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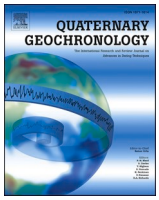

Testing the effect of oxidizing pre-treatments on amino acids in benthic and planktic foraminifera tests

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ABSTRACT

Amino acid racemization (AAR) is a geochronological method that uses the ratio of D- to L-configurations in optically active amino acids from carbonate fossils to determine the time elapsed since the death of an organism. Although AAR techniques have been widely applied to foraminiferal tests, there have been limited dedicated assessments of the potential of isolating a bleach-resistant, intra-crystalline fraction of proteins to improve the reliability of AAR in this biomineral system. In this study, we evaluate the effect of two oxidative pre-treatments (hydrogen peroxide and bleach) on amino acid concentrations and D/L values in sub-modern benthic foraminifers (*Ammonia* spp. and *Haynesina germanica*) and well-preserved mid Holocene and mid Pleistocene planktic foraminifers (*Pulleniatina obliquiloculata*, *Globorotalia truncatulinoides*, and *Globorotalia tumida*). The oxidative pre-treatments successfully reduced the amino acid content of the foraminiferal tests to a residual fraction, and with the exception of *Ammonia* spp., neither pre-treatment substantially affected the relative proportion of individual amino acids. The bleaching pre-treatment does not consistently alter D/L values when compared to peroxide pre-treatment, but it does tend to reduce the subsample variability in D/L values, albeit only to a small degree in an inconsistent fashion. Therefore, we recommend that a relatively weak oxidative pre-treatment with 3% hydrogen peroxide is sufficient for foraminifera-based AAR applications.

1. Introduction

Amino acid racemization (AAR) is a diagenetic process that has been used as a geochronological tool for decades. AAR is the interconversion of amino acids from their L- to D-enantiomeric configuration, which starts upon the death of an organism and subsequent breakdown of proteins (Bada and Schroeder, 1975; Wehmiller and Hare, 1971). The D- to L-ratio of amino acids (D/L) is thus a measure of the extent of racemization and increases with time (and temperature), from values of ~0 in modern materials to equilibrium values of ~1 (for most amino acids), when the forward and reverse reaction rates are equal (e.g., McCoy, 1987; Miller and Mangerud, 1985). AAR is applicable to a wide range of fossiliferous material (e.g., mollusks, foraminifera, eggshell, and ostracods), depositional environments (e.g., marine, lacustrine, and fluvial) as well as to determine stratigraphic relationships (e.g., correlations, the reworking of fossils, and detecting unconformities) (Miller et al., 2013; Walker, 2005). Since the 1970s, AAR has been used as a relative (e.g., Kaufman and Miller, 1992; Miller et al., 1979; Penkman et al., 2011, 2013; Walker, 2005; Wehmiller, 1977, 2013) or absolute (e.g., Goodfriend, 1989; Hearty et al., 2004; Kosnik et al., 2013; Macko and Aksu, 1986; Sejrup et al., 1984) dating tool for Quaternary deposits. In order to reliably use D/L values in fossil material to calculate the time elapsed since the death of the organism (i.e., as a geochronometer), it is essential that external influences on racemization rates and the effects of modern contamination are minimized.

To this end, one advancement in AAR geochronology is the development of the intra-crystalline protein diagenesis (IcPD) approach, which uses a chemical oxidant, bleach (NaOCl), to remove the inter-crystalline protein "mesh" between the biomineral crystallites while leaving behind proteins that are embedded within the crystallites

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(Penkman et al., 2008; Sykes et al., 1995; Towe, 1980). This approach provides more consistent results in mollusks, corals, and eggshells than more gentle cleaning methods involving sonication in deionized water or weak oxidation with dilute hydrogen peroxide (3% H_2O_2) (Crisp et al., 2013; Demarchi et al., 2013a, 2013b; Hendy et al., 2012; Ortiz et al., 2015, 2018; Penkman et al., 2008, 2011). While bleaching pre-treatments have been used during the preparation of foraminiferal tests for AAR (Kaufman et al., 2013; Nicholas, 2012), dedicated assessments of the IcPD approach to this class of biominerals are limited (Hearty et al., 2004; Stathoplos and Hare, 1993). Recently, a study detailing the effects of bleach on the tests of the planktic foraminifer *Neogloboquadrina pachyderma* (sinistral) suggested that the IcPD approach reduces the influences of open-system behavior when compared to a sonication-only pre-treatment (Wheeler et al., 2021). However, peroxide pre-treatments were not considered by Wheeler et al. (2021) for comparison with the bleach pre-treatment.

In this study, we test the effects of different oxidative pre-treatments on the amino acid composition and D/L values for several benthic and planktic foraminiferal species. Specifically, we assess the effects of sonication, various peroxide concentrations, and the addition of bleach on the composition of amino acids, the D/L values within individual amino acids, and the inter-sample variability of D/L values. This study merges data generated in two separate efforts: one focusing on benthic foraminifera from surface sediments (0–5 cm, sub-modern) and the other on planktic foraminifera from deep sea sediment cores (~6 ka to ~410 ka). The primary goal in the former study was to establish whether an oxidation-resistant protein fraction can be isolated from benthic foraminiferal tests and to assess the effects of these pre-treatments on amino acid composition and D/L values. In the latter study, the original research question asked whether or not pre-treatment with bleach had a significant effect on amino acid composition, inter-species differences in D/L, and D/L variability of previously peroxide-cleaned planktic foraminiferal tests ranging in age from the mid Holocene through the mid Pleistocene. Although the study designs were independent, both approaches have similar implications for understanding the effect of oxidative pre-treatment on foraminiferal proteins, thereby warranting integration into a common framework within this paper.

