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RE: Revised Manuscript No.: bg-2016-391

Dear Marcel,

Please find below the revised version of our manuscript, "Identification of reworking in Eocene to Miocene pollen records from offshore Antarctica: a new approach using red fluorescence and digital imaging", by Strother et al., along with a point-by-point response to the reviewer comments and the revised manuscript track-changes version

Our major changes include additional:

- chapter 2 explaining the conceptual model
- chapter 3 describing sedimentology (incl. new Figure 2)
- paragraphs in the method section to better explain selection of pollen grains and statistical approach
- chapter 6.1 to explain advantages of our new approach
- chapter 7 to address applications and limitations
- revised Figure three with photos of reworked pollen grains
- additional tables and data in Supplementary Information

We fully agree with your comment, that it would be good to test our approach on different records. However, this is beyond the scope of this study, but we have included your suggestion in the concluding remarks section. We did not change Figure 4 (previously 3) and marked the potentially reworked pollen grains/data points, as this would falsely suggest that the method produces thresholds suitable for identifying reworking of single pollen grains. Please note that our samples at a given depth can only include reworked palynomorphs from older strata, not from younger strata (if we assume that the age model is correct).

We would like to thank you and the reviewers for the thorough review and comments and hope that you will find the revised paper suitable for publication in "Biogeosciences".

I am looking forward to hearing from you.

Best regards,

Ulrich
(on behalf of the co-authors)

RESPONSE TO REV#1:

We would like to thank Reviewer #1 for their very constructive comments which helped to further improve this manuscript. Before we address each "line comment" separately, we will respond to the main two concerns (1. Burial History/Conceptual Model and 2. Sample Size) Rev#1 raised in the "General Comment" section:

GENERAL COMMENTS

1. Burial History/Conceptual Model

We added two new sections to our manuscript about the approach and related hypotheses (Section 2), sedimentology (Section 3), and additional paragraphs and figure in order to address REV#1 comments regarding the missing information on burial history and the conceptual model. There are some misunderstandings which we hope to have clarified in the revised version:

a) While we agree with REF#1 that our manuscript will benefit from a more detailed description of the theoretical model and assumptions to better guide the reader on how to determine reworking, we would like to reiterate that our approach is not designed to estimate absolute ages from fluorescence values. We therefore think that conceptual models assuming linear or exponential changes, as described in Rev#1 are not useful, as they would suggest a potential to estimate absolute ages from fluorescence measurements. At least from the Oligocene onwards, the burial history of site U1356 is, like many other sites in Antarctica, dominated by repeated and partly abrupt retreats and advances of glaciers and therefore too complex to be described with a simple linear or exponential function (explained in detail in section 2 and 3).

b) Many comments made by REV#1 refer to how we compared mean values and how mean value and variance might be affected by the low sample size. There seems to be a misunderstanding as we did not compare the mean values of our fluorescence measurements. We instead avoided working with mean values as this might require a larger sample size. We might have confused REV#1 by using the term "mean" in the original manuscript for the fluorescence values produced by the imaging software. The software measures fluorescence by drawing a contour around the pollen grain and measuring multiple spots from this contour image. The program provides the mean value of the grain (i.e. each red fluorescence pixel value from around the contour of the grain). We clarified this in the revised manuscript and avoided using the term "mean". Finally, we would like to thank REV#1 for the suggestion to use variances as a tool to assess the degree of reworking. Unfortunately, our sample number is too low and variable to further explore this approach. However, we have added this suggestion to our discussion for future research.

2. Sample Size

We fully agree with Rev#1 that a much higher pollen count would be better and allow for a more detailed statistical analysis and discrimination between the different sediment layers. Unfortunately, the Wilkes Land sediment samples have, like all other comparable Antarctic cores (e.g. ANDRILL or SHADRILL), a low pollen concentration. This is particularly the case for post Eocene samples where the pollen deposition is affected by both glacial sedimentation history and reduced pollen production on land. Measuring 500-1000 pollen grain, as suggested by Rev#1, is therefore unfortunately not possible.

However, our method is designed to work with low pollen counts and we are confident that our approach produces statistically robust results. We therefore disagree with REV#1' s comment that our work is "under-sampled" and sample number (n=30) "barely sufficient". The Mann-Whitney U Test is applicable to all samples sizes and may be used with as few as four measurements in each sample (*Fowler et al. 2009. Practical Statistics for Field Biology. 259 p.*) A methodological "disadvantage" of this test it that it reacts very sensitive to small samples sizes and normally indicates no differences if the sample size tends to be low. The fact that in our study the Mann-Whitney U Test shows statistically significant differences, despite our low sample size, gives us even higher confidence in our results and indirectly demonstrates that our sample size was actually large enough. We also think that grouping all pollen into a single group, in addition to test each single taxon, is indeed useful. With this approach we increased the sample size and, by excluding species variation, we tested one group and one explanatory variable only.

We are unsure about REV#1's comments on changes in mean value and variance as the Mann-Whitney U Test does not compare sample means. The confusion might be caused by the use of "mean values" in our manuscript, which refer to the image-processing software data (see comment above). We rephrased the relevant section to avoid further misunderstanding and also added additional paragraphs to the Method and Discussion section explaining the implications of low samples numbers for this approach as suggested by the reviewer.

LINE COMMENTS
(Line numbers are original line numbers before corrections)

Rev#1: L67 suggest "are subjected to" instead of "confronted"

Response: Done

Rev#1: L70 to follow from the previous point, this needs to be qualified with something along the lines of "if burial histories are the same, fluorescence change could be used as an indicator of age"

Response: Previous sentences have been revised to clarify the fluorescence colour can change with burial over geological timescales. However, the change of fluorescence colour cannot be used as a determination of age.

Rev#1: L83 "each should come with" = "we hypothesise"?

Response: Done

Rev#1: L105 A summary of what is known of the the burial history would be helpful here – is there any constraint or estimate of the amount eroded at eh disconformities – i.e. is there any possibility the Eocene pollen was buried to a greater depth before Oligocene time etc. . . if these sort of effects relate to only 10s of meters of extra burial, this is useful for the reader to know

Response: Following Rev#1 suggestions we added a new section, the Sedimentology (Section 3) of Wilkes Land, detailing the complex burial history, sedimentation rates and glacial influence throughout the core.

Rev#1: L132 modern name for Nothofagus fusca type trees has been changed to Fuscospora

Response: We are aware of the discussion started by Heenan & Smissen (2013) to split *Nothofagus* into four genera (Phytotaxa, 146 (1): 1–31). However, in order to be consistent with previous published palynological research at site U1356, Wilkes Land (e.g. Pross et al. 2012. Nature, 488, 73-77; Contreras e al. 2013. Rev Palaeobot Palyn., 197, 119-142) we prefer to keep the "old" genus name when describing the fossil record (see also discussion in Hill et al. 2015 Australian Systematic Botany, 28, 190–193).

Rev#1: *L147, This seems sensible. So why do you then combine them for your statistics?*

Response: The Pearson's correlation values were initially combined to determine which fluorescence values (red, blue, green, brightness, saturation and intensity) show a strong statistical correlation with age to further assess the fluorescence behavior of taxa. Red fluorescence showed the strongest statistical relationship with age to test against geological ages. Due to variations in the chemical composition of the exine affecting the fluorescence of grains, the red fluorescence statistical relationship was then determined for each taxon. There is a different reason to combine the red fluorescence values for the Mann-Whitney U test. As outlined under Sample Size in the General Comments section, the reason for combining the samples in the Mann-Whitney test was to increase sample size and test for one explanatory variable only.

