time of deposition (Cornaldesi et al., 2008;
264 2011). DNA extraction methods have been developed to target either of these DNA fractions
265 (Cornaldesi et al., 2005; Taberlet et al., 2012b; Alawi et al., 2014). However, it is difficult to
266 prove at what time in the past the organism died, and its DNA became extracellular.
267 Furthermore, the extra- and intracellular DNA pool may not always be clearly distinguishable
268 as genetic material present in the environment might have been taken up by competent
269 bacteria (Demanèche et al., 2001; Dell’Anno et al., 2004) and even by eukaryotes (Overballe-
270 Petersen and Willerslev, 2014). It is also important to note that if only the extracellular pool
271 was to be studied, the paleontological value of dormant yet ancient DNA (e.g., from cysts
272 deposited far back in time) will be lost. Due to these issues, extraction techniques targeting
273 only the extracellular portion are currently not recommended for marine *seDNA* studies.
274 Alternatively, bioinformatics approaches that can clearly identify ancient signals (Ginolhac et
275 al., 2011; Kistler et al., 2017) are preferred options for authenticating aDNA sequences
276 (Jónsson et al., 2013).

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278 2.2.5 Vertical DNA movement in marine sediment cores

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

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652 287 indicating that the extent of leaching depends on the taxonomic source (Haile et al., 2007). In
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654 288 Previous studies from lake sediments have shown that leaching is not a factor (Parducci et
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656 289 al., 2017), and in seafloor sediments DNA it seems to play a minor role as aDNA and lipid
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658 290 biomarkers derived from the same microbial source were found to co-exist or to be both below
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660 291 detection limit in marine sediments just centimetres apart (Boere et al., 2009; Coolen et al.,
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662 292 2006; 2009; 2013). In the latter studies it therefore appears that the pore size of the laminated
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664 293 sediments was too small for intracellular DNA to migrate, and that all extracellular plankton
665
666 294 DNA was adsorbed to the mineral matrices. Recent studies showing *upwards* vertical pore
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668 295 fluid movement also demonstrate the potential for vertical migration of relict or intact DNA
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670 296 within sediments (Torres et al., 2015), and should likewise be considered. Vertical migration
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672 297 of relict or intact DNA is expected to be especially a concern in sediments with micron scale
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674 298 pore sizes and/or a low clay content and a poor capacity to adsorb extracellular DNA. Future
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676 299 experimental research is required to quantify DNA leaching and/or migration through marine
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678 300 sediments, acknowledging the challenge of replicating a complex environmental system
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680 301 varying widely in hydrodynamics and sediment type.

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683 684 303 2.2.6 Cross validation of marine aDNA and palaeo-environmental proxies

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686 304 In addition to using proper contamination controls, downcore changes in past plankton
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688 305 compositions inferred from marine *seda*DNA can be validated through a complementary
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690 306 analysis of independent biological (e.g., microfossils, lipid biomarkers) and geochemical
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692 307 proxies (indicative of the prevailing paleoenvironmental conditions) (Boere et al., 2009; Coolen
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694 308 et al., 2004; 2006; 2013; Hou et al., 2014; More et al., 2018). The most detailed comparison
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696 309 between past ecosystem changes using marine *seda*DNA and the paleo-depositional
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698 310 environment to date has been performed on Holocene sediments from the permanently anoxic
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700 311 and sulfidic Black Sea (Coolen, 2011; Coolen et al., 2006; 2009; 2013; Giosan et al., 2012;
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702 312 Manske et al., 2008). The anoxic and laminated sediments of this semi-isolated sea are devoid
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704 313 of bioturbation and form high-resolution archives of climate-driven hydrological and

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711 314 environmental changes (Calvert et al., 1987; Hay, 1988). Episodes of postglacial sea-level
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713 315 rise ~9,000 years ago (Major et al., 2006) and sea surface salinity increase ~5,200 years ago
714
715 316 (Giosan et al., 2012) have been associated based on *sedaDNA* with freshwater to
716
717 317 brackish/marine planktonic community transitions (Coolen et al., 2013). For example, the
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719 318 gradual increase in sea surface salinity coincided with the arrival of marine copepods (*Calanus*
720
721 319 *euxinus*), which could only be identified through *sedaDNA* analysis (Coolen et al., 2013) as
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723 320 these important zooplankton members generally do not leave other diagnostic remains in the
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725 321 fossil record besides difficult to distinguish resting eggs (Marcus et al., 1996).

726
727 322 *Vice versa*, paleoenvironmental conditions inferred from more traditional geochemical
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729 323 and micropaleontological proxies have been verified from parallel *sedaDNA* analysis. By way
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731 324 of example, Black Sea sediments deposited since the last 2,500 years contain coccoliths from
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733 325 the calcified marine haptophyte *Emiliania huxleyi* whereas haptophyte-derived diagnostic long
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735 326 chain alkenones in the absence of coccoliths were abundant in up to 7,500-year-old sediments
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737 327 (Hay et al., 1991; Coolen et al., 2009). Paired analysis of long-chain alkenones and *sedaDNA*
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739 328 analysis (18S rRNA) revealed that that the first haptophytes that colonized the Black Sea
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741 329 ~7,500 years ago were initially a mixture of *E. huxleyi* and a highly diverse suite of previously
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743 330 overlooked non-calcified haptophytes related to alkenone-producing brackish *Isochrysis*
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745 331 species. *E. huxleyi* remained the only alkenone producer after 5,200 years BP when salinity
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747 332 reached modern day levels (Coolen et al., 2009). It was concluded that while calcite dissolution
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749 333 prevented the preservation of *E. huxleyi* coccoliths in sediments older than 2,500 years ago,
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751 334 their molecular fossils (DNA fragments and long-chain alkenones) survived much longer and
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753 335 showed that in reality this marine haptophyte entered the Black Sea already shortly after the
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755 336 marine reconnection which occurred ~9,000 years ago (Coolen et al., 2009; 2013). Even more
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757 337 detailed analyses of *E. huxleyi* (targeting 250-bp-long mitochondrial cytochrome oxidase
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759 338 subunit I; mtCOI) indicate a series of transitions from possibly low-salinity to high-salinity
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761 339 adapted strains of *E. huxleyi* in the Black Sea (7.5 – 5.2 ka BP), to a different suite of strains
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763 340 during the most marine stage (5.2 – 2.5 ka BP), returning to low salinity strains after 2.5 ka
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765 341 BP. The latter transition coincides with the onset of the cold and wet Subatlantic climate
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342 (Coolen, 2011) when the Black Sea experienced re-freshening from 32 to 18 ppt (Van der
343 Meer et al., 2011; Giosan et al., 2012; Coolen et al., 2013). The analysis of similar length
344 preserved sequences of viral major capsid protein (mcp) genes revealed a continuous co-
345 existence of *E. huxleyi* and coccolithoviruses in the Black Sea since the last 7,000 years and
346 that the same *E. huxleyi* strains, which occurred shortly after the marine reconnection returned
347 with the same viral strains after the re-freshening during the Subatlantic climate thousands of
348 years later (Coolen, 2011). More recently, detailed sedimentary 18S rDNA profiling targeting
349 the shorter (130 bp) V9 region revealed that long-term expansion of past oxygen minimum
350 zones (OMZ) created isolated habitats for unicellular eukaryotes (protists) capable of
351 sustaining oxygen depletion either by adapting a parasitic life cycle (e.g., apicomplexans) or
352 by establishing mutualistic connections with others (e.g., radiolarians and mixotrophic
353 dinoflagellates). These examples show that *sed* aDNA can be used to identify biological
354 sources of lipid biomarkers, to verify the reliability of paleoenvironmental information inferred
355 from more traditional proxies, and to reconstruct past ecosystems at multiple trophic levels.

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

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829 369 spanning the last glacial-interglacial cycle (Orsi et al., 2017). Further studies are required to
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831 370 determine as to how far the persistence of this phenomenon extends with increased depth in
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833 371 the biosphere. Nevertheless, these examples show that the complementary analysis of marine
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835 372 *seda*DNA-inferred past plankton composition and biological and geochemical proxies is a
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837 373 powerful tool to reconstruct palaeo-environments.
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843 375 **3 aDNA research in the International Ocean Discovery Program (IODP) framework**

845 376 3.1 IODP infrastructure

847 377 IODP is the global community's longest marine geoscience program, operating for 51 years.
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849 378 Its scientific strategy has been to answer globally-significant research questions about the
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851 379 Earth's structure, and the processes that have, and continue to, shape our planet and its
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853 380 climatic history. More recently, additional focus has been cast on biological evolution and
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855 381 limits, particularly in the sub-seafloor environment, under the new Biosphere Frontiers theme
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857 382 (Bickle et al., 2011). This theme has been inspired by the rapidly evolving knowledge and
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859 383 technical capabilities across the multiple merging fields of molecular biology, microbiology,
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861 384 organic and inorganic geochemistry, and micropalaeontology and includes scope for the
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863 385 integration of marine *seda*DNA research. IODP is currently serviced through three platforms,
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865 386 the United States of America's research vessel *JOIDES Resolution*, Japan's *Chikyu* and by
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867 387 the European consortium's Mission Specific Platforms (MSP). In recent years, the laboratories
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869 388 and storage facilities on the ships were modified, or purpose built, to ensure addressing Deep
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871 389 Biosphere questions was possible. As a result, the latest IODP decadal plan considered
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873 390 options to enable access to uncontaminated samples, their processing and preservation on-
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875 391 board. The latter has led to new coring technologies such as the Half-Length Advanced Piston
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877 392 Corer (HLAPC) allowing a coring depth extension of the conventionally used Advanced Piston
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879 393 Corer (APC), and the use of chemical contamination tracers such as perfluorocarbon tracers
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881 394 (PFTs) (see Sections 3.2 and 3.3, respectively). Particularly useful to aDNA studies may be
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395 the development of remotely controlled instruments allowing sediment sampling at ambient
396 pressure (MeBo; Pape et al., 2017) and a rock-drilling device (RD2; Früh-Green et al., 2015).
397 Notable achievements under the new Deep Biosphere theme include the finding of millions of
398 years old active microbial community from coal beds buried at 2.5 km below the seafloor
399 (Inagaki et al., 2015), and the preservation of an imprint of the Chicxulub impact catastrophe
400 (Cockell et al., 2017). A lot remains to be understood before this theme and its challenges are
401 satisfactorily addressed and it is clear that scientists engaging in Biosphere Frontiers will push
402 methodological, technological and multidisciplinary studies.