2. Methods

2.1. Sample selection

The first set of samples involves benthic foraminifers from surface (0–5 cm) sediments collected in the Humber Estuary, a mid-latitude, coastal plain estuary formed by the Ouse and Trent rivers in the northeastern United Kingdom (Table 1). Sample collection was carried out in February 2018. These samples were not independently dated, but they most likely represent a modern assemblage of foraminifera. Although reworking of the salt-marsh sediment is a possibility (Figueira and Hayward, 2014), radiocarbon dating of basal peats in the area gives a maximum age of a few thousand years (Long et al., 1998). To reflect the uncertainty in their age, we refer to these samples as "sub-modern." Abundant foraminiferal species include several belonging to *Ammonia* and *Haynesina germanica*. The speciation of *Ammonia* is challenging; therefore, we refer to them collectively as "*Ammonia* spp." (Hayward et al., 2004). The extent of AAR in *Ammonia* spp. has been used to date Quaternary sea-level deposits (e.g., Blakemore, 2014; Dowling et al., 1998; Nicholas, 2012; Scourse et al., 1999), but to the authors' knowledge, *H. germanica* has not previously been used in foraminiferal-based AAR studies.

The second set of samples involves planktic foraminifers that were picked from three Ocean Drilling Program (ODP) sites (Sites 1056, 1059, and 1062) and one piston core (KNR140 JPC-37) located on the Blake Bahama Outer Ridge (BBOR) in the northwestern subtropical Atlantic Ocean (Table 1). We use three species, *Globorotalia truncatulinoides, Globorotalia tumida*, and *Pulleniatina obliquiloculata,* because of their relatively high abundance, large tests, resistance to dissolution (e.g., Hemleben et al., 1989), and their demonstrated applicability to AAR research (e.g., Hearty et al., 2004; Kaufman, 2006; Kaufman et al., 2013; Stathoplos and Hare, 1993; Wehmiller and Hall, 1997). By analyzing multiple species, we also assess potential inter-species differences in amino acid concentration, composition, and variability in amino acid D/L values.

At the BBOR, samples span the mid Holocene through mid Pleistocene (~6–410 ka, Table 1). Mid-Holocene samples from the BBOR were initially located in the cores using published age models (Grützner et al., 2002). Early Holocene through mid Pleistocene-aged samples from Site 1056 and KNR140 JPC-37 were identified using the cores' $\delta^{18}O$ stratigraphy (Billups et al., 2004; Hagen and Keigwin, 2002, respectively).

Table 1
Site locations and sampling intervals.

Location	Longitude, Latitude	Water Depth (m)	Core Intervals (cm)	Sediment Depth (mcd)[a]	Age (ka)[b]	Age (ka)[c]
Sub-modern						
Humber Estuary	54°N, 0°E	–	0–5	0.00–0.05	–	–
Mid Holocene						
1056D, 1H-1	32°N, 76°W	2178	62, 64, 68, 70	0.62–0.70	6.0–6.5	4.8–5.0
1059A, 1H-1	32°N, 75°W	2997	44, 50, 54	0.44–0.56	6.0–6.5	5.0–5.4
1062B, 1H-1	28°N, 74°W	4780	62	0.62–0.64	7.1	7.0
Early Holocene						
KNR140 JPC37	31°N, 75°W	2997	150	1.50	10.5	–
Late Pleistocene						
KNR140 JPC37	32°N, 75°W	2997	1112	11.12	51.5	–
Mid Pleistocene						
1056B, 5H-7	32°N, 76°W	2178	44	35.34–35.36	410.0	–

[a] MCD stands for meters composite depth.
[b] Stratigraphic ages (see Section 2.1).
[c] AMS^{14}C dates. For Site 1062, this is the average of two ages (6.68 and 7.23 ka).

The time equivalency of the mid Holocene samples from the three BBOR sites was subsequently confirmed to within ~1–2 ka (Table 1) using rapid, low precision, ^{14}C analyses (Bush et al., 2013; Watson, 2019). These were conducted on mono-specific planktic foraminiferal tests at the Keck AMS facility at the University of California Irvine with calibration to calendar ages following the convention of Stuiver and Polach (1977).

2.2. Experimental methods

Experiments involving benthic foraminifera from the Humber Estuary were carried out at the Northeast Amino Acid Racemization (NEaar) laboratory (University of York, UK). Experiments (Fig. 1a) consisted of a series of extended oxidation pre-treatments using hydrogen peroxide (3% and 30% w/v H_2O_2) or bleach (12% w/v NaOCl), compared to "unbleached" samples treated only by sonication in purified water.

Experiments involving planktic foraminifera from the BBOR were conducted at the University of Delaware. These experiments compared foraminifer tests oxidized for 2 h with 3% w/v H_2O_2 (referred to as "3% H_2O_2") to those additionally oxidized for 48 h with 12% w/v NaOCl (referred to as "12% NaOCl-addition") (Fig. 1b). The pre-treatments used by the University of Delaware are akin to a subset of the pre-treatments used by the University of York.

2.2.1. Sequential pre-treatment experiments (University of York)

Tests of *Ammonia* spp. and *H. germanica* from the surface sediments of the Humber estuary tidal flats were picked from the 63–500 μm fraction and whole tests were cleaned prior to pre-treatment by sonicating repeatedly with purified water (18.0 MΩ) until the water was clear. Each replicate sample consisted of 30–50 monospecific tests with 3–4 replicates for each species and pre-treatment. Each replicate was prepared separately in sterilized 0.1 mL conical-bottomed micro-reaction vials (Thermo Scientific). Tests were crushed in the vials with a clean needle before a second round of sonication (3 × 1 min, rinsing with purified water between each sonication). For "unbleached" samples, no further pre-treatment was carried out prior to analysis.

Oxidative pre-treatments were carried out for various durations (2–192 h) using hydrogen peroxide (3% and 30% w/v H_2O_2) and bleach (12% w/v NaOCl) (Fig. 1a). An oxidizing agent (20 μL) was added to each replicate sample, with periodic agitation to ensure complete exposure of the biomineral to the oxidizing agent. The oxidizing agent was then removed by pipette and the biomineral rinsed with purified water six times and HPLC-grade methanol once then air dried prior to demineralization.