Rev#1: L157, suggest remove "in situ"… All you can infer is they are "not obviously reworked"… that distinction is critical for this paper!

Response: In order to address Rev#1 concerns we replaced "in situ" in the manuscript by "non-reworked"

Reviewer: L160, I suggest that a clear description of conceptual models of reworking is really important about here – to provide some context and reason for the statistics in the next section…the reasons for wanting to know why correlations against age and significant difference between mean values must be laid out.

Response: Two additional sections detailing the burial history and conceptual model (Section 2 and 3) have been added.

Rev#1: L171, what do you mean "set" the p-value? Is this a threshold you have adopted to accept or reject a hypothesis? If so, at least this should be acknowledged/highlighted in Table 1 – perhaps bold the results with acceptable p-values?

Response: For determining if results are statistically relevant, we used the highest significance level of p-values (0.01), the 99 percentile. We deleted the "set threshold" and revised in Table 1 to bold results with p-values indicating the highest significant correlation (0.01).

Rev#1: L175, the meaning of U-values this test generates should be explained…If this is a threshold score, describe what it is, where it is from and what it means, and make this clear in your Table 2 – including same comments on p-values as above.

Response: Additional sentences have been added to section 4.3 to describe the meaning of U-values and a further explanation of U-values have been included in the Table 2 caption.

Rev#1: L175, Once you get into multiple sequential significance tests of this sort, perhaps describe why some sort of Bonferroni – type correction is not appropriate?

Response: The Bonferroni correction was not applied because this type of correction comes at the cost of increasing the probability of producing a false negative, i.e. reducing the statistical power of the test.

Rev#1: L180, Are these results tabulated?

Response: Following Rev#1 suggestion, we added a table with the ANOSIM results to the Supplementary Information (Table S2). In addition, section 3.3 has been reworded to clarify the ANOSIM results.

Rev#1: L200, could you plot these visual data, to demonstrate there really is an advantage to using the digital data? The ranges you quote seem to overlap about as much as the fluorescence red values? The visual data does not appear in your supplementary data?

Response: The number associated with Yeloff and Hunt (2005) colour chart classification has been added to the supplementary material (Table S1). The ranges listed are the visual colours identified through the observations of each pollen and spore grain measured for fluorescence. Additional sentences have been added to help explain the colour classification chart and the implication of visual fluorescence colours overlapping through time.

Rev#1: L230, The visual fluorescence data are not shown or plotted – how can you demonstrate that then that the digital measurements are better or worse at allowing differentiation of mean values between epochs?

Response: A table with results from visual assessment has been added to the supplementary material (Table S1). Additional sentences have been added to Section 6.1 to demonstrate why digital measurements are an advantage for differentiation of mean red values between epochs. We also showed that the subjective colour comparison of fluorescence alone could not distinguish between

Oligocene and Miocene grains. We thereby demonstrated that the digital measurement does in contrast to the visual assessment, not only produce objective and reproducible data but also more accurate results than the visual assessment. Additional sentences have been added to the discussion and result section to make this clearer.

Rev#1: L240, where are these results shown?

Response: ANOSIM table S2 has been added to the Supplementary Information.

Rev#1: L245, following burial models discussed above – it is really not clear to me how demonstrating a linear relationship as you have done is an indicator or otherwise of reworking. This needs to be described more clearly.

Response: REV#1 is correct: we used the correlation to select the best parameter. We deleted this sentence and rewrote the entire section to clarify.

Rev#1: L252, how? What is your threshold value or test to conclude that the sample or stage has enough in situ pollen for reconstruction?

Response: Our "threshold value" is a statistical significant difference in fluorescence colour between different pollen assemblages/depth. We added an improved and more detailed explanation of our approach in section 2 and the discussion.

Rev#1: L259, "applied" rather than "adhered"?

Response: Done.

RESPONSE TO REV#2:

We would like to thank Reviewer #2 Michael Hannah for his very constructive and helpful comments. In response to this we have taken the following actions:

Rev#2: After applying standard laboratory processing techniques, thirty specimens from each sample were analysed. I am not a statistician – but this number seems low. Can the authors demonstrate that this level of counting is statistically relevant?

Response: The Mann-Whitney U Test is applicable to all samples sizes and may be used with as few as four measurements in each sample (Fowler et al. 2009. Practical Statistics for Field Biology. 259 p.) A methodological "disadvantage" of this test it that it reacts very sensitive to small samples sizes and normally indicates no differences if the sample size tends to be low. The fact that in our experiment the Mann-Whitney U Test shows statistically significant differences, <u>despite</u> our low sample size, gives us even higher confidence in our results and indirectly demonstrates that our sample size was actually large enough.

Rev#2: Five species of terrestrial palynomorphs were focused on. Were these the only ones analysed? Also, there has been a major revision of the genus *Nothofagus*.

Response: Sentences have been updated to make clear only five common pollen and spore taxa were used for fluorescence analysis.

We are aware of the discussion started by Heenan & Smissen (2013) to split Nothofagus into four genera (Phytotaxa, 146 (1): 1–31). However, in order to be consistent with previous published palynological research at site U1356, Wilkes Land (e.g. Pross et al. 2012. Nature, 488, 73-77; Contreras e al. 2013. Rev Palaeobot Palyn., 197, 119-142) we prefer to keep the "old" genus name when describing the fossil record (see also discussion in Hill et al. 2015 Australian Systematic Botany, 28, 190–193).

Rev#2: One of the concerns I have involves the determination of in situ material prior to fluorescence analysis and its implications. As I understand the method, it is assumed that all of the specimens examined are in situ. Reworked material was identified and rejected based on a visual examination of each specimen. Of course, this is the only approach that can be used – but I think that the text should spell out clearly the limitations that this brings to the study.

Response: We do not assume that all of the specimen examined are "in situ". We added text and photos to Fig. 3 in order to better explain the "pre-selection process". We also added a new section 2 and additional paragraphs to the Discussion section where we provide a more detailed explanation of our approach and its limitations.

Rev#2: *I have to admit, however, that I am at a loss to understand figure 3. It needs a fuller, more detailed, caption explaining exactly what was the diagram is showing – as it stands I can't make the link between the diagram and the results outlined in the text.*

Response: The Figure 3 caption has been updated with additional explanation.

Rev#2: *My uneasiness with the assumption that all the specimens measured are in situ as outlined above is addressed to a degree in the discussion. But the argument appears to be somewhat circular. Analysis suggests that a shift to red indicates age and probable reworking, an in line 247 the authors state that the mean red fluorescence indicates that a "considerable proportion of the specimens are in situ." But how do we know that the rest aren't also in situ and the technique has failed? The authors seem to suggest that the answer lies in looking at the total assemblage and deciding whether or not sufficient numbers of individuals are in situ to trust the palynological analysis. But I'm not convinced that this gets around the circularity of the argument.*

Response: The fluorescence signal of the "rest" of the specimen overlap with those from previous samples and therefore indicate that the sporomorphs have been reworked into the younger layers. A considerable portion of non-reworked sporomorphs is required to produce a signal which is significantly different from the previous. The principle of our approach is not fundamentally different from previous methods using autofluorescence in Palynology. We just propose using digital imaging (to measure the signal) and statistical analysis to make this process reproducible and independent from subjective classification of the analyst. We hope to have clarified our approach by adding a new section (Sect 2) to better explain the conceptual model and underlying assumptions.