403

404 3.2 Coring strategies suitable for marine *seda*DNA retrieval

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

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

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

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438 3.3 Contamination tracing during coring

439 Deep ocean coring requires the lowering of coring instruments through hundreds to thousands
440 of metres of seawater before the seafloor is reached, hence exposes the instruments to
441 contamination by modern DNA (Fig. 1). This unavoidable issue has called for the development
442 of methods for environmental DNA contaminant tracing during coring operations. One
443 approach has been to compare biological material found in the contaminating source material
444 (e.g., seawater, drilling fluid) to that of sub-seafloor communities, and to exclude all signals
445 occurring in either from the final analyses (e.g., Expedition 330 Scientists, 2012; Cox et al.,
446 2018). This approach can be implemented for either piston coring or rotary core barrel drilling,
447 provided other sampling constraints associated with these coring systems can be

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

453 Another approach has been the introduction of fluorescent microspheres, which are particulate
454 tracers of 0.2 - 1.0 mm in diameter physically mimicking contaminating organisms. The
455 microspheres have been introduced near the coring head, i.e., where the sediment enters the
456 corer and coring pipe, spreading across the outside of the core (inside the pipe) while drilling,
457 simulating particle movement (Expedition 330 Scientists, 2012; Orcutt et al., 2017).
458 Microscopy has been used to quantify the number of microspheres at the periphery and in the
459 centre of the core to assess contamination (Expedition 330 Scientists, 2012; Orcutt et al.,
460 2017). Similar methods using other perfluorocarbon tracers (PFT’s) including
461 perfluoromethylcyclohexane (PMCH) have been developed for the USA drilling vessel
462 *JOIDES Resolution* (Smith et al., 2000) already in the early phases of IODP. Later, PMCH-
463 based contamination tracing has also been applied during riser drilling on the *Chikyu* (Inagaki
464 et al., 2015). During the IODP Expedition 357 (Atlantis Massif Serpentinization and Life), the
465 PMCH tracer delivery system was further developed to fit the seafloor-based drilling systems
466 MeBO (Pape et al., 2017) and RD2 (Früh-Green et al., 2015) (see Section 3.1). **PMCH is**
467 **highly volatile which can lead to false positive measurements in uncontaminated samples,**
468 **therefore, more recent investigations during IODP expeditions have moved to the use of the**
469 **heavier chemical tracer perfluoromethyldecalin (PFMD, 512.09 g mol⁻¹) (e.g., Fryer et al.,**
470 **2018).**

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472 3.4 Subsampling after core acquisition

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1065 473 Key to enable interdisciplinary sampling and correlations of independent measurements is a
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1067 474 detailed sampling plan, specifying sample types as well as the sequence in which these
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1069 475 samples are to be collected. Sampling for aDNA is time-sensitive (to avoid exposure to
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1071 476 oxygen, high temperatures and contamination), thus should be conducted immediately after
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1073 477 core retrieval on an untreated core-half (i.e., prior to any type of scanning such as by X-Ray).
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1075 478 The laboratory in which subsampling for aDNA is carried out should be clean and
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1077 479 workbenches and surfaces decontaminated with bleach (considered to be most efficient at
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1079 480 removing contaminating DNA) and, if applicable, ethanol (to prevent corrosion of metal after
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1081 481 bleach-treatment). Detailed records on whether molecular and amplification techniques (i.e.,
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1083 482 PCR) have been employed in on-board laboratories and which organisms were targeted
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1085 483 should be kept on record within IODP to ensure sampling for aDNA can be spatially separated
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1087 484 from these laboratories. While most vessels are not currently equipped for complete DNA
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1089 485 decontamination, such records may be invaluable for post-expedition aDNA data analyses.
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1093 487 Two sampling approaches are the most feasible on board IODP ships and MSP's: cutting
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1095 488 whole round cores or direct subsampling after core cutting into 1.5 m long sections. The choice
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1097 489 of approach needs to be made on a case-by-case basis, and depends on the specific facilities,
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1099 490 consumables, chemicals and researcher expertise available during each mission. It is
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1101 491 recommended that cutting or subsampling are performed under filtered air, e.g., a portable
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1103 492 type of a horizontal laminar flow clean air system as described in Morono and Inagaki (2016).
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1105 493 Additionally, subsampling should be conducted from the bottom to the top of the core (ancient
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1107 494 to modern), using clean (e.g., bleach and ethanol treated) sampling tools for each sample to
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1109 495 avoid any form of cross-contamination. Most commonly, soft sediments acquired by piston
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1111 496 coring are used for *seda*DNA analyses, therefore, we focus on subsampling procedures of the
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1113 497 latter here, subsequently briefly outlining sampling recommendations for hard rock material.
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1116 499 If the sampling decision is in favour of whole round core samples, the newly acquired core
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1118 500 sections are cut into 5 - 50 cm sections (preferably under cold conditions), which should be
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1124 501 packed in sterile bags or wrap and transferred directly into a fridge or freezer. Although quick
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1126 502 and providing a large amount of material for later sub-sampling, this approach has the
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1128 503 disadvantage that a lot of freezer space is required, and post-expedition transport can be
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1130 504 costly due to the high sample volume and weight.
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1134 506 An alternative to whole round core cutting is direct subsampling immediately after core cutting,
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1136 507 either directly from the centre of the top or bottom of each unsplit core section (usually 1.5 m
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1138 508 long), or after or splitting the core sections into two halves. In any case the core liner should
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1140 509 be cleaned with bleach to remove potential contamination from seawater, and core cutters
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1142 510 and splitting-wires, usually metal and sensitive to bleach should be cleaned with ethanol. If
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1144 511 sampling from uncut sections, surface material (~0.5 cm) should be removed with bleach and
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1146 512 ethanol-treated scrapers before sampling, which is most easily done with sterile cut-tip
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1148 513 syringes, placed into sterile plastic bags and stored frozen.
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1152 515 If sampling is undertaken on split core halves, simultaneous visual sedimentological
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1154 516 assessments are possible that enable more targeted sampling at specific depths of interest.
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1156 517 Using DNA-clean tools, the top 0.5 cm of the core surface should be scraped off perpendicular
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1158 518 to the core pipe using sterile scrapers (from bottom to top of the core). Alternatively, the core
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1160 519 half to be sampled can be covered with plastic wrap, followed by powdered dry ice, which will
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1162 520 result in the top 0.5 cm to become solid frozen. After 5 min, the frozen outer sediment layer
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1164 521 can be lifted at one edge with a sterile scalpel creating a contaminant-free surface, from which
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1166 522 subsamples can be taken (Coolen and Overmann, 1998). Then, subsampling should be
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1168 523 undertaken using sterile (e.g., gamma-irradiated) plastic syringes or centrifuge tubes (e.g.,
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1170 524 capacity of ~15 mL). Cut-tip syringes have the advantage that more sediment can be collected
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1172 525 as no pressure builds up when pushing the syringe into the sediment (the filled syringe should
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1174 526 be placed into a sterile plastic bag immediately, e.g., Whirl-Pak®). Alternatively, sterile
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1176 527 centrifuge tubes can be used as is to collect 'plunge-samples', usually providing ~1 - 3 cc of
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1178 528 sediment material. The outside of the 'mini-cores' should be cleaned with bleach and placed
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529 into sterile plastic bags to avoid cross-contamination between samples. For subsamples,
530 storage at -20 °C or -80 °C is recommended as freezing has been shown to facilitate
531 phytoplankton cell-lysis during DNA extractions (Armbrecht et al., *in prep.*). Sub-samples can
532 also be collected by transferring a small amount of sediment into a sterile microcentrifuge tube
533 using clean metal or disposable spatulas (particular care needs to be taken to avoid cross-
534 contamination when using the same sampling tool for different samples). The latter approach
535 may be a good solution when only a few small samples are required, e.g., to supplement other
536 scientific questions of an ongoing expedition. For replication purposes it is recommended that
537 duplicate samples are taken at each depth.

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

3.5 Marine aDNA sample processing and analysis

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

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

558 As on-board subsampling, DNA extractions should be carried out from the most ancient to
559 most recent samples, to prevent modern DNA inadvertently being carried to ancient samples.

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

571 While it would be ideal to find one extraction method that will yield the best quality data and
572 enable standardisation across ancient marine sediment studies, the type of sediment or target
573 organisms may require some adjustments of standard protocols (Hermans et al., 2018).

574 Extraction methods can bias the diversity observed due to differential resilience of taxa to the
575 cell-lysis method (Zhou et al., 1996; Young et al., 2015) and DNA binding capacities of
576 different soil and sediment types (Lorenz and Wackernagel, 1994; Miller et al., 1999). As a
577 result, the aDNA extraction efficiency can be poor and the detection of an aDNA signal lost.

578 To date, a variety of commercial kits have been successfully used to isolate aDNA from
579 sediments (Table 3). To further increase the yield of very low amounts of highly fragmented
580 aDNA several studies have been utilising extraction protocols that include a liquid-silica DNA
581 binding step (e.g., Brotherton et al., 2013 and Weyrich et al., 2017 for non-sediment samples)
582 or ethylenediaminetetraacetic acid (EDTA) cell-lysis step (Slon et al., 2017; utilising cave-

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1301 583 sediment samples). Other studies have replaced the Bead Solution in the DNeasy extraction
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1303 584 kits (Qiagen; Table 2) by 1M sodium phosphate pH 9 - 10 and 15 vol% ethanol to efficiently
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1305 585 release clay-adsorbed DNA, and to prevent DNA released from intact cells from adsorbing to
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1307 586 clay minerals during the extraction (Direito et al., 2012; Orsi et al., 2017; More et al., 2018).
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1309 587 The latter is especially important when working with low organic, high carbonate rocks and
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1311 588 sediments (Direito et al., 2012).