For the hydrolysis step, all samples were demineralized in the micro-reaction vials with 10 μL 7 M HCl, flushed with nitrogen, and heated at 110°C for 24 h. Samples were then dried under vacuum and stored at room temperature prior to rehydration for analysis. Samples were rehydrated with 8 or 10 μL of rehydration fluid (indicating that some samples had to be diluted and reanalyzed), comprising 0.01 M HCl, 1.5 mM NaN_3, and 0.01 mM L-*homo*-arginine (L-*h*Arg), an internal synthetic amino acid standard used to quantify the abundance of amino acids in each vial. Amino acid enantiomers were resolved by reversed phase high performance liquid chromatography (HPLC) at the NEaar lab using a slightly modified method of Kaufman and Manley (1998) with the additions of Kaufman (2000) for analyzing microfossils. Of the total sample volume, 2 μL of the rehydrated samples were injected into the HPLC where they underwent pre-column derivatization with *o*-phthaldialdehyde (OPA) and N-isobutyryl-L-cysteine (IBLC) followed by chiral separation with a C_{18} stationary phase (Hypersil BDS, 5 μm) and fluorometric detection (Kaufman and Manley, 1998). Procedural blanks were analyzed for each experiment to quantify background levels of amino acid contamination. Due to the limited number of replicates analyzed for each pre-treatment (Fig. 1), statistical comparisons were not carried out for this series of experiments.

2.2.2. Comparison between oxidative pre-treatments (University of Delaware)

Bulk sediment samples from the three ODP core sites on the BBOR (Table 1) were processed using standard techniques with disaggregation in a buffered sodium metaphosphate solution, washing over a 63 μm sieve with deionized water, and leaving to air dry. The sediments from the piston core were already processed according to Hagen and Keigwin (2002). From each core interval (n = 11), we aimed to pick about 150 individuals of *P. obliquiloculata*, *G. truncatulinoides*, and *G. tumida* from the >350 μm fraction.

The 150 species-specific tests from each core interval (n = 19) were placed into individual 16 mm glass culture tubes. Each tube was filled with deionized water and sonicated briefly to loosen surface contaminants. To remove organic contaminants, the tests were soaked in 3% w/

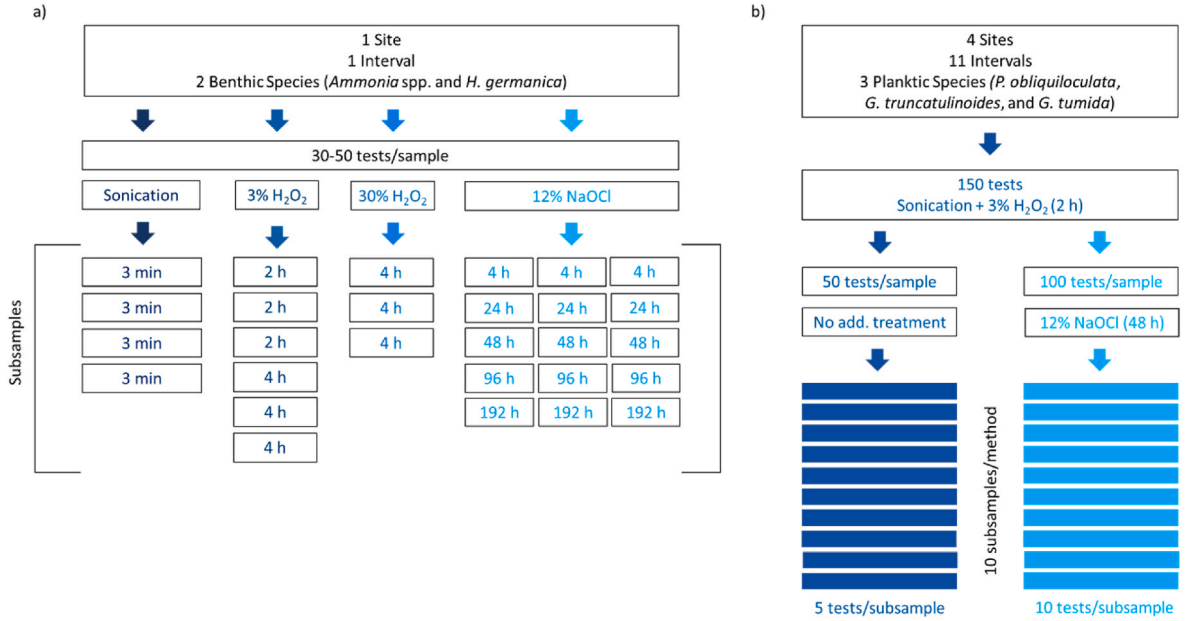

Fig. 1. Visual representation of the pre-treatment methodology for a) the sequential pre-treatment experiments (University of York) and b) the comparison between 3% H_2O_2-only and the 12% NaOCl-addition pre-treatments (University of Delaware). Discussion of these methods can be found in Sections 2.2.1 and 2.2.2.

v H_2O_2 for 2 h, rinsed three times with reagent grade H_2O, decanted, and air dried under laminar flow; this is a common cleaning step in other foraminiferal-based AAR studies (e.g., Kaufman et al., 2008). Once clean, the 150 species-specific tests for each core interval were split into samples of 50 and 100 tests. The samples containing 50 tests were then subdivided into 10 subsamples composed of 5 individuals on average, which were placed into sterilized, conical bottomed 0.1 mL micro-reaction vials (e.g., Fig. 1b). Subsamples were hydrolyzed, rehydrated, and analyzed using a similar procedure to the one used by the University of York (Section 2.2.1). The difference is that samples were hydrolyzed with 7 μL 6 M HCl, flushed with nitrogen, and heated at 110°C for 6 h, in order to minimize hydrolysis-induced racemization in the older samples. This heating time is shorter than the NEaar hydrolysis protocol, but the difference will not impact the paired comparisons as the D/L values are not being directly compared between the two studies (e.g., Dungworth 1975; Williams and Smith, 1977). Samples were then rehydrated with 4 μL of the rehydration fluid, and all 4 μL of the sample was injected into the column for analysis. Amino acid enantiomers were resolved by reversed phase HPLC at the Amino Acid Geochronology Lab (Northern Arizona University, AZ) using the same parameters as above (Section 2.2.1).