Rev#2: But I would like to have seen a short section outlining how this approach may be applied in a practical sense.

Response: The practical applications have been addressed in the newly added concluding remarks (7) and section 2, where we explain the conceptual model and underlying assumptions.

Identification of reworking in Eocene to Miocene pollen records from offshore Antarctica: a new approach using red fluorescence and digital imaging

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Abstract. Antarctic palaeoclimate evolution and vegetation history after the formation of a continent-scale cryosphere at the Eocene/Oligocene boundary, 33.9 million years ago, has remained a matter of controversy. In particular, the reconstruction of terrestrial climate and vegetation has been strongly hampered by uncertainties in unambiguously identifying non-reworked*in situ* as opposed to reworked sporomorphs that have been transported into Antarctic marine sedimentary records by waxing and waning ice sheets. Whereas reworked sporomorph grains over longer non-successive geological time scales are easily identifiable within younger sporomorph assemblages (e.g., Permian sporomorphs in Pliocene sediments), distinguishing non-reworked*in situ* from reworked material in palynological assemblages over successive geological time periods (e.g., Eocene sporomorphs in Oligocene sediments) has remained problematic. This study presents a new quantitative approach to identifying non-reworked pollen assemblages *in situ* grains from a marine sediment corescore from circum-Antarctic waters. We measured the fluorescence signature, including and mean red, green and blue, brightness, intensity and saturation values, of selected pollen and spore taxa from Eocene, Oligocene and Miocene sediments from the Wilkes Land margin Site U1356 (East Antarctica) recovered during Integrated Ocean Drilling Program (IODP) Expedition 318. Our study identified statistically significant differences in mean red fluorescence values of non-reworked*in situ* sporomorph taxa against age. We conclude that red fluorescence is a reliable parameter to identify the presence of non-reworked*in situ* pollen and spores in Antarctic marine sediment records from the circum-Antarctic realm that are influenced by glaciation and extensive reworking. Our study provides aan essential new tool required to accurately reconstruct Cenozoic terrestrial climate change on Antarctica using fossil pollen and spores.

Keywords: Fluorescence, pollen, spores, Antarctica, reworking, vegetation, climate reconstruction

1 Introduction

Antarctica plays a key role in understanding past and future global climate change due to the impact its large ice sheets exert on sea level as well as on oceanic and atmospheric circulation. Throughout the last 65 million years, the Antarctic continent has undergone a drastic change from a greenhouse environment in the early Paleogene towards an icehouse world in the late Paleogene and Neogene (e.g., Askin and Raine, 2000; Prebble et al., 2006; Bijl et al., 2013~~2009~~; Anderson et al., 2011; Pross et al., 2012; Passchier et al., 2013). The analysis of fossil pollen and spores is one of the most important tools for reconstructing and quantifying past vegetation and terrestrial climate change. For Antarctica the lack of long and well-dated sediment records puts considerable constraints on a detailed spatial and temporal reconstruction of terrestrial environmental change. Macro~~*In situ* macro~~ and microfossil evidence for vegetation cover from continental sections of Antarctica is often difficult to date and in general sparse due to past and present ice cover (e.g., Birkenmajer and Zastawniak, 1989; Pole et al., 2000; Lewis et al., 2008; Warny et al., 2016). Therefore, most reconstructions of climate and vegetation on the Antarctic continent are based on palynological records from marine, circum-Antarctic sediment cores. However, the waxing and waning of Antarctic ice sheets throughout the Oligocene and Miocene caused reworking of terrestrial material into marine sediments, ultimately leading to a combination of non-reworked~~*in situ*~~ and reworked palynomorphs in palaeorecords that are difficult to differentiate especially over short geological time scales (e.g., Askin and Raine, 2000; Raine and Askin, 2001; Prebble et al., 2006; Salzmann et al., 2011; Griener et al., 2015).

The unambiguous identification of~~differentiation between *in situ* and~~ reworked palynomorphs in palaeorecords is essential to establish reliable climate and vegetation reconstructions for the Antarctic continent. However, a quantitative approach to differentiate non-reworked~~*in situ*~~ from reworked pollen assemblages~~sporomorphs~~ over relative short~~shorter~~ geological time scales (e.g. Oligocene to Miocene) has not yet been established. Previous palynological studies in Antarctica have identified reworked Cenozoic sporomorphs ~~based on approaches~~ using the thermal alteration of grains (e.g., Askin and Raine, 2000; Raine and Askin, 2001; Prebble et al., 2006, Griener et al., 2015; Warny et al., 2016). This approach~~These approaches~~ only takes~~take~~ into account reworked pollen grains that have been exposed to significantly~~strongly~~ different taphonomical conditions than the non-reworked sporomorphs~~*in situ* material~~. However, submarine reworking of shelf material can only have small impacts on preservation quality, hampering the unambiguous identification of reworked palynomorphs using light microscopy (e.g., Salzmann et al. 2011).

Subjective fluorescence microscopy has been applied in Antarctic palynology~~pollen studies~~ to help remedy the issue of reworking. Raine (1998) and Salzmann et al. (2011) used autofluorescence to identify reworked Permian and Mesozoic sporomorphs within Cenozoic sediments from the Cape Roberts cores in the Ross Sea and James Ross Island, Antarctica. Qualitative attempts to separate reworked and non-reworked~~*in situ*~~ sporomorphs based on their fluorescence colours through geological time have been shown to work (Phillips, 1972; Bujak and Davies, 1982). However, these methods are highly subjective~~; being dependent upon the observer and difficult to reproduce. The~~

~~fluorescence signal from fossil pollen and spores comes from the sporopollenin in the exine, which contains heteroatomic compounds (Yeloff and Hunt, 2005). Over geological timescales pollen and spores in sediments are confronted with elevated temperatures and pressures after burial, and the less resistant compounds of the sporopollenin shift to the red end of the colour spectrum and ultimately towards no fluorescence (Van Gijzel, 1967; Bujak and Davies, 1982; Yeloff and Hunt, 2005). This suggests that the amount of fluorescence changes with burial time: sporomorphs from old sediments show little to no fluorescence, and pollen and spores from the oldest section of a core show fluorescence predominantly on the red end of the spectrum. Critically, the process of fluorescence loss is irreversible, meaning that fluorescence cannot be re-gained by the sporomorphs at any time.~~ , being dependent upon the observer, and difficult to reproduce. ~~This behaviour provides an opportunity to assess whether sporomorphs are *in situ* or reworked from older strata.~~

By using fluorescence microscopy this study aims to develop a new systematic and quantitative approach to identify non-reworked ~~*in situ*~~ pollen and spore assemblages in marine sediments from Antarctica. We measured the fluorescence signature, including ~~and mean~~ red, green, blue, brightness, intensity and saturation values, of the most common pollen and spore taxa under ultra-violet (UV) light in Eocene, Oligocene and Miocene sediments. All samples were taken from the Wilkes Land margin sediment record at IODP Site U1356 (Fig. ~~Figure~~ 1), and cover the early Eocene through the mid-Miocene with two hiatuses from the mid- Eocene to the early Oligocene (~47 – 33.6 Ma) and from the latest Oligocene to early Miocene (~23.12 – 16.7 Ma) (Escutia et al., 2011; Tauxe et al., 2012). This provides us with four time intervals ~~three timeintervals~~ , all yielding abundant sporomorphs (Escutia et al., 2011; Pross et al., 2012, Contreras et al., 2013; Sangiorgi et al., in review) in which we hypothesise ~~each should come with~~ different fluorescence behaviour. The Cenozoic sediment record of Site U1356 provides a unique opportunity to compare the fluorescence of the same pollen taxa through the Eocene to Miocene, i.e., before and during the impact of large-scale glaciation. The aim of our study is to provide a simple quantitative approach to identify the presence of non-reworked palynomorphs in a given sediment layer that can be used to reliably reconstruct past vegetation and climate for the time interval during which the sediment was deposited. ~~in order to unambiguously identify reworked palynomorphs.~~