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1313 589 Two points are particularly important to prevent contamination during extractions. Firstly, as
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1315 590 with the samples themselves, it is crucial that all tools and reagents undergo rigorous
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1317 591 sterilisation procedures before utilisation, such as by bleach and UV treatment of any packing
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1319 592 material before entering ancient DNA facilities. Secondly, blank controls should be included
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1321 593 for every step of the laboratory process, i.e., extraction/library preparation blank controls,
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1323 594 sequencing and bioinformatic analysis controls (Ficetola et al., 2016). Controlling and
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1325 595 monitoring contamination is particularly important when analysing bacterial diversity due to
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1327 596 their presence in all laboratory environments and reagents (Weyrich et al., 2015). Optimally,
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1329 597 extraction blanks are included in a 1:5 ratio (Willerslev and Cooper, 2005), with a bare
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1331 598 minimum of one control with each set of extractions. Aside from bioinformatically removing
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1333 599 any organisms determined in such extraction blanks from the investigated sample material,
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1335 600 the contaminants should be tracked within a laboratory, and contaminant lists published
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1337 601 alongside the data for reasons of data transparency and authenticity.

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1339 602 Post-extraction, many marine aDNA studies have employed methods that are routinely used
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1341 603 for modern marine DNA analysis. Although modern DNA work is not exempt from precautions,
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1343 604 there are several issues with aDNA work: (i) as outlined in Sections 2.1. and 2.2. aDNA is
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1345 605 highly fragmented and degraded and any small amount of modern DNA present in the sample
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1347 606 (from reagents, labs or living cells that were present in the sediment sample) will amplify over
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1349 607 the aDNA; (ii) sampling and extraction controls are often not included in the sequencing
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1351 608 sample; (iii) PCRs are often inhibited due to the co-extraction of humic substances, pigments
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1353 609 and heavy metals along with DNA (Webster et al., 2003 and references therein), requiring
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610 adequate removal of these impurities (e.g., Coolen et al., 2009); (iv) successful PCRs are
611 prone to bias due to random amplification in reactions that contain very low amounts of DNA
612 template, thus PCR drift (stochastic variation in the first PCR cycles) can occur (Wagner et al.,
613 1994; Polz and Cavanaugh, 1998; Webster et al., 2003). More importantly, the number of,
614 e.g., bacterial 16S and eukaryotic 18S rRNA operons can greatly vary between per genome
615 and per cell and can cause a biased representation of the past community structure (e.g.,
616 Klappenbach et al., 2001). The above biases can be reduced and the detection limit lowered
617 when PCR approaches selectively, amplifying particular groups of organisms indicative of
618 environmental changes, are paired with independent geochemical proxies (e.g., Coolen et al.,
619 2004; 2006; 2009). However, we strongly advocate for the use of strict aDNA methodologies
620 and facilities in order to achieve the generation of authentic marine *seda*DNA data, following
621 the guidelines in this review.

622 Shotgun metagenomics are currently widely accepted and the least biased method to analyse
623 the broad diversity of ancient environmental samples (e.g., Slon et al., 2017). Although only a
624 small portion of the generated sequence data might be attributable to the ancient organism in
625 question (Morard et al., 2017), next generation sequencing (NGS) generates large quantities
626 of data that enable meaningful statistics, with the additional benefit of preserving the relative
627 proportion of detected taxa. To analyse aDNA sequence data, robust bioinformatic pipelines
628 (e.g., Paleomix, Schubert et al., 2014) have been developed and are available for the
629 application to marine *seda*DNA, integrating damage detection algorithms (e.g., Ginolhac et al.
630 2011; Kistler et al., 2017) that enable the distinction between ancient and modern signals.
631 Determining the extent of cytosine residues deamination (C to T and G to A, Weyrich et al.
632 2017) should also be considered to assess authenticity of aDNA sequences, especially when
633 the data was generated from mixed communities, such as from marine *seda*DNA. It is
634 furthermore crucial to carefully screen sequencing data for any low-complexity reads, which
635 may get incorrectly assigned to taxa during alignments against genetic databases, as well as
636 ensuring that taxonomic assignments in the database of choice are correct. Bioinformatic

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1419 637 pipelines removing such misidentification-derived errors do not currently exist and should be
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1421 638 the focus of future research, as well as the comparison of shotgun and amplicon marine
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1423 639 *seda*DNA data to accurately determine biases and analysis strategies best suited to this new
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1425 640 discipline.

1428 641 1429 1430 1431 642 **4 Future marine aDNA sampling considerations**

1434 643 4.1 Equipment and installations required aboard IODP platforms

1435
1436 644 In addition to the recent upgrades and investments IODP has made to enable sediment
1437
1438 645 sampling suitable for Biosphere Frontiers theme (Section 3.1) we suggest the following items
1439
1440 646 to facilitate contamination-free sediment sampling and the tracing of contaminants.

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1443 647 (i) Laboratories in which sampling for aDNA is undertaken should be carefully chosen to
1444
1445 648 minimise contamination. Rapid transport of the core from the deck to the lab, thorough
1446
1447 649 decontamination measures (see Section 3.4), and easy access to fridges or freezers are
1448
1449 650 crucial. While a positively air-pressured lab (standard for aDNA laboratories) may not be
1450
1451 651 feasible, air-flow can be reduced by keeping all doors shut and fans off during aDNA sampling.
1452
1453 652 Contamination by human DNA from analysts can be greatly reduced by wearing adequate
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1455 653 protective clothing (gloves, facemask, freshly laundered/disposable lab coat/overall). A
1456
1457 654 detailed record of any molecular work undertaken in ship-board labs should be maintained by
1458
1459 655 IODP, and under no circumstances should aDNA sampling be conducted in labs used
1460
1461 656 previously to run PCRs (see Section 3.4). Alternatively, the equipment of a shipping container
1462
1463 657 exclusively dedicated to aDNA sampling could be a good solution to spatially separate aDNA
1464
1465 658 sampling aboard drilling-platforms and installation could be as required during expeditions that
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1467 659 involve aDNA sampling.

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1469 660 (ii) DNA is likely to behave quite different from chemical tracers and microspheres currently
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1471 661 used to track contamination. With constantly advancing technologies in the field of synthetic

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

669
670 4.2 Ground-truthing marine aDNA research and data

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

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

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

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

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

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

695
**5 Application of marine *seda*DNA research guidelines to other contamination
susceptible environments**

698 5.1 Permafrost

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

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713 Sampling for ice and permafrost in polar regions is challenging in terms of logistics and
714 minimising contamination risks for both the sample and the sampled environment. For
715 example, permafrost soil samples are, like marine sediment cores, retrieved through drilling,
716 which can introduce microbial contaminants to the deeper permafrost soil layers as the drill
717 head and liquid pass through the top active soil layer (Bang-Andreasen et al., 2017).
718 Additionally, the cryosphere has been accumulating industrial chemicals and metals since the
719 1850's (McConnell et al., 2007), so that the present-day microbial community is now capable
720 of degrading industrial contaminants, thereby representing an anthropogenically-adapted
721 rather than an original pristine community (Hauptmann et al., 2017). With both these newly
722 adapted anthropogenic and drilling fluid communities containing characteristics for heavy
723 metal degradation, distinguishing indigenous ice core or permafrost communities from drilling
724 fluid communities will become more difficult in the future (Miteva et al., 2014). Therefore, the
725 described guidelines in this review for distinguishing ancient from modern and contaminant
726 signals, as well as the need for aseptic sampling procedures, are highly applicable to
727 permafrost environments and, more generally, the cryosphere.

728

729 5.2 Planetary exploration

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

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740 that in 2018 is mid-way through its predicted mission (Grotzinger et al., 2014). The rover Mars
741 2020 is being designed at present to test for evidence of life in the near-surface environment.
742 It will drill, collect and cache samples from the Martian surface, which will then be returned to
743 Earth for more detailed analysis (Beatty et al., 2015). Sample return from Mars to Earth is
744 planned for the end of the 2020's (Foust, 2018). Active planning is also ongoing for possible
745 missions to land and analyse materials from the surfaces of moons such as Europa and Titan,
746 by both NASA and the European Space Agency. For example, Europa (a moon of Jupiter) is
747 known to have a global saltwater ocean below its icy crust, as well as a rocky seafloor, so is
748 one of the highest priority targets in the search for present-day life beyond Earth (Hand et al.,
749 2017). A key concern with this solar system exploration is planetary protection, which is
750 governed by the United Nations Outer Space Treaty (United Nations Office for Disarmament
751 Affairs, 2015) and the Committee on Space Research (COSPAR) of the International
752 Committee for Science. There are two important categories of planetary protection. The first
753 is "forward contamination", where Earth-derived microbial life hitches a ride on spacecraft and
754 contaminates parts of a planetary surface being explored. The second is "backward
755 contamination", where life from an explored planet or moon is inadvertently returned to Earth,
756 maybe in a spacecraft or within a rock sample. The relevance to aDNA analytical protocols is
757 in forward contamination (i.e., the risk of contaminating sample material that could lead to data
758 misinterpretations, and/or generally introducing Earth contaminants to other planets; Rummel
759 and Conley, 2017). It should be noted that if indeed there is or was life on other planetary
760 bodies, it may well not be based on a genetic code composed of DNA and RNA. Independently
761 originated Life 2.0 would be highly unlikely to have evolved exactly the same nucleic acid
762 genetic code as life on Earth (e.g., Rummel and Conley, 2017). Indeed, it has been postulated
763 that an alternative biosphere could exist as a "shadow biosphere" on Earth (Davies et al.,
764 2009). If DNA or RNA-based extant life is found on Mars, for example, then it is most likely
765 that it would represent either past natural exchange of rocks between the two planets
766 (panspermia), or anthropogenic forward contamination. Therefore, the procedures used for
767 distinguishing indigenous life in planetary exploration will need broadening to include the

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

772

773 **Conclusions**

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

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791

792 **Acknowledgements:**

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815 **Figures:**

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

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819 recommended precautions to be considered and/or controls to be taken. An impact score (1-
820 3 stars) is given to indicate the severity of potential contamination or the impact that impaired
821 data would have on the results at each step in the process. Schematic graphics are not to
822 scale.

823
824 **Figure 2:** Overview of IODP coring systems. A) Advanced piston coring system (APC), shown
825 before and after stroking; only small volumes of drill fluid can enter the space between the
826 core barrel and collar from above after stroking, greatly reducing the risk of contamination. B)
827 Extended core barrel system (XCB) and C) Rotary core barrel system (RCB); both containing
828 circulation jets at the bottom of the core barrel through which drill-fluid enters and removes
829 coring debris by transporting it upwards within the drill hole to the surface. D) Comparison of
830 rotary and piston cored sediments demonstrating the well-preserved lamination in Piston
831 cored material. Figure adapted from Sun et al. (2018) and IODP
832 (iodp.tamu.edu/tools/index.html).