The samples containing 100 tests were further treated with bleach following Penkman et al. (2008), with modifications. Each group of 100 tests were divided into 10 subsamples (e.g., Fig. 1b), each containing 10 tests on average, and placed into separate glass test tubes. To isolate the intra-crystalline fraction, tests in each subsample were gently broken and 12% NaOCl was added to fill three fourths of the tube. The tubes were then agitated, left for 24 h, re-agitated to ensure complete exposure to the bleach, and soaked for another 24 h. After 48 h, the bleach was pipetted off and samples were rinsed with purified water 3–5 times before being left to dry under laminar flow. The dry fragments were transferred to conical bottomed 0.1 mL micro-reaction vials and hydrolyzed, rehydrated, and analyzed using the same procedure as the peroxide-cleaned samples.

2.3. Data screening

In this study, we focus on the D/L values of aspartic acid and glutamic acid, which are two of the most abundant amino acids in foraminiferal protein and best resolved chromatographically (Kaufman et al., 2013). However, during hydrolysis, asparagine and glutamine are irreversibly hydrolyzed to aspartic acid and glutamic acid respectively; therefore, these amino acids are analytically indistinguishable and reported as Asx and Glx (Hill, 1965). The integrity of AAR data can be assessed using a range of diagnostic criteria, such as serine (Ser) abundance, depth or age trajectories, covariance of concentrations or D/L values, and variability of replicate subsamples (Kosnik and Kaufman, 2008). Cut-offs for sample exclusion are determined empirically and are known to depend on age and taxonomy (Kaufman, 2006; Kosnik and Kaufman, 2008). Here we followed the three-step screening procedure from Kosnik and Kaufman (2008) to systematically identify subsample outliers that could influence the resulting mean D/L values for each sample.

First, the concentration of Ser, a labile amino acid which should be present only in low concentrations within fossils due to its rapid rate of decomposition, was used to identify subsamples contaminated by modern amino acids (Kaufman, 2006; Kaufman et al., 2013; Kosnik and Kaufman, 2008). Although this criterion is not applicable to the sub-modern material from Humber Estuary, because the low extent of protein breakdown means that Ser concentrations remain high, individual subsamples from the BBOR cores with L-Ser/L-Asx values ≥ 0.9 were rejected. Second, the covariance between the D/L values of Glx and Asx was visually assessed to identify subsamples with D/L values that substantially deviate from the linear relationship of all subsamples within a species (Kaufman, 2006; Kaufman et al., 2013). Third, subsamples with Asx or Glx D/L values that fell beyond $\pm 2\sigma$ of the mean of the rest of the group were rejected (Kaufman, 2006; Kaufman et al., 2013).

No outliers were identified in the estuarine data set, likely due to the small number of replicates for each pre-treatment (n = 3–4). Screening of the BBOR data (n = 381) resulted in the rejection of 46 (12.1%) individual data points. Of these, 14 (3.7%) were rejected based on high Ser content, 7 (1.8%) were rejected as outliers based on the expected linear Asx D/L vs. Glx D/L trend for a species, and 25 (6.6%) were rejected due to falling beyond the $\pm 2\sigma$ of the mean of the group. The rejection rate is slightly lower for the samples pretreated with hydrogen peroxide (19 out of 196 subsamples rejected, 9.7%) as opposed to samples pretreated with bleach (27 out of 185 subsamples rejected, 14.6%).

3. Results

3.1. Amino acid concentration

Our results show that the choice of pre-treatment affects the concentration of total hydrolysable amino acids ([THAA]) in both benthic (Fig. 2) and planktic foraminifer tests (Fig. 3). Regarding the benthic foraminifer tests, sequential oxidation illustrates that when treated with 12% bleach, the total concentration of amino acids decreases by ~90% in the first 4 h of bleaching, then remains at consistently low levels for the remainder of the oxidation period, regardless of species (Fig. 2). This suggests that the residual fraction of proteins isolated within 4 h of bleach exposure represents a bleach-resistant intra-crystalline protein fraction. The results of the H_2O_2 pre-treatment experiments are more species dependent. In *Ammonia* spp., the variability of [THAA] in the unbleached samples precludes a quantitative comparison of the proportion of amino acids removed by each oxidation pre-treatment, although a smaller proportion of amino acids are removed by the 4 h 3% H_2O_2 and 30% H_2O_2 treatments than the 4 h 12% NaOCl treatment (Fig. 2a). This suggests that H_2O_2 is not a strong enough oxidizing agent to isolate the bleach-resistant intra-crystalline fraction, at least within 4 h, in *Ammonia* spp. Conversely, the 4 h treatments with 3% H_2O_2, 30% H_2O_2 and 12% NaOCl all remove a similar proportion of amino acids in *H. germanica* (Fig. 2b). The concentration of THAA in *Ammonia* spp. remains stable up to 192 h, while there appears to be a further gradual loss of amino acids after prolonged bleaching (>96 h) in *H. germanica,* indicating possible etching of the mineral matrix (Dickinson et al., 2019; Penkman et al., 2008).