2 Conceptual Model for identifying non-reworked palynomorphs in Antarctic sediments

Various factors such as burial depth and geological age contribute to the fluorescence of sporomorphs. These ~~**2 Materials and Methods**~~

~~**2.1 Sampling and sedimentology**~~

factors reflect the ultimate determining factor of fluorescence alteration, which is heat flow and the length of time the sporomorphs are exposed to this heat (Waterhouse et al., 1998). The fluorescence signal from fossil pollen and spores comes from the sporopollenin in the exine, which contains heteroatomic compounds (Yeloff and Hunt, 2005). Over geological timescales pollen and spores in sediments are subjected to elevated temperatures and pressures after burial, and the less resistant compounds of the sporopollenin shift to the red end of the colour spectrum and ultimately towards no fluorescence (Van Gijzel, 1967; Bujak and Davies, 1982; Yeloff and Hunt, 2005). This

suggests that the colour of fluorescence changes with burial time: sporomorphs from older sediments (Cretaceous and older) show little to no fluorescence, and pollen and spores from older epochs of the Cenozoic (Paleocene, Eocene and Oligocene) show fluorescence predominantly on the red end of the spectrum with additional fluorescence colour (orange, yellow, blue and green) variations including red fluorescence continuing through to modern (Bujak and Davies, 1982). Critically, the process of fluorescence loss is irreversible, meaning that fluorescence cannot be re-gained by the sporomorphs at any time.

The above described predictable change in colour and intensity of fluorescence in relation to burial time and depth, provides an opportunity to assess whether sporomorphs in a sediment record are non-reworked or reworked from older strata. Antarctic Cenozoic sediments typically show a complex burial history with episodes of glacial erosion and rapid sediment deposition. The Wilkes Land Site U1356 shows shifts in the delivery of sediments to the site (e.g., shift in depocenters, changes in the amount and type of sediment delivered to the site) and erosion during two major events near the Eocene to Oligocene and Oligocene to Miocene transgression (see also Sect. 3). The alternating phases of erosion, accumulation and rapid deposition have a strong impact on the fluorescence of each individual palynomorph and the assemblage. We addressed these factors by building our experimental approach on the following assumptions:

(i) Each palynomorph assemblage contains a strongly varying percentage of reworked pollen and spores, which can originate from multiple sources and ages.
(ii) Changes in fluorescence are site- and sediment-specific, which prevents the use of fluorescence data for absolute age determinations and a comparison of the fluorescence values between sites.
(iii) Fluorescence values in consecutive sediment layers can overlap and are strongly variable.
(iv) Discrimination of non-reworked palynomorphs can only be based on relative measurements and not on absolute values taken from single strata and single grains.

We hypothesise that the presence of non-reworked palynomorphs in a sediment core will result in a fluorescence signal that continuously declines with decreasing depth. We further hypothesise that significantly different values between depths indicate the presence of a sufficiently high number of non-reworked pollen and spores to be used for a meaningful environmental reconstruction.

3 Sedimentology of Site U1356 and potential source of reworking

Pollen and spores were examined from Eocene, Oligocene and Miocene sediments from IODP Site U1356 located ~ 300 km off Wilkes Land, East Antarctica (63°18.6138'S, 135°59.9376'E) ~~taken~~ at the transition between the continental rise and the abyssal plain (Fig.~~Figure~~ 1; Escutia et al., 2014). The Wilkes Land margin formed during the late Cretaceous as part of~~during~~ a non-volcanic rift, with Oligocene-Eocene shelf sediments exposed today on the continental shelf proximal to Site U1356 (Close et al., 2009; Expedition 318 Scientists, 2011). ~~Pollen taxa were analysed from 28 samples between 106.62 and 998.99 mbsf. The early (53.9 – 51.9 Ma) to mid-Eocene (49.3 – 46 Ma) sediments covered depths between approximately 1000.08 and 893 mbsf (Expedition 318 Scientists, 2011). The lowermost Eocene interval consists of clay mineral assemblages mainly containing smectite and kaolinite~~

~~(Expedition 318 Scientists, 2011). This lithology points to chemical weathering under warm and humid conditions and a shallow-water depositional environment (Expedition 318 Scientists, 2011). Graded sandstones units~~
~~suggesting mass transports comprise the upper Eocene sediments (Expedition 318 Scientists, 2011). The WL-U3 unconformity separated the non-glacial Eocene strata from the Oligocene strata influenced by glacial deposition (Escutia et al., 2005). Approximately 455 m of sedimentary strata were recovered from Site U1356 (440.7 – 895.41 mbsf) and dated as early to late Oligocene (~33 – 23 Ma) (Escutia et al., 2011; Tauxe et al., 2012~~The sediments at Site U1356 (Fig. 2) record a complex history in the development of this margin that is deeply influenced by the growth of a continental-scale ice sheet during the Eocene-Oligocene Transition (EOT) and by ice-ocean interactions since the earliest Oligocene.~~). The Wilkes Land Oligocene sedimentary deposits reside in a distal setting (lowermost rise abyssal plain) with evidence of iceberg activity indicated by dropstones suggesting a glaciated environment (Escutia et al., 2011).~~

During ~~The lithology of~~ the early and middle Eocene, sedimentation took place in a shallow-water environment largely influenced by delivery of sediments from the continent. The lowermost interval (949-1006 metres below seafloor (mbsf)) dated early Eocene consists of bioturbated claystones likely deposited by hemipelagic sedimentation. Rare laminated siltstone and sandstone interbeds indicate sporadic gravity flows or bottom current activity reaching the site. Sedimentary environments during the middle Eocene (895-949 mbsf) are characterized by the presence of sandstones with clasts and contorted bedding, diamictites, and micaceous fining-upwards sandstones and siltstones, which point to deposition in a shallow-water environment. The clay fraction in all Eocene sediments contains smectite and kaolinite (Expedition 318 Scientists, 2011), which points to chemical weathering under warm and humid conditions (Expedition 318 Scientists, 2011).

During the EOT, subsidence and the glacial isostatic adjustment of the margin resulted? in partial eustatic recovery on the continental shelf and erosive currents on deeper parts of the margin (Stocchi et al., 2013). At Site U1356, the non-glacial middle Eocene strata are separated from early~~Lower~~ Oligocene glacimarine strata by the WL-U3 regional unconformity (Fig. 2; Escutia et al., 2005; Escutia et al., 2011). This unconformity represents a 13 million year (Ma) hiatus (Escutia et al., ~~shows evidence of terrestrial sediment influence from contorted diamictites along with contorted~~ 2011; Tauxe et al., 2012) suggested to predominately be caused by an extreme erosion event associated with the growth of a continental-wide ice sheet during the EOT leading to the Oi-1 event (Escutia et al., 2011; 2014). On the continental shelf, approximately 300 - 600 m of sediment were eroded during the Oi-1 event (Eittreim et al., 1995), but partial eustatic recovery provided accommodation space for early Oligocene sediments (Escutia et al., 2011) and potentially late Eocene sediments (Stocchi et al., 2013) to accumulate.