833
834 **Table 1:** Terms commonly used in marine aDNA research and their definition. aDNA terms
835 are listed hierarchically, all other terms are listed alphabetically.

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

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841 and intracellular DNA from a single marine sediment sample. Journal of microbiological
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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.

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3 **1 Title:**
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8 *3 sample handling and data generation*
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102
103 42 **Abstract**
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106 43 The study of ancient DNA (aDNA) from sediments (*sedaDNA*) offers great potential for
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108 44 paleoclimate interpretation, and has recently been applied as a tool to characterise past
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110 45 marine life and environments from deep ocean sediments over geological timescales. Using
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112 46 *sedaDNA*, palaeo-communities have been detected, including prokaryotes and eukaryotes
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114 47 that do not fossilise, thereby revolutionising the scope of marine micropalaeontological
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121 48 research. However, many studies to date have not reported on the measures taken to prove
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123 49 the authenticity of *seda*DNA-derived data from which conclusions are drawn. aDNA is highly
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125 50 fragmented and degraded and extremely sensitive to contamination by non-target
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127 51 environmental DNA. Contamination risks are particularly high on research vessels, drilling
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129 52 ships and platforms, where logistics and facilities do not yet allow for sterile sediment coring,
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131 53 and due consideration needs to be given to sample processing and analysis following aDNA
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133 54 guidelines. This review clarifies the use of aDNA terminology, discusses common pitfalls and
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135 55 highlights the urgency behind adopting new standards for marine *seda*DNA research, with a
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137 56 focus on sampling optimisation to facilitate the incorporation of routine *seda*DNA research into
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139 57 International Ocean Discovery Program (IODP) operations. Currently available installations
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141 58 aboard drilling ships and platforms are reviewed, improvements suggested, analytical
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143 59 approaches detailed, and the controls and documentation necessary to support the
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145 60 authenticity of aDNA retrieved from deep-sea sediment cores is outlined. Beyond practical
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147 61 considerations, concepts relevant to the study of past marine biodiversity based on aDNA,
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149 62 and the applicability of the new guidelines to the study of other contamination-susceptible
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151 63 environments (permafrost and outer space) are discussed.

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156 65 **Keywords:** ancient DNA; marine sediments; deep biosphere; phytoplankton; contamination;
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158 66 seafloor; IODP; biomarkers; Mars

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163 68 **Abbreviations:** aDNA, ancient DNA; APC, Advanced Piston Corer; HLAPC, Half-Length
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165 69 Advanced Piston Corer; IODP, International Ocean Discovery Program; mbsf, metres below
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167 70 seafloor; MSP, Mission Specific Platforms; NGS, Next generation Sequencing; PCR,
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169 71 polymerase chain reaction; PFT, perfluorocarbon tracer; PMCH, perfluoromethylcyclohexane;
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171 72 PFMD, perfluoromethyldecalin; *seda*DNA, sedimentary ancient DNA

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73 1 Introduction

74 Past marine environments have generally been investigated using a suite of methodological
75 approaches and interdisciplinary research fields, such as geology, organic and inorganic
76 geochemistry, paleoceanography and micropaleontology. Discoveries in all of these
77 disciplines have contributed greatly to our understanding of the climatic history of Earth and
78 the evolution and responses of its inhabitants. However, to date, it has not been possible to
79 achieve a detailed picture of all living organisms that have occupied global oceans in the past,
80 restricting estimates of past environmental conditions and climate. The techniques that have
81 traditionally been applied to reconstruct marine palaeo-communities are limited, such as
82 microscopy to investigate the microfossil record (e.g., Winter et al., 2010; Armbrecht et al.,
83 2018). Due to dissolution and degradation of phytoplankton and microzooplankton while
84 sinking to the seafloor post-mortem, only the most robust skeletons and shells are preserved
85 within a complex geological record (Loucaides et al., 2011). Often, these microfossils are
86 broken, altered by chemical processes and unrecognizable. In the absence of well-preserved
87 diagnostic morphological features, lipid biomarkers can provide supplementary information on
88 biological sources in sediment records (Volkman et al., 1998; Coolen et al., 2004; Sinninghe
89 Damste et al., 2004; Brocks et al., 2011), however, the majority of plankton members do not
90 possess highly diagnostic biomarkers.

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

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

106 In the last decade, marine palaeo-research has been reinvigorated by genomic techniques
107 that enable the analysis of ancient DNA (aDNA) molecules from long-dead organisms. Past
108 prokaryotic and eukaryotic plankton communities have been reconstructed using aDNA
109 sequencing approaches (e.g., Coolen and Overmann, 1998; 2007; Coolen et al., 2004; 2008;
110 2013; Bissett et al., 2005; D'Andrea et al., 2006; Boere et al., 2009; Lejzerowicz et al., 2013;
111 Hou et al., 2014; Randlett et al., 2014; More et al., 2018). These studies have confirmed that
112 phyto- and zooplankton are good targets for aDNA-based studies, while also being particularly
113 relevant for ecosystem-climate reconstructions. It is reasonable to assume that obligate
114 photosynthetic plankton (phytoplankton) and/or zooplankton do not survive and reproduce
115 after burial in deep sediments, and represent uncommon lab contaminants (e.g., Lejzerowicz
116 et al., 2013; Hou et al., 2014; More et al., 2018). aDNA analysis has shown that even after
117 their voyage through the water column plankton-derived particles that had settled on the
118 seafloor still reflect the global biogeographic patterns of living species (Morard et al., 2017).
119 Notably, the reconstruction of past marine communities using aDNA is possible using just a
120 few grams of sediment, facilitating sediment sample collection, transport and storage for the
121 purpose of aDNA analyses.

122 The marine aDNA archive extends back to the Pleistocene, as shown by studies of genomic,
123 18S rRNA gene markers targeting various eukaryotic groups. For example, aDNA has been
124 recovered from various eukaryotic plankton taxa in 43,000-year-old Arabian Sea sediments
125 (More et al., 2018). Taxon-specific approaches targeting small, degraded DNA fragments
126 allowed the retrieval of foraminiferal aDNA from ~800-year-old fjord sediments (Pawlowska et

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127 al., 2014) and ~30,000-year-old deep-sea sediments with the additional benefit of enabling
128 the detection of rare taxa (Lejzerowicz et al., 2013). However, if a targeted approach is used,
129 the origin and fate of the DNA in question must be carefully considered, especially for very old
130 claims, such as the retrieval of 1.4 million years old DNA from chloroplasts (Kirkpatrick et al.,
131 2016), which are subject to kleptoplasty (sequestration and maintenance of chloroplasts;
132 Bernhard and Bowser, 1999). While Kirkpatrick et al. (2016) used thorough contamination
133 control, the finding of >1 million years old DNA remains to be replicated using adapted control
134 measures (e.g., sediment core decontamination and metagenomic sequencing, as outlined in
135 this review). Most studies to date have involved well-dated sediment records and used a cross-
136 validation through paired analysis of aDNA and diagnostic lipid biomarkers as well as
137 geochemical proxies (e.g., Coolen et al., 2006; 2009). Yet, the absence of modern
138 contaminants in analysed samples was not always verified through sequencing analysis of
139 negative sampling and/or extraction controls, which is crucial for the interpretation of aDNA
140 data even if DNA values measured following amplification (by polymerase chain reaction;
141 PCR) are zero (as DNA may be present but simply be below detection limit). To date, the
142 oldest authenticated aDNA records are from ~400,000-year-old cave sediments (Willerslev et
143 al., 2003) and ~700,000-year-old permafrost mammal bones (Orlando et al., 2013).

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

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357 155 understanding sub-seafloor communities. Our guidelines for deep-ocean *sedaDNA* isolation
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359 156 are applicable to any low-biomass and setting, including permafrost regions or planet Mars.
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363 157 **2 Definitions and pre-sampling considerations**

364 365 158 2.1 Ancient DNA (aDNA), sedimentary ancient DNA (sed aDNA), and palaeo-environmental 366 367 159 DNA (PalEnDNA)

368
369 160 aDNA research involves the biomolecular study of non-modern genetic material preserved in
370
371 161 a broad range of biological samples (Shapiro und Hofreiter, 2012; Table 1). When an organism
372
373 162 dies, mechanisms that ensure DNA repair in the cell are no longer active, leaving the DNA to
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375 163 degrade over time (Allentoft et al., 2012). Eventually, DNA from dead specimens becomes
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377 164 ancient. aDNA is highly fragmented to an average length of less than 100 base pairs (bp), for
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379 165 example, an average length of 48 bp has been determined in the oldest microbial genome
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381 166 assembled to date - from a 48,000-year-old Neandertal (Weyrich et al., 2017). aDNA is
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383 167 affected by post-mortem oxidative and deamination damage, such as thymine enrichment at
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385 168 the end of DNA sequences (Briggs et al., 2007; Ginolhac et al., 2011). Both fragmentation and
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387 169 damage patterns can be used to authenticate aDNA, and damage can even be used to predict
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389 170 its age in certain scenarios (Kistler et al., 2017).

390
391 171 aDNA research mainly focuses on organismal DNA extracted from some tissue remnants of
392
393 172 a wide range of single specimen (e.g., tooth, bone, hair, eggshell, feather). In contrast,
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395 173 environmental DNA (eDNA) focuses on disseminated genetic material found in environmental
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397 174 samples such as soil, sediment, water and ice (Taberlet et al., 2012a). Such samples contain
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399 175 complex mixtures of DNA from taxonomically diverse organisms (e.g., bacteria, archaea,
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401 176 plants, animals). In addition to aDNA and eDNA, the term sedimentary aDNA (*sedaDNA*) has
402
403 177 been coined to describe aDNA that is exclusively recovered from sediments (Willerslev et al.,
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405 178 2003; Jørgensen et al., 2012). The term fossil DNA has also been used in pioneer studies
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407 179 where sedimentary plankton DNA and lipid biomarkers (i.e., “chemical fossils”) derived from
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409 180 the same historical source organisms were analysed in parallel to validate the ancient DNA
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181 results (e.g., Coolen and Overmann, 1998; 2007; Coolen et al., 2004). To a lesser degree,
182 ‘palaeo-environmental DNA’ (PalEnDNA) has also been used to describe disseminated
183 genetic material in a broad range of ancient environmental samples including sediments as
184 well as soil, paleosols, coprolites, water and ice (Rawlence et al., 2014). Modern sequencing
185 technologies and bioinformatic tools ease the analysis of these complex environmental aDNA
186 samples and of the biological responses to human or climate change, with investigations
187 having focussed on terrestrial settings (Jørgensen et al., 2012; Giguët-Covex et al., 2014;
188 Willerslev et al., 2014; Alsos et al., 2015; Pansu et al., 2015). In this review, we use the term
189 ‘marine *seda*aDNA’, which specifically refers to aDNA recovered from ocean sediments. A
190 detailed list of terms frequently used in aDNA research and their definitions is given in Table
191 1.