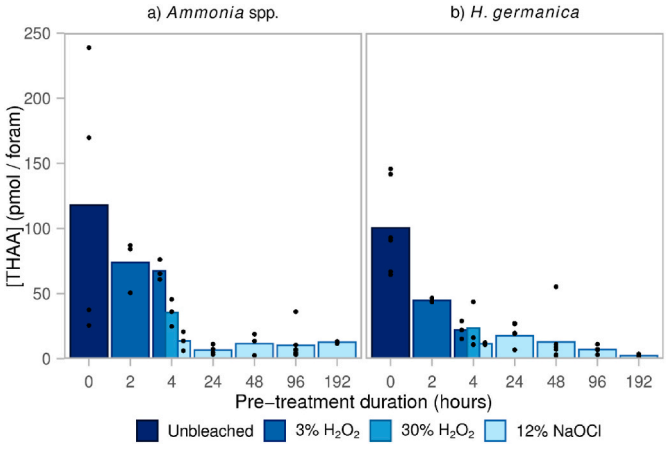

Fig. 2. Concentration of total hydrolysable amino acids ([THAA]) in unbleached (i.e., H_2O sonication only) and oxidized (i.e., H_2O_2 or NaOCl) samples of modern benthic species a) *Ammonia* spp. and b) *H. germanica* from surface sediments of the Humber Estuary. The colored bars show the average concentration at each time point. Due to the small number of replicates analyzed at each time point in this suite of experiments, all data points are shown as solid black points.

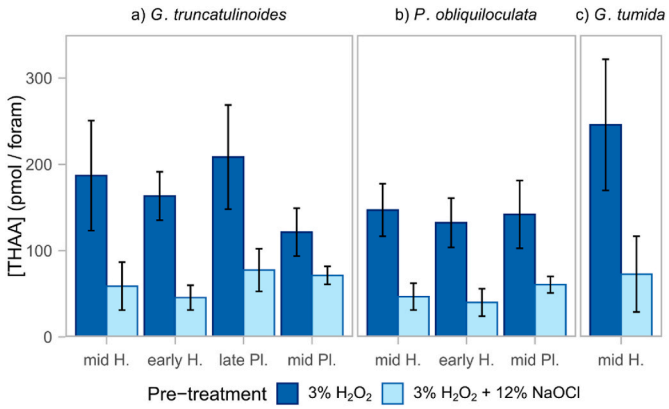

Fig. 3. Concentration of total hydrolysable amino acids ([THAA]) for a) *G. truncatulinoides*, b) *P. obliquiloculata*, and c) *G. tumida* pre-treated with 3% H_2O_2 only or 3% H_2O_2 plus 12% NaOCl. Mean [THAA] is separated based on sample age (mid Holocene, early Holocene, late Pleistocene, and mid Pleistocene) for each pre-treatment. Error bars represent the ±1σ around the mean. All differences are statistically significant at the 95% confidence interval (P-values < 0.05).

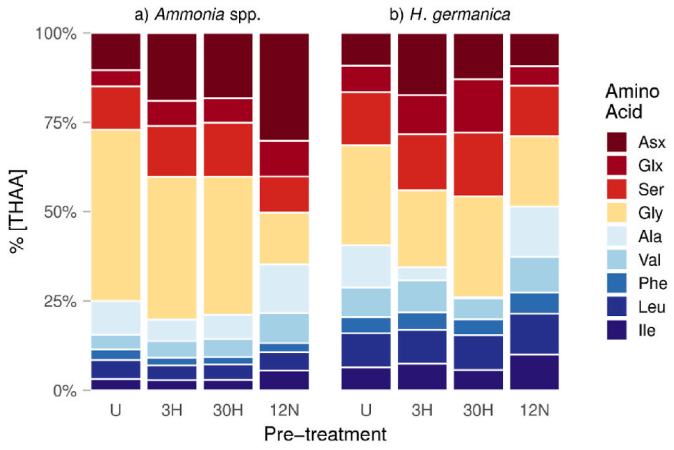

Fig. 4. Relative proportion of amino acids (%THAA) in benthic foraminifer tests from estuarine surface samples for a) *Ammonia* spp. b) *H. germanica*. Four different pre-treatments were used: H_2O sonication only (Unbleached, U), 4 h 3% H_2O_2 (3H), 4 h 30% H_2O_2 (30H), and 4 h 12% NaOCl (12N).

In comparison to the benthic foraminifer species (Fig. 2), the planktic foraminifer species (Fig. 3) have higher amino acid concentrations when treated with 3% H_2O_2. This observation most likely corresponds to the larger size of the planktic species. The 24 h bleach pre-treatment reduces [THAA] by ~90% in the sub-modern, benthic foraminifera, whereas [THAA] is reduced by 41–72% in the down-core, planktic foraminifera. This difference could be explained by the sample age difference. For *G. truncatulinoides* and *P. obliquiloculata*, bleach reduces [THAA] by 68–72% in mid to early Holocene-aged samples but only 41–57% in mid Pleistocene-aged samples. Thus, it appears bleach removes a smaller proportion of amino acids as the age of the samples increase, indicating a loss of open-system inter-crystalline amino acids during the early stages of diagenesis. This pattern has been observed in a variety of mollusk species (e.g., Ortiz et al., 2015, 2017; Penkman et al., 2008; Sykes et al., 1995).

In sum, bleaching foraminifer tests more effectively reduces [THAA] as opposed to either peroxide treatment, in both benthic and planktic foraminifer species. This reduction is more drastic in the sub-modern benthic foraminifera, where little to no diagenesis has taken place to remove the easily accessible inter-crystalline amino acids.

3.2. Amino acid composition

The pre-treatment methods do not have a consistent effect on the relative proportion of individual amino acids among the foraminifera species used in the two experiments. The largest effect is observed for the benthic species *Ammonia* spp. (Fig. 4a), where increasing the strength of the oxidizing agent increases the proportion of Asx, Glx, and Ala but decreases the proportion of Gly, especially for the 12% NaOCl treatment. In *H. germanica*, samples treated with 3% and 30% H_2O_2 have a lower %Ala and higher %Asx than the unbleached samples, but compositionally the samples treated with 12% NaOCl are more similar to the unbleached samples (Fig. 4b). This may be due to the large inter-replicate variability of composition, especially for Asx and Gly (Fig. S1). The more similar composition of *H. germanica* protein between the different pre-treatments is consistent with the similar concentrations for this species for the 4 h treatments at different oxidizing agent strengths, in contrast to *Ammonia* spp. (Fig. 2).