High sedimentation rates during the Oligocene resulted in approximately 455 m (440.7 – 895.41 mbsf) of sedimentary strata being deposited at Site U1356 and dated as early to late Oligocene (~33.6 – 23 Ma) (Escutia et al., 2011; Tauxe et al., 2012). Oligocene sediments all denote deposition in deep-water environment with occasional reach of iceberg activity indicated by dropstones (Escutia et al., 2011). Sedimentation rates during the early Oligocene (723.5-895 mbsf) section are 20 m/m.y. and lithologies are characterized by interbedded laminated claystones, bioturbated claystones and contorted diamictites and convoluted bedded mudstones (Expedition 318

Scientists, 2011). These sediments indicate that times of hemipelagic sedimentation with the influence of bottom-currents alternate with times dominated by Mass Transport Deposits (MTDs). The presence of turbidites and MTDs points to times with an increased contribution of transported sediment from shallow-marine and ultimately terrestrial sources, indicating a strong likelihood of reworked palynomorphs in the record. The late Oligocene (459.4-723.5 mbsf) is characterized by a sharp increase in sedimentation rates (89 m/m.y.). The sedimentary section is comprised by an alternation of bioturbated claystones with laminated claystones indicative of hemipelagic deposition influenced by bottom-currents of varying velocities. Interbedded with the claystones are diamictites, gradded siltstones and sandstones indicative of the deposition of end members of subaqueous density flows (turbidite, debris flow, MTDs), pointing to an~~The Upper Oligocene indicates a further~~ increase in terrigenous sediment to the site~~with bioturbated claystones, siltstones, sandstones, and contorted diamictites~~ (Expedition 318 Scientists, 2011).

The late~~Upper~~ Oligocene and early~~Lower~~ to middle~~Middle~~ Miocene (23.12 – 16.7 Ma) are separated by a ~6 m.y. long hiatus (Escutia et al., 2011). The~~ Lower to Middle/Upper~~ Miocene sedimentary section includes bioturbated claystones, siltstones and sandstones (Expedition 318 Scientists, 2011). The late-middle ~~encompassed depths of ~459.4 mbsf to 3 mbsf and~~ Miocene section is characterized by diatom ooze and laminated ~~lithologies document fine-grained terrestrial input with bioturbated claystones, siltstones and sandstones (Expedition 318 Scientists, 2011). The Upper Miocene shows evidence of ice rafting with gravel in~~ diatom-rich silty clay indicating high-biogenic and low-terrigenous hemipelagic sedimentation dominates in a ~~clays and oozes (Expedition 318 Scientists, 2011). The Oligocene to Miocene sediments are indicative of~~ relatively high productivity environment, also affected by bottom-current activity. The pebble-sized clasts and coarse sand clusters or interbeds likely indicate ice rafting. The middle Miocene section below 278 mbsf shows an increase in bioturbation and lack of gravel-sized clasts suggesting minimal iceberg rafting. Sedimentation rates during Miocene are around 80 m/m.y. but significantly decrease at around 12 Ma. Miocene sediments younger than 12.7 Ma are devoid of any palynomorphs.~~deep-water, sea-ice-influenced setting (Escutia et al., 2011). The lithologies recognised at Site U1356 suggest a large contribution of transported sediment from the shelf, indicating a strong likelihood of reworked palynomorphs in the record.~~

4 Methods

4.1 Palynological sampling and preparation

Pollen taxa were analysed from 28 samples between 106.62 and 998.99 mbsf (see Supplementary Table S1). ~~**2.2 Palynology**~~

All samples were processed at the Laboratory of Palaeobotany and Palynology, Utrecht University, The Netherlands, using their standard palynological processing method for marine sediments (e.g., Bijl et al., 2013). Samples were treated with 10% HCl and cold 38% HF to dissolve carbonates and silicates, respectively, and again with 10% HCl to eliminate silica gel and sieved with a 10-micrometre mesh. Residues were mounted on glass microscope slides using glycerine jelly, and the edges were sealed with nail polish. The nail polish seems to limit the

fluorescence of the underlying palynomorphs due to the additional medium diminishing the intensity of the brightness. Therefore, we chose to consider only those palynomorphs that were away from the edges of the slide. The use of acids such as HF and HCl can alter the fluorescence of grains towards the red end of the spectra (Van Gijzel, 1971; Waterhouse, 1998). However, the same palynological processing techniques were uniformly used for all samples. ~~For each sample~~ 30 pollen and spore grains ~~with no obvious signs of reworking (e.g., colour, corrosion)~~ were randomly selected for the Miocene, late Oligocene, early Oligocene and Eocene to determine the fluorescence signatures through geological time. The aim of our study is to identify reworking in palynological assemblages over successive geological time periods. We therefore performed a pre-selection by removing obviously reworked, older-than-Eocene sporomorphs, that were extremely dark to almost opaque under a light microscope and with very little to no fluorescence under UV excitation (Fig. 3 -6a/b). We only selected those pollen and spore grains as Eocene-Miocene palynomophs that were light and translucent under a light microscope with strong fluorescence under UV excitation, and not covered by other material on the slide (i.e. organic matter). This examination was done on all slides studied from the Eocene, early Oligocene, late Oligocene and Miocene. Five common pollen and spore taxa, which are abundant in most Antarctic pollen records and also found in the majority of the Wilkes Land samples, were selected for analysis.~~.~~ Dependent on availability the number of different taxa per time slice varied. These taxa include (name in brackets indicates~~indicate~~ potential nearest living relative after Raine et al., 2011 and Contreras et al., 2013) *Cyathidites minor* (*Cyathea*), *Myricipites harrisii* (Casuarinaceae or Myricaceae), *Nothofagidites flemingii*~~*fleminigii*~~ (*Nothofagus*), *N. lachlaniae* (*Nothofagus*), and *Podocarpidites ellipticus* (*Podocarpus*).

4.

2.~~3~~ Fluorescence microscopy

~~Various factors such as burial depth and geological age contribute to the fluorescence emission of sporomorphs. However, these factors reflect the ultimate determining factor of fluorescence alteration, which is heat flow and the length of time the sporomorphs are exposed to this heat (Waterhouse et al., 1998).~~ The five pollen and spore taxa were examined under light and UV-fluorescence using an Olympus BX40F microscope with a high-pressure mercury burner, dichronic mirror with a 330 – 385nm exciter filter and 420nm long-pass barrier filter. The biochemical fluorescence emitted in Cenozoic sporomorphs ranges through the red, green and blue light intensity spectrum (Bujak and Davies, 1982). Factors such as intensity, saturation and brightness also affect the fluorescence emission and were measured to test whether these variables changed with age and depth.