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193 2.2 Authenticity of marine aDNA

194 2.2.1 Environments favourable for marine aDNA preservation

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

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

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218 2.2.2 Marine *seda*aDNA degradation and fragment length

219 18S rRNA gene fragments of past dinoflagellates, diatoms, and haptophytes as long as 500
220 bp in length have been amplified and sequenced (e.g., Coolen et al., 2004), after DNA was
221 isolated from sediments exhibiting characteristics favourable for aDNA preservation (Section
222 2.2.1). Up to 20% of genomic DNA from haptophyte algae has been reported to still be of high
223 molecular weight after 2,700 years of deposition in Black Sea sediments, and the ratio
224 between 500 bp-long haptophyte 18S rDNA fragments and the concentration of haptophyte-
225 diagnostic long-chain alkenones did not vary substantially for at least 7,500 years after
226 deposition, indicative that both types of biomolecules from the same plankton source were
227 equally well preserved (Coolen et al., 2006). This contradicts the generalised view that aDNA
228 is characterised by fragment lengths of <100bp. Nevertheless, studies that report the recovery
229 of exceedingly long aDNA fragments should be viewed with scepticism especially in the
230 absence of sampling and extraction controls, where there is no indication on whether the data
231 might reflect modern signals. However, to date, no data are available on average aDNA
232 fragment length for deep-sea sediments, which could be obtained from metagenomic shotgun
233 sequencing. Gaining insights into the latter should be the focus of future research as this

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

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237 2.2.3 Contamination sources by modern DNA

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

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254 2.2.4 Intracellular vs. extracellular DNA

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

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260 adsorption or within clay aggregates (Levy-Booth et al., 2007). Extracellular DNA represent
261 an archive of taxa that were autochthonous at the time of deposition (Cornaldesi et al., 2008;
262 2011). DNA extraction methods have been developed to target either of these DNA fractions
263 (Cornaldesi et al., 2005; Taberlet et al., 2012b; Alawi et al., 2014). However, it is difficult to
264 prove at what time in the past the organism died, and its DNA became extracellular.
265 Furthermore, the extra- and intracellular DNA pool may not always be clearly distinguishable
266 as genetic material present in the environment might have been taken up by competent
267 bacteria (Demanèche et al., 2001; Dell'Anno et al., 2004) and even by eukaryotes (Overballe-
268 Petersen and Willerslev, 2014). It is also important to note that if only the extracellular pool
269 was to be studied, the paleontological value of dormant yet ancient DNA (e.g., from cysts
270 deposited far back in time) will be lost. Due to these issues, extraction techniques targeting
271 only the extracellular portion are currently not recommended for marine *seda*DNA studies.
272 Alternatively, bioinformatics approaches that can clearly identify ancient signals (Ginolhac et
273 al., 2011; Kistler et al., 2017) are preferred options for authenticating aDNA sequences
274 (Jónsson et al., 2013).

275 276 2.2.5 Vertical DNA movement in marine sediment cores

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

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652 287 al., 2017), and in seafloor sediments DNA it seems to play a minor role as aDNA and lipid
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654 288 biomarkers derived from the same microbial source were found to co-exist or to be both below
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656 289 detection limit in marine sediments just centimetres apart (Boere et al., 2009; Coolen et al.,
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658 290 2006; 2009; 2013). In the latter studies it therefore appears that the pore size of the laminated
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660 291 sediments was too small for intracellular DNA to migrate, and that all extracellular plankton
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662 292 DNA was adsorbed to the mineral matrices. Recent studies showing *upwards* vertical pore
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664 293 fluid movement also demonstrate the potential for vertical migration of relict or intact DNA
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666 294 within sediments (Torres et al., 2015), and should likewise be considered. Vertical migration
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668 295 of relict or intact DNA is expected to be especially a concern in sediments with micron scale
669
670 296 pore sizes and/or a low clay content and a poor capacity to adsorb extracellular DNA. Future
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672 297 experimental research is required to quantify DNA leaching and/or migration through marine
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674 298 sediments, acknowledging the challenge of replicating a complex environmental system
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676 299 varying widely in hydrodynamics and sediment type.

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680 301 2.2.6 Cross validation of marine aDNA and palaeo-environmental proxies

681
682 302 In addition to using proper contamination controls, downcore changes in past plankton
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684 303 compositions inferred from marine *seda*DNA can be validated through a complementary
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686 304 analysis of independent biological (e.g., microfossils, lipid biomarkers) and geochemical
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688 305 proxies (indicative of the prevailing paleoenvironmental conditions) (Boere et al., 2009; Coolen
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690 306 et al., 2004; 2006; 2013; Hou et al., 2014; More et al., 2018). The most detailed comparison
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692 307 between past ecosystem changes using marine *seda*DNA and the paleo-depositional
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694 308 environment to date has been performed on Holocene sediments from the permanently anoxic
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696 309 and sulfidic Black Sea (Coolen, 2011; Coolen et al., 2006; 2009; 2013; Giosan et al., 2012;
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698 310 Manske et al., 2008). The anoxic and laminated sediments of this semi-isolated sea are devoid
699
700 311 of bioturbation and form high-resolution archives of climate-driven hydrological and
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702 312 environmental changes (Calvert et al., 1987; Hay, 1988). Episodes of postglacial sea-level
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704 313 rise ~9,000 years ago (Major et al., 2006) and sea surface salinity increase ~5,200 years ago

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711 314 (Giosan et al., 2012) have been associated based on *sedaDNA* with freshwater to
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713 315 brackish/marine planktonic community transitions (Coolen et al., 2013). For example, the
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715 316 gradual increase in sea surface salinity coincided with the arrival of marine copepods (*Calanus*
716
717 317 *euxinus*), which could only be identified through *sedaDNA* analysis (Coolen et al., 2013) as
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719 318 these important zooplankton members generally do not leave other diagnostic remains in the
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721 319 fossil record besides difficult to distinguish resting eggs (Marcus et al., 1996).
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723 320 *Vice versa*, paleoenvironmental conditions inferred from more traditional geochemical
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725 321 and micropaleontological proxies have been verified from parallel *sedaDNA* analysis. By way
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727 322 of example, Black Sea sediments deposited since the last 2,500 years contain coccoliths from
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729 323 the calcified marine haptophyte *Emiliana huxleyi* whereas haptophyte-derived diagnostic long
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731 324 chain alkenones in the absence of coccoliths were abundant in up to 7,500-year-old sediments
732
733 325 (Hay et al., 1991; Coolen et al., 2009). Paired analysis of long-chain alkenones and *sedaDNA*
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735 326 analysis (18S rRNA) revealed that that the first haptophytes that colonized the Black Sea
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737 327 ~7,500 years ago were initially a mixture of *E. huxleyi* and a highly diverse suite of previously
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739 328 overlooked non-calcified haptophytes related to alkenone-producing brackish *Isochrysis*
740
741 329 species. *E. huxleyi* remained the only alkenone producer after 5,200 years BP when salinity
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743 330 reached modern day levels (Coolen et al., 2009). It was concluded that while calcite dissolution
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745 331 prevented the preservation of *E. huxleyi* coccoliths in sediments older than 2,500 years ago,
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747 332 their molecular fossils (DNA fragments and long-chain alkenones) survived much longer and
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749 333 showed that in reality this marine haptophyte entered the Black Sea already shortly after the
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751 334 marine reconnection which occurred ~9,000 years ago (Coolen et al., 2009; 2013). Even more
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753 335 detailed analyses of *E. huxleyi* (targeting 250-bp-long mitochondrial cytochrome oxidase
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755 336 subunit I; mtCOI) indicate a series of transitions from possibly low-salinity to high-salinity
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757 337 adapted strains of *E. huxleyi* in the Black Sea (7.5 – 5.2 ka BP), to a different suite of strains
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759 338 during the most marine stage (5.2 – 2.5 ka BP), returning to low salinity strains after 2.5 ka
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761 339 BP. The latter transition coincides with the onset of the cold and wet Subatlantic climate
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763 340 (Coolen, 2011) when the Black Sea experienced re-freshening from 32 to 18 ppt (Van der
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765 341 Meer et al., 2011; Giosan et al., 2012; Coolen et al., 2013). The analysis of similar length
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342 preserved sequences of viral major capsid protein (mcp) genes revealed a continuous co-
343 existence of *E. huxleyi* and coccolithoviruses in the Black Sea since the last 7,000 years and
344 that the same *E. huxleyi* strains, which occurred shortly after the marine reconnection returned
345 with the same viral strains after the re-freshening during the Subatlantic climate thousands of
346 years later (Coolen, 2011). More recently, detailed sedimentary 18S rDNA profiling targeting
347 the shorter (130 bp) V9 region revealed that long-term expansion of past oxygen minimum
348 zones (OMZ) created isolated habitats for unicellular eukaryotes (protists) capable of
349 sustaining oxygen depletion either by adapting a parasitic life cycle (e.g., apicomplexans) or
350 by establishing mutualistic connections with others (e.g., radiolarians and mixotrophic
351 dinoflagellates). These examples show that *sed* aDNA can be used to identify biological
352 sources of lipid biomarkers, to verify the reliability of paleoenvironmental information inferred
353 from more traditional proxies, and to reconstruct past ecosystems at multiple trophic levels.

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

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

373 **3 aDNA research in the International Ocean Discovery Program (IODP) framework**

374 3.1 IODP infrastructure

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

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

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402 3.2 Coring strategies suitable for marine *seda*DNA retrieval

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

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

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

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436 3.3 Contamination tracing during coring

437 Deep ocean coring requires the lowering of coring instruments through hundreds to thousands
438 of metres of seawater before the seafloor is reached, hence exposes the instruments to
439 contamination by modern DNA (Fig. 1). This unavoidable issue has called for the development
440 of methods for environmental DNA contaminant tracing during coring operations. One
441 approach has been to compare biological material found in the contaminating source material
442 (e.g., seawater, drilling fluid) to that of sub-seafloor communities, and to exclude all signals
443 occurring in either from the final analyses (e.g., Expedition 330 Scientists, 2012; Cox et al.,
444 2018). This approach can be implemented for either piston coring or rotary core barrel drilling,
445 provided other sampling constraints associated with these coring systems can be
446 accommodated. However, this procedure does not account for potential "false negative" DNA
447 signals that might indeed occur in both ancient sediments *and* modern contaminating material.