For the planktic foraminifera, the amino acid composition is relatively similar between pre-treatments and across species and ages (Fig. 5). With the addition of 12% NaOCl, there is a slight reduction in the %Gly and %Phe with an increase in %Ala observed for *G. truncatulinoides*, *P. obliquiloculata*, and *G. tumida*. The small differences observed in the THAA composition of the older planktic species (in contrast to the sub-modern benthic species) could be due to the loss of easily accessible inter-crystalline proteins during the early stages of diagenesis, where afterwards the THAA composition becomes relatively stable against aging and oxidative effects.

3.3. D/L differences between pre-treatments

D/L values for Asx and Glx were investigated because these amino acids are the most common for AAR age or temperature reconstructions (Goodfriend et al., 1996; Kaufman, 2003, 2006; Kaufman et al., 2008, 2013; Miller et al., 2013). Within the data scatter, there are no robust trends for pre-treatment conditions in the D/L values of the benthic foraminifer samples, regardless of whether or not the samples were treated beyond sonication with H_2O (Fig. 6). In *H. germanica*, there may be a slight decrease in Asx and Glx D/L values between the sonication-only samples and samples subjected to short oxidation times (2–4 h), followed by a slight trend of increasing D/L between 4 and 192 h of bleach exposure (Fig. 6b). This pattern has been observed in various mollusk shells (Penkman et al., 2008) and ostracods (Bright and Kaufman, 2011), and could indicate a catalytic effect of NaOCl on racemization following the initial removal of inter-crystalline amino acids. This combined with the slight decrease in amino acid concentration in *H. germanica* after 92 h (Fig. 2b) suggests that the fraction of amino acids isolated by the initial bleach exposure is not resistant to chemical influences. However, given the small number of replicate analyses (n = 3–4) and relatively large overall variability in D/L values, this observation should be treated with caution.

For the planktic foraminifera, the addition of bleach to peroxide treated samples does not have a consistent impact on the D/L values of Asx or Glx. The differences in mean D/L between the pre-treatments are fairly small (e.g., average of 7% and 11% for Asx and Glx, respectively) and are not consistent in direction (Figs. 7 and 8; Table 2). The relatively large number of subsamples measured at the BBOR afford statistical analyses to support these results. Welch's independent t-tests for the mid Holocene samples show that 8/14 mean Asx D/L values and 5/14 mean Glx D/L values had significant differences (P < 0.05) between the two pre-treatments (Fig. 7; Table 2). Unlike the mid Holocene-aged samples, the early Holocene to mid Pleistocene-aged samples show a lack of significant change in the mean D/L values between the two differing treatment methods. The *t*-test results show that only 1/5 mean Asx D/L values and 1/5 mean Glx D/L values are significantly different (Table 2). This indicates that the D/L of older samples (>10.5 ka) are less affected by the bleaching pre-treatment. Nevertheless, a pattern did emerge for

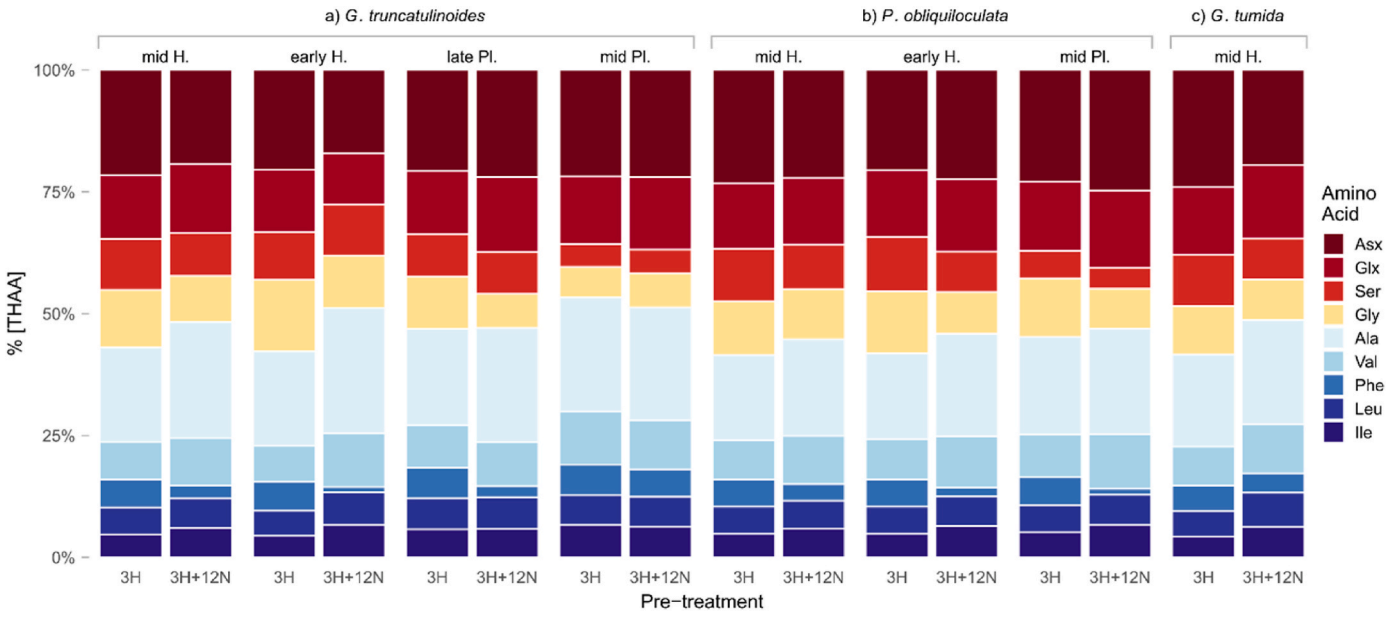

Fig. 5. Relative proportion of amino acids (%THAA) in planktic foraminifer tests from deep marine sites aged mid Holocene (H) to mid Pleistocene (Pl) for a) *G. truncatulinoides*, b) *P. obliquiloculata*, and c) *G. tumida*. Two different pre-treatments were used: 3% H_2O_2 only (3H) and 3% H_2O_2 + 12% NaOCl (3H + 12N).