~~Sporomorphs were examined under light and UV-fluorescence using an Olympus BX40F microscope with a high-pressure mercury burner, dichronic mirror with a 330 – 385nm exciter filter and 420nm long-pass barrier filter.~~ Pollen and spores emit fluorescence ranging from blue (400nm) to red (700nm), and the preservation of the exine helps to determine the fluorescence colour (Van Gijzel, 1971). For an initial qualitative colour classification of the investigated pollen and spores, the colour chart based on UV-fluorescence by Yeloff and Hunt (2005) was used. When correlating the UV-fluorescence signal of sporomorph grains, only comparison between the same sporomorph taxa can be done. This is due to variations in the chemical composition of the exine that affects the fluorescence

colour of the grains (Hunt et al., 2007). The gain and exposures were standardised for all measured grains throughout the analysis to allow for accurate representation of the ~~mean~~ red, green and blue (RGB) values measured. For the light microscope the gain was 1.00x, and the exposure 20 ms (+2.0 EV), while under fluorescence the gain stayed at 1.70x and the exposure was 100 ms (+2.0 EV). The white balance (1.30, 1.00, 2.00) was constant through the entire process. Pictures were taken using a Nikon DS-Fil camera and analysed in image processing software (NIS-Elements Basic Research 3.0 program). ~~A pre-selection was performed removing obviously reworked, older-than-Eocene sporomorphs that were extremely dark to almost opaque under a light microscope and with very little to no fluorescence under UV-excitation. This allowed for the investigation of the fluorescence signature of grains that are *in situ* or not identified as obviously reworked. This examination was done on all slides studied from the Eocene, early Oligocene, late Oligocene and Miocene.~~ The~~The mean~~ RGB, intensity, saturation and brightness values were measured for each grain under light and UV-fluorescence. This was in relation to a greyscale from 0 (no light) to 256. The ~~mean~~ values were taken from each grain through an autodetect tool, which draws a contour around the grain. From this contour the program provides a mean value of the grain, i.e. each fluorescence pixel value from around the contour of the grain. The total fluorescence value for each individual grain was then applied to statistical evaluation~~, and the fluorescence values were only measured from this contour image~~.

~~2.~~4.3 Statistical analyses~~: factors influencing the fluorescence signature of the sporomorphs~~

To quantitatively assess the fluorescence behaviour of the five taxa from Site U1356, three different statistical approaches were used:

(i) A Pearson's correlation coefficient (r) was calculated to determine whether the fluorescence measurements of sporomorphs correlate with age. This correlation coefficient shows the strength of the ~~linear~~ relationship from independently measured fluorescence variables against age through the Eocene to the Miocene. Coefficient values that are closer to 1 or -1 show a better linear agreement. The most statistically significant fluorescence value, ~~mean~~ red, was then measured for each taxon to indicate the correspondence of taxa ~~mean~~ red values against age. This was undertaken in IBM SPSS Statistic version 22, for determining if correlation results are statistically relevant,~~and~~ the highest significance level of~~threshold was set at 0.01 (99%) for all measured~~ p-values (0.01) was used.

(ii) The Mann-Whitney *U* test was performed in PAST (Hammer et al., 2001) to compare whether two datasets that are not normally distributed are statistically different from one another. Like other marine cores from the Antarctic shelf (e.g. Askin and Raine, 2000; Anderson et al., 2011), the sediments from U1356 have a comparatively low pollen concentration, in particular in the Oligocene and Miocene sections deposited after the growth of a continental-wide ice sheet during the EOT. The overall number of palynomorphs suitable for fluorescence measurements was therefore limited and varied between samples. However, to assess statistically significant differences between samples, we have chosen the Mann-Whitney U Test, which is applicable to all samples sizes and may be used with as few as four measurements in each sample (Fowler et al. 2009). The datasets being compared are sporomorph

fluorescence measurements (red, brightness and intensity) of successive geological time slices (e.g., late Oligocene vs. Miocene red values). For each value in one sample, the number of values in a second sample are counted when the value is smaller than the first sample (ties are counted as 0.5), the total of these counts are the U statistic (Hammer et al., 2001). For larger sample values (n=30), an asymptotic approximation to p-value based on normal distribution is used. Smaller values of the U statistic would support the separation of fluorescence measurements between time steps, and larger U values would support the null hypothesis indicating the groups are similar (Hammer et al., 2001).~~The datasets being compared are sporomorph fluorescence measurements (mean red, mean brightness and mean intensity) of successive geological time slices (e.g., late Oligocene vs. Miocene mean red values).~~ This tests if a statistically significant fluorescence signature can be identified to separate sporomorphs over subsequent geological epochs in the Wilkes Land core. The fluorescence variables ~~mean~~ red, ~~mean~~ brightness and ~~mean~~ intensity were chosen because these values had the strongest linear relationship against age (high r values) from the Pearson correlation tests.

(iii) To determine if similar fluorescence values of palynomorphs can be grouped by age, burial depth, taxonomy or fluorescence colour, a series of 1-way Analysis of Similarities tests (ANOSIM) with 999 permutations were conducted using PRIMER 6 (Clarke and Gorley, 2006). The raw fluorescence data for the 120 measured palynomorphs was first pre-treated with a square root transformation and then a resemblance matrix was constructed using the Bray-Curtis similarity algorithm. Using ANOSIM tested if palynomorphs with similar fluorescence values (~~mean~~ intensity, ~~mean~~ RGB, ~~mean~~ saturation and ~~mean~~ brightness) could be grouped into categorical factors: Age – Eocene, Early Oligocene, Late Oligocene, Miocene; Burial Depth; Taxonomy (*Cyathidites minor*, *Myricipites harrisii*, *Nothofagidites flemingii*, *N. lachlaniae*, *Podocarpidites ellipticus*); fluorescence light colour (yellow, orange and red). ANOSIM tests the null hypothesis that there are no fluorescence differences between samples grouped by the levels of a factor (e.g. fluorescence values for an Eocene sample would be distinct from a Miocene sample). If the Global R is close to 0 then fluorescence values characterised by different levels of a factor (e.g. Eocene, Early Oligocene, Late Oligocene, Miocene) are similar and the hypothesis that the age of the sample determines the fluorescence (in this example) can be rejected. Conversely, the closer the Global R value is to 1, the more strongly that factor explains the separation of the similar fluorescence values (Clarke and Gorley, 2006).

5~~3~~ Results

5~~3~~.1 Subjective assessment of sporomorphs through fluorescence colours

A subjective assessment of the sporomorphs fluorescence signature revealed that a purely visual estimate~~assessment~~ of fluorescence colour only allows a limited identification of reworking and separation of geological ages. Following the colour chart classification of Yeloff and Hunt (2005), each colour was assigned a number with Eocene pollen and spores generally graded from an orange/red (46 – 49) while Oligocene grains fluoresced an orange/yellow colour (43 – 46) and Miocene grains showed similar yellow/light orange fluorescence (42 – 45) (see

also supplementary material). The visual examination and identification of the fluorescence colour of a pollen or spore grain can vary dependent upon the observer, and the fluorescence colour of sporomorphs overlaps through time (Bujak and Davies, 1982).~~).~~ The visible red colour fluorescence clearly distinguishes Eocene sporomorphs from Oligocene and Miocene grains in the Site U1356 material, shown from the contrast between pollen and spore grains and the slide background under red filter (Fig. 3~~Figure 2~~). However, the subjective colour comparison of fluorescence alone could not distinguish between Oligocene and Miocene grains (Fig. 3; see Supplementary Table S1~~Figure 2~~).