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

451 Another approach has been the introduction of fluorescent microspheres, which are particulate
452 tracers of 0.2 - 1.0 mm in diameter physically mimicking contaminating organisms. The
453 microspheres have been introduced near the coring head, i.e., where the sediment enters the
454 corer and coring pipe, spreading across the outside of the core (inside the pipe) while drilling,
455 simulating particle movement (Expedition 330 Scientists, 2012; Orcutt et al., 2017).
456 Microscopy has been used to quantify the number of microspheres at the periphery and in the
457 centre of the core to assess contamination (Expedition 330 Scientists, 2012; Orcutt et al.,
458 2017). Similar methods using other perfluorocarbon tracers (PFT’s) including
459 perfluoromethylcyclohexane (PMCH) have been developed for the USA drilling vessel
460 *JOIDES Resolution* (Smith et al., 2000) already in the early phases of IODP. Later, PMCH-
461 based contamination tracing has also been applied during riser drilling on the *Chikyu* (Inagaki
462 et al., 2015). During the IODP Expedition 357 (Atlantis Massif Serpentinization and Life), the
463 PMCH tracer delivery system was further developed to fit the seafloor-based drilling systems
464 MeBO (Pape et al., 2017) and RD2 (Früh-Green et al., 2015) (see Section 3.1). PMCH is
465 highly volatile which can lead to false positive measurements in uncontaminated samples,
466 therefore, more recent investigations during IODP expeditions have moved to the use of the
467 heavier chemical tracer perfluoromethyldecalin (PFMD, 512.09 g mol⁻¹) (e.g., Fryer et al.,
468 2018).

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470 **3.4 Subsampling after core acquisition**

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

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1065 474 oxygen, high temperatures and contamination), thus should be conducted immediately after
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1067 475 core retrieval on an untreated core-half (i.e., prior to any type of scanning such as by X-Ray).
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1069 476 The laboratory in which subsampling for aDNA is carried out should be clean and
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1071 477 workbenches and surfaces decontaminated with bleach (considered to be most efficient at
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1073 478 removing contaminating DNA) and, if applicable, ethanol (to prevent corrosion of metal after
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1075 479 bleach-treatment). Detailed records on whether molecular and amplification techniques (i.e.,
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1077 480 PCR) have been employed in on-board laboratories and which organisms were targeted
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1079 481 should be kept on record within IODP to ensure sampling for aDNA can be spatially separated
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1081 482 from these laboratories. While most vessels are not currently equipped for complete DNA
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1083 483 decontamination, such records may be invaluable for post-expedition aDNA data analyses.
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1087 485 Two sampling approaches are the most feasible on board IODP ships and MSP's: cutting
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1089 486 whole round cores or direct subsampling after core cutting into 1.5 m long sections. The choice
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1091 487 of approach needs to be made on a case-by-case basis, and depends on the specific facilities,
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1093 488 consumables, chemicals and researcher expertise available during each mission. It is
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1095 489 recommended that cutting or subsampling are performed under filtered air, e.g., a portable
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1097 490 type of a horizontal laminar flow clean air system as described in Morono and Inagaki (2016).
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1099 491 Additionally, subsampling should be conducted from the bottom to the top of the core (ancient
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1101 492 to modern), using clean (e.g., bleach and ethanol treated) sampling tools for each sample to
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1103 493 avoid any form of cross-contamination. Most commonly, soft sediments acquired by piston
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1105 494 coring are used for *sedaDNA* analyses, therefore, we focus on subsampling procedures of the
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1107 495 latter here, subsequently briefly outlining sampling recommendations for hard rock material.
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1111 497 If the sampling decision is in favour of whole round core samples, the newly acquired core
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1113 498 sections are cut into 5 - 50 cm sections (preferably under cold conditions), which should be
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1115 499 packed in sterile bags or wrap and transferred directly into a fridge or freezer. Although quick
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1124 501 disadvantage that a lot of freezer space is required, and post-expedition transport can be
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1126 502 costly due to the high sample volume and weight.
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1130 504 An alternative to whole round core cutting is direct subsampling immediately after core cutting,
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1132 505 either directly from the centre of the top or bottom of each unsplit core section (usually 1.5 m
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1134 506 long), or after or splitting the core sections into two halves. In any case the core liner should
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1136 507 be cleaned with bleach to remove potential contamination from seawater, and core cutters
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1138 508 and splitting-wires, usually metal and sensitive to bleach should be cleaned with ethanol. If
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1140 509 sampling from uncut sections, surface material (~0.5 cm) should be removed with bleach and
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1142 510 ethanol-treated scrapers before sampling, which is most easily done with sterile cut-tip
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1144 511 syringes, placed into sterile plastic bags and stored frozen.
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1148 513 If sampling is undertaken on split core halves, simultaneous visual sedimentological
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1150 514 assessments are possible that enable more targeted sampling at specific depths of interest.
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1152 515 Using DNA-clean tools, the top 0.5 cm of the core surface should be scraped off perpendicular
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1154 516 to the core pipe using sterile scrapers (from bottom to top of the core). Alternatively, the core
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1156 517 half to be sampled can be covered with plastic wrap, followed by powdered dry ice, which will
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1158 518 result in the top 0.5 cm to become solid frozen. After 5 min, the frozen outer sediment layer
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1160 519 can be lifted at one edge with a sterile scalpel creating a contaminant-free surface, from which
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1162 520 subsamples can be taken (Coolen and Overmann, 1998). Then, subsampling should be
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1164 521 undertaken using sterile (e.g., gamma-irradiated) plastic syringes or centrifuge tubes (e.g.,
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1166 522 capacity of ~15 mL). Cut-tip syringes have the advantage that more sediment can be collected
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1168 523 as no pressure builds up when pushing the syringe into the sediment (the filled syringe should
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1170 524 be placed into a sterile plastic bag immediately, e.g., Whirl-Pak®). Alternatively, sterile
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1172 525 centrifuge tubes can be used as is to collect 'plunge-samples', usually providing ~1 - 3 cc of
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1174 526 sediment material. The outside of the 'mini-cores' should be cleaned with bleach and placed
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1176 527 into sterile plastic bags to avoid cross-contamination between samples. For subsamples,
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1178 528 storage at -20 °C or -80 °C is recommended as freezing has been shown to facilitate
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1183 529 phytoplankton cell-lysis during DNA extractions (Armbrecht et al., *in prep.*). Sub-samples can
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1185 530 also be collected by transferring a small amount of sediment into a sterile microcentrifuge tube
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1187 531 using clean metal or disposable spatulas (particular care needs to be taken to avoid cross-
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1189 532 contamination when using the same sampling tool for different samples). The latter approach
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1191 533 may be a good solution when only a few small samples are required, e.g., to supplement other
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1193 534 scientific questions of an ongoing expedition. For replication purposes it is recommended that
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1195 535 duplicate samples are taken at each depth.

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1199 537 If the material is hard rock or similar, subsamples are most easily collected from whole round
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1201 538 or split cores. The same decontamination procedures as outlined above should be considered
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1205 540 tools with bleach and ethanol, sampling under cold conditions and filtered or low air-flow,
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1207 541 packing of samples into sterile bags before storage). A de-contaminated metal cutter or a
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1209 542 hammer and chisel are best used to remove the outer layer of the exposed sediment, at least
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1211 543 at those depths where subsampling is anticipated.

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1217 546 3.5 Marine aDNA sample processing and analysis

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1219 547 Marine aDNA samples should be processed in a specialised aDNA laboratory to prevent
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1221 548 contamination with modern DNA. Such a laboratory is generally characterised by creating a
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1223 549 low-DNA environment, with a clear separation of no-DNA (e.g., buffer preparation) and DNA-
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1225 550 containing (e.g., DNA extraction) workflows, regular and thorough sterilisation procedures,
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1227 551 positive air pressure, and protective clothing of the analyst (lab coat/suit, gloves, facemask,
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1229 552 visor). Details on optimised laboratory set-up, techniques and workflows have been reviewed
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1231 553 before (Cooper and Poinar, 2000; Pedersen et al., 2015). The introduction of aDNA samples
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1233 554 into such facilities is relatively straight-forward, as the outer packaging and surface of the
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1235 555 sample can be easily sterilised (e.g., using bleach and/or UV).

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

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

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

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

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

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610 template, thus PCR drift (stochastic variation in the first PCR cycles) can occur (Wagner et al.,
611 1994; Polz and Cavanaugh, 1998; Webster et al., 2003). More importantly, the number of,
612 e.g., bacterial 16S and eukaryotic 18S rRNA operons can greatly vary between per genome
613 and per cell and can cause a biased representation of the past community structure (e.g.,
614 Klappenbach et al., 2001). The above biases can be reduced and the detection limit lowered
615 when PCR approaches selectively, amplifying particular groups of organisms indicative of
616 environmental changes, are paired with independent geochemical proxies (e.g., Coolen et al.,
617 2004; 2006; 2009). However, we strongly advocate for the use of strict aDNA methodologies
618 and facilities in order to achieve the generation of authentic marine *seda*DNA data, following
619 the guidelines in this review.

620 Shotgun metagenomics are currently widely accepted and the least biased method to analyse
621 the broad diversity of ancient environmental samples (e.g., Slon et al., 2017). Although only a
622 small portion of the generated sequence data might be attributable to the ancient organism in
623 question (Morard et al., 2017), next generation sequencing (NGS) generates large quantities
624 of data that enable meaningful statistics, with the additional benefit of preserving the relative
625 proportion of detected taxa. To analyse aDNA sequence data, robust bioinformatic pipelines
626 (e.g., Paleomix, Schubert et al., 2014) have been developed and are available for the
627 application to marine *seda*DNA, integrating damage detection algorithms (e.g., Ginolhac et al.
628 2011; Kistler et al., 2017) that enable the distinction between ancient and modern signals.
629 Determining the extent of cytosine residues deamination (C to T and G to A, Weyrich et al.
630 2017) should also be considered to assess authenticity of aDNA sequences, especially when
631 the data was generated from mixed communities, such as from marine *seda*DNA. It is
632 furthermore crucial to carefully screen sequencing data for any low-complexity reads, which
633 may get incorrectly assigned to taxa during alignments against genetic databases, as well as
634 ensuring that taxonomic assignments in the database of choice are correct. Bioinformatic
635 pipelines removing such misidentification-derived errors do not currently exist and should be
636 the focus of future research, as well as the comparison of shotgun and amplicon marine

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637 *sedaDNA* data to accurately determine biases and analysis strategies best suited to this new
638 discipline.