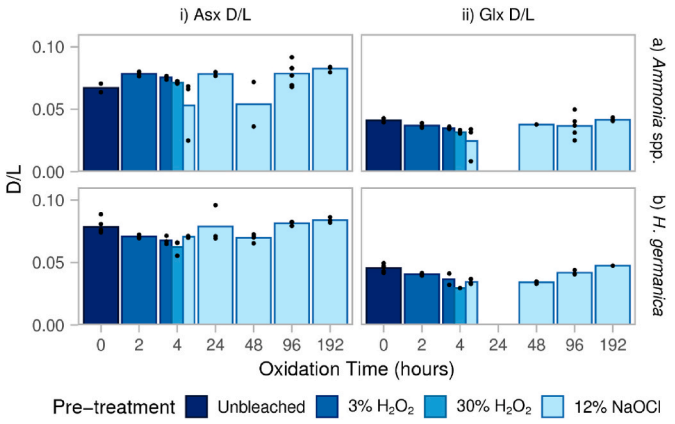

Fig. 6. Trends in i) Asx and ii) Glx THAA D/L with increasing oxidation time for a) *Ammonia* spp. and b) *H. germanica* from the estuarine surface. The top of the bar reflects the average concentration derived from individual measurements (solid black circles). Note that the gap at 24 h indicates that no Glx D/L values were collected for that particular oxidation step due to low amino acid concentrations.

species specific effects regardless of sample age. For samples that showed significant differences in their mean D/L values, bleach increased the D/L values of all *P. obliquiloculata* samples (5/5), decreased the D/L values of most *G. truncatulinoides* samples (4/5), and both increased and decreased the D/L values of *G. tumida* samples (Figs. 7 and 8).

Due to the increase in the familywise error rate when carrying out individual t-tests, a three-way analysis of variance (ANOVA) was used to confirm the results for normally distributed data, whereas individual Kruskal-Wallis tests were used for not normally distributed data (Table 3). The ANOVA evaluated three variables that can contribute to differences in the D/L ratios: 1) pre-treatment, 2) planktic foraminifer species, and 3) ODP site (i.e., sample age and water depth). The ANOVA and Kruskal-Wallis results align with those from the t-tests, indicating that the D/L of some Holocene-aged samples are significantly different between the two pre-treatments, whereas the D/L of Pleistocene-aged samples are not significantly different. As a whole, the Kruskal-Wallis tests for samples aged 6.0–410.0 ka show that the pre-treatment may have a larger effect on the D/L of Glx, possibly due to the slower racemization rate of this amino acid. The statistical results also suggest that there are significant differences in the mean D/L arising from the species used (further explored in Section 3.4) and sampling site, which is expected due to varying sample age and water temperature at each site.

3.4. D/L differences between foraminifer species

At the genus level, differences in the rate of amino acid racemization in foraminifera are well documented in the literature (Kaufman, 2006; Kaufman et al., 2013; King and Neville, 1977). Here we evaluate the effect of oxidative pre-treatments on foraminifer D/L values at the species level. We obtained sufficient tests from *P. obliquiloculata*, *G. truncatulinoides,* and *G. tumida* from six mid Holocene to late Pleistocene intervals to evaluate species differences in D/L values (Table 4). However, we were able to obtain enough tests for only one species comparison from both the early Holocene and late Pleistocene sample sets and none from the mid Pleistocene interval.

Akin to the ANOVA and Kruskal-Wallis results (Table 3), the t-tests show that significant differences in the D/L values tend to exist between co-occurring planktic species in the majority of intervals (Table 4). For Asx D/L, 8/10 intervals of both peroxide-treated and additional bleach-treated comparisons show significant differences, although not for the same species pair (Table 4). For Glx D/L, about half of the comparisons show significant differences for the peroxide (5/10 intervals) versus the additional bleach treatments (6/10 intervals). These latter comparisons could illustrate that differences in Glx D/L are less species-specific than Asx D/L in this range, and adding bleach does not change this result appreciatively. Alternatively, the slower racemization rate of Glx could result in smaller D/L differences between different species in this age range.

Focusing on the individual Asx D/L comparisons where species differences are more apparent, there is no consistent change in the species differences with a particular pre-treatment method. The difference in Asx D/L in *P. obliquiloculata* versus *G. truncatulinoides* is significant in 5/6 pairs whether or not bleach is added and is significant in the sixth comparison after bleach is added. The *P. obliquiloculata* versus *G. tumida* comparison, on the other hand, is inconclusive as the addition of bleach results in a significant difference in one of the two analysis pairs (1056D,