5~~3~~.2 Variation of fluorescence values through the Eocene to Miocene

The Pearson's correlation (r) coefficient indicates a moderate to strong relationship between fluorescence values ~~mean~~ red, ~~mean~~ intensity, ~~mean~~ brightness and geological age. Red~~Mean red~~ values showed the strongest statistical correlation with age values, r = -0.46~~459~~ (p < 0.0001) (Table 1a; Fig. 4a~~Figure 3a~~). Due to the moderate to very strong relationship between total ~~mean~~ red values and age, a Pearson's correlation was also performed on each taxon's ~~mean~~ red value (Table 1b; Fig. 4b~~Figure 3b~~). A very strong agreement includes *Cyathidites minor* (r = -0.69~~687~~, p = 0.004) and *Nothofagidites lachlaniae* (r = -0.661~~663~~, p = 0.002~~001~~), with *Podocarpidites ellipticus* (r = -0.505~~503~~, p = 0.0007) showing a moderate correlation between ~~mean~~ red values and age (Table 1b; Fig. 4b~~Figure 3b~~). Brightness and intensity had a moderate correlation with age, r = -0.323, but high significance (p = 0.0003), whereas~~). The mean~~ saturation values showed~~had~~ no relationship with age (~~,~~r = -0.22). Green ~~and was not significant (p = 0.118). Mean green~~ (r = -0.308, p = 0.0006) and ~~mean~~ blue values (r = -0.269, p = 0.003) had a weak relationship to age with high significance. Pearson's correlation indicates ~~mean~~ red, ~~mean~~ intensity and ~~mean~~ brightness as the most statistically significant fluorescence signatures to separate non-reworked~~*in situ*~~ sporomorphs in the Wilkes Land core.

The Mann-Whitney *U* tests show statistically highly significant (p < 0.0001) changes of fluorescence ~~mean~~ red, ~~mean~~ intensity and ~~mean~~ brightness values from the Eocene to late Oligocene and the Eocene to Miocene (Table 2). However, there is no statistically considerable difference between the Eocene to early Oligocene ~~mean~~ red values (p = 0.2772), whereas significant differences exist between the ~~mean~~ intensity (p = 0.0014) and brightness (p = 0.0014) (Table 2). The early Oligocene to Miocene (p = 0.0009) and early Oligocene to late Oligocene (p = 0.0067) ~~mean~~ red signals can be distinguished from one another (Table 2), however the intensity and brightness cannot. The late Oligocene to Miocene ~~mean~~ red (p = 0.2772), ~~mean~~ intensity (p = 0.0451) and ~~mean~~ brightness (p = 0.0459) cannot be differentiated from each other. This indicates that measuring the ~~mean~~ red fluorescence values from the Wilkes Land samples can distinctively separate the fluorescence signal from sporomorphs in the Eocene, Oligocene and Miocene. The Mann-Whitney *U* test indicates that non-successive intervals in geological time, e.g., Eocene to Miocene and Eocene to late Oligocene, show more distinctive differences in fluorescence ~~mean~~ red, ~~mean~~ intensity and ~~mean~~ brightness values. However, unlike the subjective fluorescence colour comparison, the Mann-Whitney *U* test shows Oligocene and Miocene grains can now be separated based on the digital quantitative measurement of their ~~mean~~ red fluorescence signature.

53.3 Factors potentially influencing fluorescence sporomorph signals

To understand if geological age~~certain factors: stage~~ (e.g., Eocene, Oligocene and Miocene), fluorescence colour of palynomorphs, burial depth and number of taxa had any influence on the similarity of fluorescence values an ANOSIM analysis was done calculating Global R values for each factor.~~.~~ The ANOSIM tests demonstrated that the age (Eocene, Oligocene and Miocene) of a sample (Global R = 0.145, P = 0.001) and the burial depth (Global R = 0.315, P = 0.001) could explain the separation of samples with similar fluorescence into factors (see Supplementary Table S2).~~.~~ This shows that both age and depth (age being a function of depth in this study) influence the fluorescence of palynomorphs. The taxonomy of the samples (i.e. number of taxa) could not explain any similarity in fluorescence values (Global R = 0.006, P = 0.385) and neither could the fluorescence colour of the palynomorph (Global R = 0.085 P = 0.011).

64 Discussion

64.1 The advantage~~Importance and application~~ of applying~~mean red fluorescence~~

~~Our study demonstrates that~~ a ~~qualitative, subjective assessment of fluorescence colour alone cannot be used to separate *in situ* and reworked pollen and spores in the Wilkes Land core over successive geological time scales. Our~~ quantitative approach **and red fluorescence**

Our approach provides a new essential and simple tool to identify non-reworked~~identified the mean red fluorescence colour of pollen and spores as the most reliable indicator for reworking, showing a strong linear correlation with age (Figure 3). This clear statistical grouping of red fluorescence values over consecutive time scales indicates a considerable portion of the measured pollen and spore taxa are *in situ*. For Site U1356, the record is expected to have significant reworking in the Neogene section due to the submarine exposure of Eocene sediments close to the site. However, the mean red fluorescence values from this approach clearly separate the Eocene to Miocene grains (Table 2) indicating with some certainty a major influence of reworking is not present in the Wilkes Land record. This does not completely disregard the influence of reworking in a sample, but ensures a sample has enough *in situ* pollen of a taxa to be used for environmental reconstruction. Our study therefore provides a new essential and simple tool to distinguish *in situ*~~ palynological assemblages in marine sediment records from the high latitudes that are influenced by glaciation and extensive reworking. Our study also highlights~~However, it must be noted that~~ the importance of using a quantitative~~red fluorescence values in our new~~ approach in combination with digital imaging software~~are not absolute values, which can be transferred between cores. Because Palynology uses the entire assemblage of taxa and never a single grain~~ to ~~reconstruct vegetation communities, our approach has not been designed to determine if a single grain is *in situ* or reworked. In order to successfully~~ identify non-reworked palynomorph assemblages over~~if a sample contains an *in situ* pollen taxa assemblage for environmental reconstructions, the measurement of red fluorescence colour needs to be adhered to individual coring sites covering~~ successive geological time scales. The quantitative approach does not only offer a reproducible and transferable method. By detecting significant differences between the Oligocene and Miocene in the Wilkes Land core, this

method has proven to be able to identify non-reworked palynomorph assemblages, which cannot be identified by using qualitative~~epochs.~~

~~Our approach offers an opportunity to resolve difficulties in differentiating *in situ* and reworked palynomorphs in Antarctic palynological assemblages from the Ross~~ and subjective colour comparisons alone.~~Weddell Sea (e.g., Raine, 1998; Askin, 2000; Askin and Raine, 2000; Warny and Askin, 2011).~~ Subjective fluorescence microscopy has been applied~~use~~ to separate non-reworked~~*in situ*~~ grains from recycled Permian-lower Mesozoic sporomorphs in the early Miocene Cape Roberts Project (CRP) 1 core (Raine, 1998). Both~~However, both~~ transmitted light (yellow to yellow-brown) and fluorescence colour (yellow to orange) comparisons could not discern Cenozoic pollen and spores (Raine, 1998; Askin and Raine, 2000; Raine and Askin, 2001). There is no apparent pattern of variation found in the fluorescence colour of sporomorphs in assemblages from the Ross Sea, emphasizing the importance of taking quantitative fluorescence measurements.

Our quantitative approach identified among the various fluorescence values (i.e. red, blue, green, brightness, saturation and intensity), the red fluorescence of pollen and spores as the most reliable indicator to differentiate reworking in successive geological epochs. From the Eocene through the Miocene, each time step shows a clear statistical grouping of red fluorescence values of pollen and spores. While individual taxa do show varying overlaps in red fluorescence values (Fig. 4b), all five pollen and spore taxa show a moderate to strong relationship with age (Fig. 4), reducing the likelihood of large amounts of reworked grains being present in the respective taxa assemblages. The Neogene sediment section of Site U1356 is expected to have significant reworking due to the submarine exposure of Eocene sediments close to the site. However, by using red fluorescence our approach was able to clearly distinguish between the Paleogene and Neogene pollen assemblages.