639
640 **4 Future marine aDNA sampling considerations**

641 4.1 Equipment and installations required aboard IODP platforms

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

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

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

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

667
668 4.2 Ground-truthing marine aDNA research and data

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

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

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

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

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

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

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

693
694 **5 Application of marine *seda*DNA research guidelines to other contamination**
695 **susceptible environments**

696 5.1 Permafrost

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

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

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713 example, permafrost soil samples are, like marine sediment cores, retrieved through drilling,
714 which can introduce microbial contaminants to the deeper permafrost soil layers as the drill
715 head and liquid pass through the top active soil layer (Bang-Andreasen et al., 2017).
716 Additionally, the cryosphere has been accumulating industrial chemicals and metals since the
717 1850's (McConnell et al., 2007), so that the present-day microbial community is now capable
718 of degrading industrial contaminants, thereby representing an anthropogenically-adapted
719 rather than an original pristine community (Hauptmann et al., 2017). With both these newly
720 adapted anthropogenic and drilling fluid communities containing characteristics for heavy
721 metal degradation, distinguishing indigenous ice core or permafrost communities from drilling
722 fluid communities will become more difficult in the future (Miteva et al., 2014). Therefore, the
723 described guidelines in this review for distinguishing ancient from modern and contaminant
724 signals, as well as the need for aseptic sampling procedures, are highly applicable to
725 permafrost environments and, more generally, the cryosphere.

726

727 5.2 Planetary exploration

728 The methodologies advocated in this review that enable aDNA in marine sediments to be
729 distinguished from modern DNA are also applicable to the search for life on other planets or
730 moons. Astrobiologists are especially interested in the possibility of detection of Life 2.0, where
731 the life has an independent genesis to that on Earth. The search for life beyond Earth has
732 been potentially possible since the 1970s, with the two Viking lander missions to Mars, but
733 there are other possible targets in our solar system, notably some of the moons around Jupiter
734 and Saturn (e.g., Europa, Titan). Space technology has now reached the point where the
735 detection of life, if it exists or existed elsewhere in the solar system, is becoming a realistic
736 possibility in the next 50 years. There have been several rovers that have carried out
737 successful exploration of the surface of Mars, including Curiosity, the Mars Science Laboratory
738 that in 2018 is mid-way through its predicted mission (Grotzinger et al., 2014). The rover Mars
739 2020 is being designed at present to test for evidence of life in the near-surface environment.

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740 It will drill, collect and cache samples from the Martian surface, which will then be returned to
741 Earth for more detailed analysis (Beatty et al., 2015). Sample return from Mars to Earth is
742 planned for the end of the 2020's (Foust, 2018). Active planning is also ongoing for possible
743 missions to land and analyse materials from the surfaces of moons such as Europa and Titan,
744 by both NASA and the European Space Agency. For example, Europa (a moon of Jupiter) is
745 known to have a global saltwater ocean below its icy crust, as well as a rocky seafloor, so is
746 one of the highest priority targets in the search for present-day life beyond Earth (Hand et al.,
747 2017). A key concern with this solar system exploration is planetary protection, which is
748 governed by the United Nations Outer Space Treaty (United Nations Office for Disarmament
749 Affairs, 2015) and the Committee on Space Research (COSPAR) of the International
750 Committee for Science. There are two important categories of planetary protection. The first
751 is "forward contamination", where Earth-derived microbial life hitches a ride on spacecraft and
752 contaminates parts of a planetary surface being explored. The second is "backward
753 contamination", where life from an explored planet or moon is inadvertently returned to Earth,
754 maybe in a spacecraft or within a rock sample. The relevance to aDNA analytical protocols is
755 in forward contamination (i.e., the risk of contaminating sample material that could lead to data
756 misinterpretations, and/or generally introducing Earth contaminants to other planets; Rummel
757 and Conley, 2017). It should be noted that if indeed there is or was life on other planetary
758 bodies, it may well not be based on a genetic code composed of DNA and RNA. Independently
759 originated Life 2.0 would be highly unlikely to have evolved exactly the same nucleic acid
760 genetic code as life on Earth (e.g., Rummel and Conley, 2017). Indeed, it has been postulated
761 that an alternative biosphere could exist as a "shadow biosphere" on Earth (Davies et al.,
762 2009). If DNA or RNA-based extant life is found on Mars, for example, then it is most likely
763 that it would represent either past natural exchange of rocks between the two planets
764 (panspermia), or anthropogenic forward contamination. Therefore, the procedures used for
765 distinguishing indigenous life in planetary exploration will need broadening to include the
766 possibility of life with a different genetic code. The protocols developed for aDNA sampling of
767 marine sediments on Earth, including the ability to distinguish from modern DNA, have

1712
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1714 768 relevance for the designing of methods to look for past life on Mars or outer solar system
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1716 769 moons using molecular biology techniques (Beaty et al., 2015; Hand et al., 2017).
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1721 771 **Conclusions**
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1724 772 Ancient DNA in marine deep-sea sediments holds the potential to open a new era of marine
1725
1726 773 palaeo-environment and -climate reconstruction. However, anti-contamination measures
1727
1728 774 central to all aDNA research have logistical constraints and are particularly poorly-suited to
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1730 775 shipboard sediment sampling and processing. For example, sterile coring equipment and
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1732 776 ultra-clean laboratories are usually not available on any type of drilling platform. Current and
1733
1734 777 future IODP drilling vessels are aware of the increasing need for improved and innovative
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1736 778 solutions to coring, non-contaminant drill fluids and appropriate laboratories and storage
1737
1738 779 facilities. Such logistical advances should go hand-in-hand with the establishment of new
1739
1740 780 criteria and standards to ensure the acquisition and preservation of sediment cores with
1741
1742 781 minimal environmental contaminants. Complementary genetic and geochemical information
1743
1744 782 currently available to date suggests that, realistically, environmental reconstructions based on
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1746 783 marine *seda*DNA from past plankton can be achieved for at least the last glacial-interglacial
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1748 784 cycle, and potentially back to ~400,000 years. These guidelines can be applied in other
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1750 785 scientific areas to facilitate and optimise research conducted in extremely remote locations,
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1752 786 contamination-susceptible environmental samples, and even during the future exploration of
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1754 787 other planets.

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1761
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1763
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813 **Figures:**

814 **Figure 1:** Schematic showing the key steps involved in acquiring deep marine sediment
815 cores, subsampling, DNA extraction, aDNA preparation for sequencing and data generation.
816 Indicated are sources of potential contamination and reduction in data quality, as well as
817 recommended precautions to be considered and/or controls to be taken. An impact score (1-
818 3 stars) is given to indicate the severity of potential contamination or the impact that impaired

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

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

831
832 **Table 1:** Terms commonly used in marine aDNA research and their definition. aDNA terms
833 are listed hierarchically, all other terms are listed alphabetically.

834
835 **Table 2:** Commonly used DNA extraction kits in aDNA studies to date.

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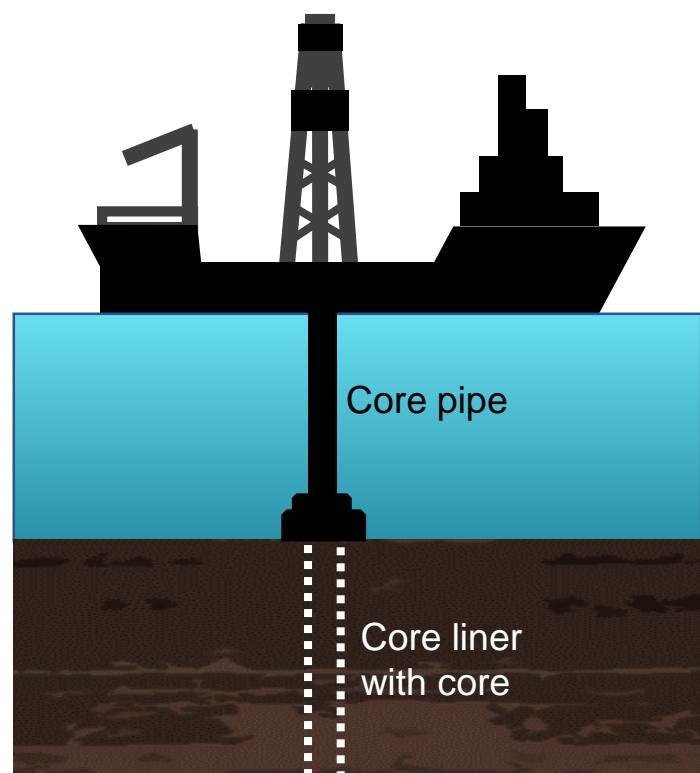
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Impact subject

Impact score

Sediment coring

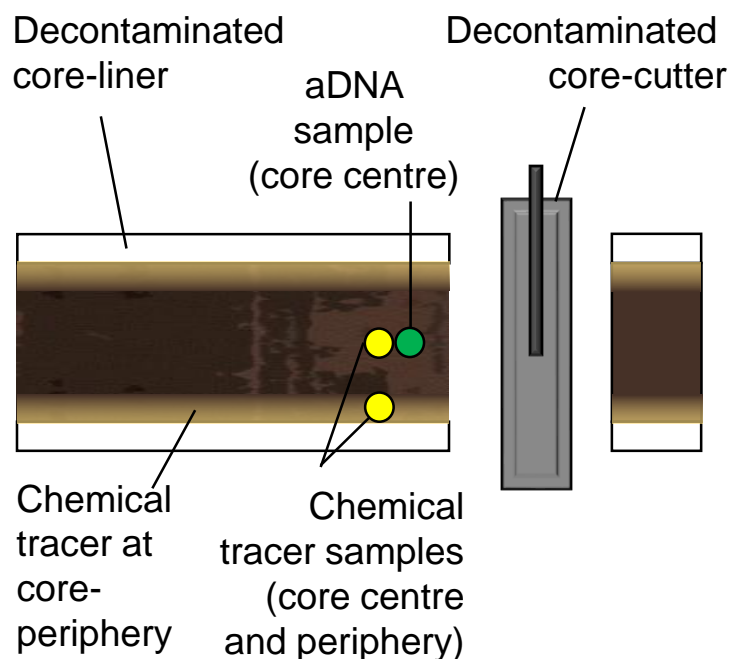


- Modern DNA in seawater
- Contaminant DNA in drilling fluid
- Surface sediment DNA pushed downwards during coring

Recommended contamination control measures: seawater control sample, application of chemical tracers



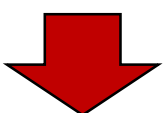
Sediment core subsampling



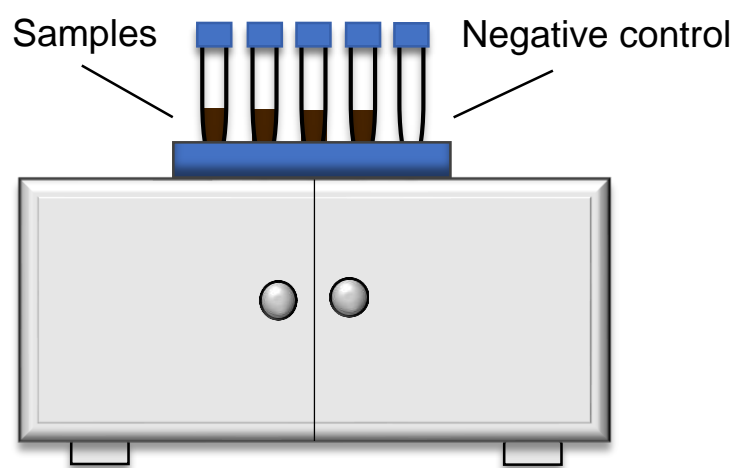
- Human DNA from analysts
- Contaminant DNA present in working area
- Cross-contamination

**

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



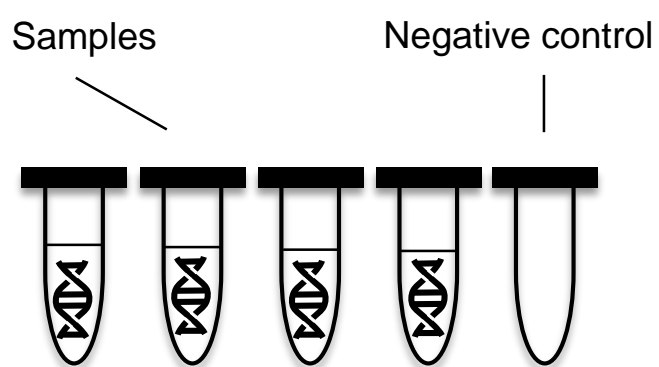
- Contaminant DNA present in reagents
- Contaminant DNA present in laboratory
- Cross-contamination

*

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)

*

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Recommended contamination control measures: working on low-DNA environment, personal protective equipment, negative controls



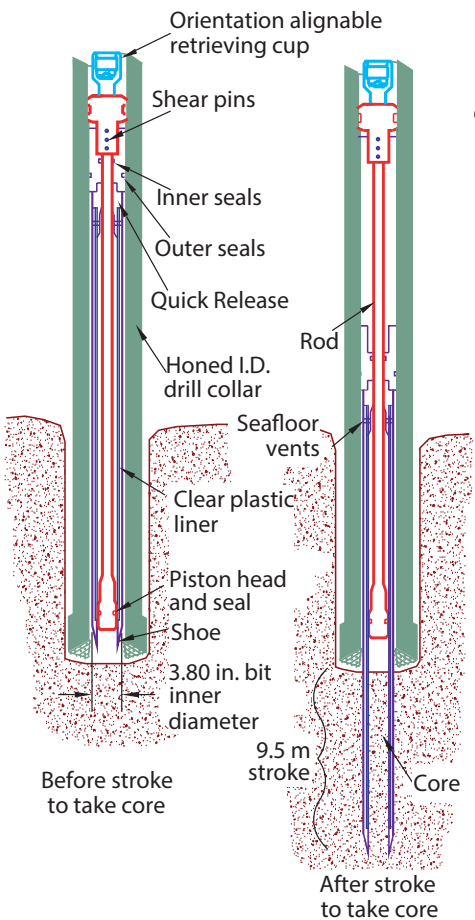
aDNA data analysis



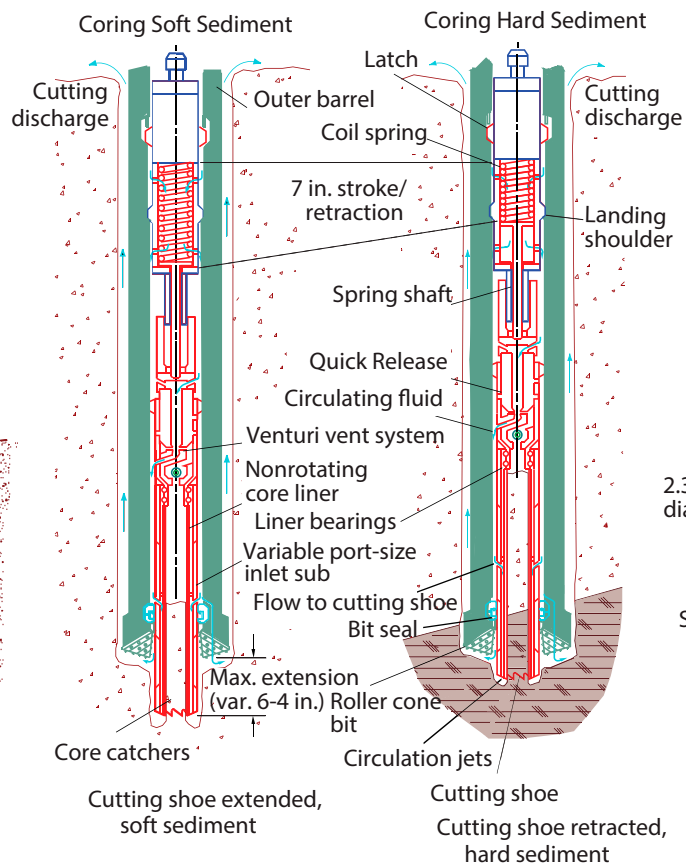
- Vigorous data filtering and quality control

Recommended contamination control measures: analysis of negative controls alongside samples

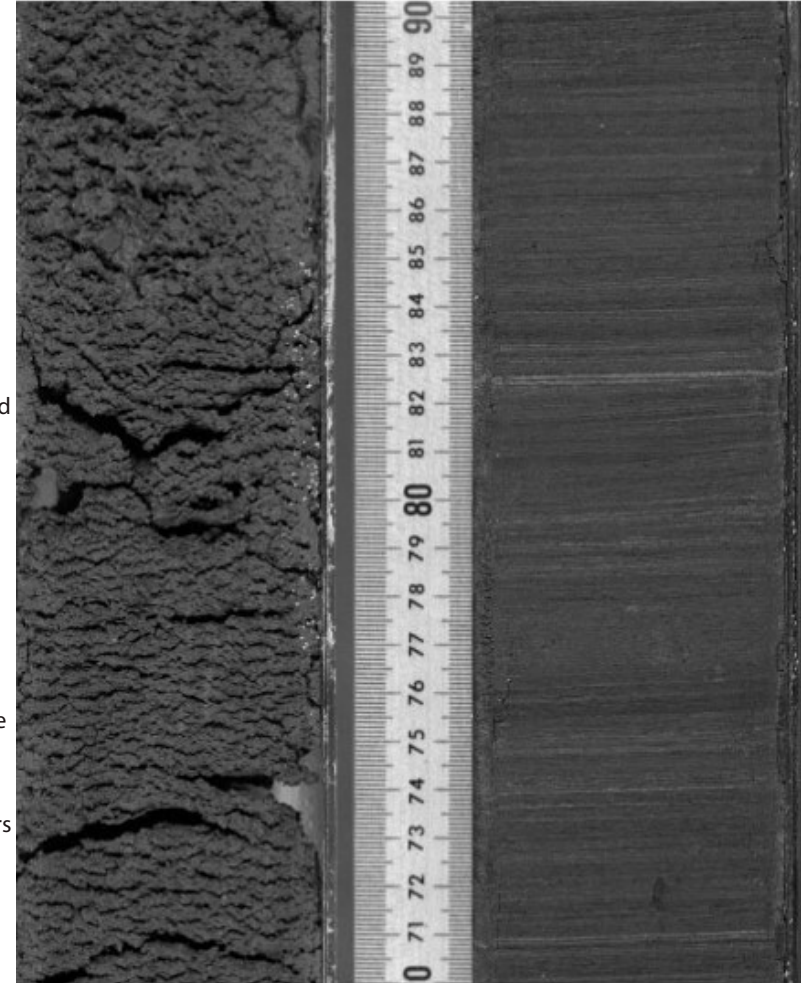
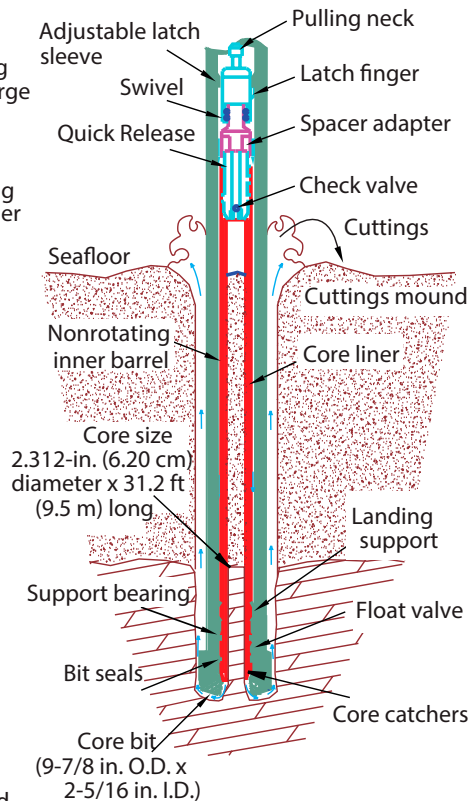
A) APC coring system



B) XCB coring system



C) RCB coring system



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