6~~4~~.2 Influence of heat flow, burial depth and hiatuses on fluorescence

In order for a distinct fluorescence signature to be unambiguously identified in a palynological assemblage an ample amount of geological time between samples is needed (e.g. Van Gijzel, 1967; Bujak and Davies, 1982). It is important to discern whether fluorescence values can be distinguished over successive geological intervals and how factors such as hiatuses and burial depth can possibly affect fluorescence. The largest differences in depth and intervals of geological time in the Wilkes Land core are between the Eocene to late Oligocene and the Eocene to Miocene and these intervals show the highest significance of ~~mean~~ red, ~~mean~~ intensity and ~~mean~~ brightness values ($p < 0.0001$; Table 2). However, geological age alone~~this~~ does not always determine~~mean geological age directly determines~~ the fluorescence signal of sporomorphs. The amount and length of exposure to burial heat ultimately establishes the fluorescence alteration of sporomorphs (Waterhouse et al., 1998). Red~~Mean red~~ fluorescence values are still significantly different~~statistically significant~~ ($p = 0.0067$; Table 2) when comparing the early and late Oligocene pollen assemblages~~samples~~. The oldest early Oligocene sample analysed was taken at 795.58 mbsf and the youngest late Oligocene sample was analysed at 555.19 mbsf. This difference in sedimentation rate and ultimately burial depth could contribute to the differentiation of ~~mean~~ red values between the early and late

Oligocene. Burial depth is shown to play an importanta role in the fluorescence of sporomorphs as also indicated by the Global R value in the ANOSIM analysis (Sect.Section 3.3).

Disruption of sporomorph exposure to burial heat is shown to have an effect on fluorescence mean red values as well. Between the mid-Eocene to early Oligocene and the late Oligocene to Miocene, the differences in mean red values are non-significantinsignificant (p = 0.2772; Table 2). There areThese mean red values correlate with two major hiatuses observed infrom the Eocene to Miocene sediment record offrom Site U1356 off Wilkes Land, East Antarctica (Escutia et al., 2011). A ~13 m.y. hiatus is found between the middle Eocene and the early Oligocene (Escutia et al., 2014). This unconformity represents extensive erosion correlating with the onset of glaciation at the Eocene-Oligocene boundary (Escutia et al., 2011; 2014; Stocchi et al., 2013). Another hiatus correlates with a regional unconformity at Wilkes Land that coincides with the Mi-1 event and extends from ~23.12 to 16.7 Ma (Escutia et al., 2005; Escutia et al., 2011). The hiatuses in the Wilkes Land core could have potentially disrupted the sporomorphs exposure to burial heat causing a less distinctive fluorescence signature between the mid-Eocene to early Oligocene and late Oligocene to Miocene. However, it is important to reiterate that our study couldthis fluorescence approach can still find significant differences inseparate the mean red fluorescence values between the entire Oligocene and Miocene (p = 0.0083; Table 2).

64.3 Fluorescence variation between taxa

In order to produce comparable values for each geological time interval, our fluorescence approach requires sediment cores spanning successive geological epochs with similar palynological assemblages. The Paleogene and Neogene Antarctic palaeovegetation are unique with a number of common taxa (e.g., *Nothofagidites* and *Podocarpidites*) still present in palynological assemblages through a major climatic change from a greenhouse to icehouse world (e.g., Truswell and Macphail, 2009; Pross et al., 2012; Griener et al., 2015). When comparing the fluorescence signature between geological time intervalsslices the same taxa must be used. Differences between the fluorescence signatures of individual taxa in the Wilkes Land assemblage are apparent (Fig. 4Figure 3; Table 1). Factors such as exine composition and differential sensitivity to thermal alteration can cause variation in fluorescence measurements between sporomorph taxa (Waterhouse et al., 1998). The differences in mean red fluorescence signature could also have occurred due to variation in the number of taxa measured for each geological time slice. The only spore taxa in this study, *Cyathidites minor* showed the strongest correlation between fluorescence mean red values ($r = -0.687$, $p < 0.004$) through geological time. This could be due to the chemical composition of *Cyathidites minor*, the thickened and complex perispore (Marquez and Morbelli, 2014) or how this spore chemistry reacts to degradation over geological time.

7 Concluding remarks: Applications and limitations of the quantitative red fluorescence approach

The unambiguous identification of non-reworked5 Conclusions

Reworking has long been a factor hindering a full appreciation of Cenozoic Antarctic palynology. The impact of ice-sheet fluctuations since the Oligocene makes identifying *in situ* pollen grains in Neogene Antarctic assemblages

~~particularly challenging (e.g., Francis and Hill, 1996; Wilson et al., 2002; Salzmann et al., 2011). However, the unambiguous identification of *in situ*~~ palynomorphs is a prerequisite to fully understand the terrestrial vegetation response to periods of extensive cooling and environmental changes on Antarctica. Our study focussed on identifying non-reworked palynological assemblages from Antarctica, although this method can theoretically be applied to all palynological records. Our approach requires a sufficiently long palynological record (e.g. Miocene to Eocene) and the consistent presence of identical pollen taxa that occur in reasonable numbers in all measured time intervals. For statistical analysis, fluorescence values of samples covering successive time scales in a single core (e.g., Eocene red values against Oligocene red values) must be measured using digital imaging software. A pre-selection removing obviously reworked grains (e.g., grains older than the successive time scales that are extremely dark to opaque under a light microscope) from the analysis needs to be performed. Suitable pollen and spore taxa that can be found throughout the core over successive time scales must be determined before fluorescence analysis. By using fluorescence microscopy this study shows that red fluorescence is~~By using fluorescence microscopy this study identifies mean red fluorescence as~~ the most reliable parameter to statistically identify reworking on million-year time scales during the Paleogene and Neogene. The study also highlights intensity and brightness as sensitive indicators.~~For Site U1356 the mean red fluorescence values measured clearly separate the Eocene to Miocene grains despite that proximal to the site, submarine exposure of Eocene sediments is present.~~ It is important to emphasize that the red fluorescence values from this study are not absolute and are specific to the Wilkes Land core. Fluorescence variation between taxa is apparent.

~~, but the mean red fluorescence measurements for each taxon (*Cyathidites minor*, *Myricipites harrisii*, *Nothofagidites flemingii*, *N. lachlaniae*, and *Podocarpidites ellipticus*) still show a strong linear relationship against age.~~ Our study offers a new quantitative approach to identify if a sediment core ~~sample~~ contains non-reworked~~an *in situ*~~ pollen taxa assemblages~~assemblage~~ to reconstruct with high confidence Cenozoic climate change and vegetation~~andvegetation~~ pre- and post-Antarctic cryosphere formation. By using the Mann-Whitney *U* test, the approach is suitable to work with low pollen concentrations that are very common in Antarctic palynology. However, a higher number of palynomorphs and fluorescence measurement would certainly increase the potential and depth of further statistical analyses. The low pollen number, for example, prevented us from exploring whether changes in the variance of the fluorescence value can be used as a measure for the degree of reworking in each sample (i.e. high variance = high number of reworked palynomorphs). Additional studies are needed to systematically explore the wider use of our red fluorescence approach for Antarctic palynology. These studies should include forthcoming IODP drilling expeditions and possibly existing sites such as ANDRILL (AND-2A) where several taxa such as *Nothofagidites brassii* group, Proteaceae and podocarp conifers were denoted with uncertainty because it is unknown when these taxa disappeared from Antarctica (Griener et al., 2015).

Data availability

The raw data analysed in this study is available as supplementary material.

Competing interests

The authors declare that they have no conflict of interest.

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