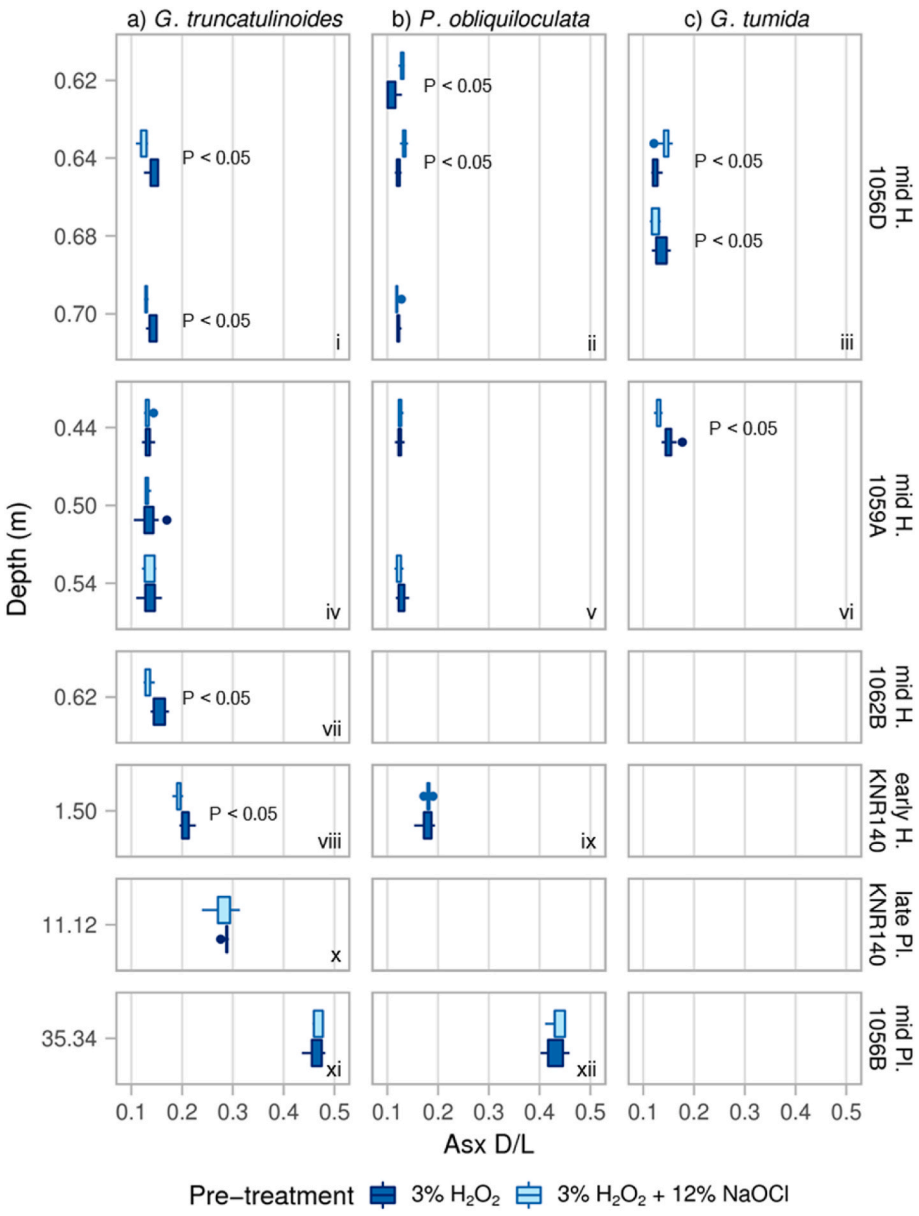

Fig. 7. Asx D/L values from ODP Sites 1056D, 1059A, 1062B, KNR140 JPC-37, and 1056B comparing the effects of bleaching on sample mean and standard deviation within a) *G.* truncatulinoides, b) P. *obliquiloculata*, and c) *G. tumida*. Samples are dated mid Holocene (H) to mid Pleistocene (Pl). The box is plotted from the first quartile to the third quartile with whiskers extending the full range and circular data points representing subsample values outside of the range by > 1.5 times the interquartile range. Pre-treatment comparisons with statistically significant differences are noted with P-values < 0.05.

0.64 m) while it removes significance in the other example (1059A, 0.44 m). The difference in Asx D/L between *G. truncatulinoides* and *G. tumida* is significant for both examples when treated with peroxide only, but adding bleach appears to diminish the difference in one (1056D, 0.64 m versus 1059A, 0.44 m, respectively). These data suggest that adding bleach does not better define existing species differences in Asx D/L with respect to samples treated with peroxide only.

Samples from the BBOR also allow an examination of species-specific differences in racemization rates. We visualize these by regressing the D/L values of the same amino acid from two species against one another (Fig. 9). For this purpose, we focus on results from *P. obliquiloculata* and *G. truncatulinoides* because they are present at three age horizons (mid Holocene, early Holocene and late Pleistocene) providing a common temporal reference frame. In this analysis, a slope of 1 reflects identical observed racemization rates (i.e., same D/L values for a given sample horizon), while deviations from a slope of 1 indicate slower or faster racemization rates, resulting in lower or higher D/L values, respectively, for a given time. *Globorotalia truncatulinoides* racemizes faster than *P. obliquiloculata* by 5% for Asx and 13% for Glx if pre-treated with peroxide only and 9% for Asx and 6% for Glx if pre-treated with peroxide and bleach (Fig. 9). The similarity of the slopes suggests that the choice of pre-treatment has little effect on species-specific racemization rates for Asx (Fig. 9a). However, bleach reduces the apparent species effect in samples with higher Glx D/L values (i.e., older tests), resulting in a decrease in the apparent racemization rate of *G. truncatulinoides* compared to *P. obliquiloculata* (Fig. 9b).

3.5. D/L variability within planktic foraminifera

The subsamples for each interval and species in the planktic foraminifer study also afford a closer look at the effects of bleach on the replicability of sample mean D/L values. To describe this subsample variability and normalize it to the mean values, we use a coefficient of variation (CV). To quantify the average change (e.g., ΔCV, Table 5) in the subsample variability between the pre-treatments for Asx and Glx, we subtract the CV for samples treated with H_2O_2-only from the CV for samples also treated with NaOCl. Accordingly, a decrease in the subsample variability is described by a decrease in the CV and indicates an improvement in replicability due to the additional bleaching pre-treatment. However, it is important to note that these statistical

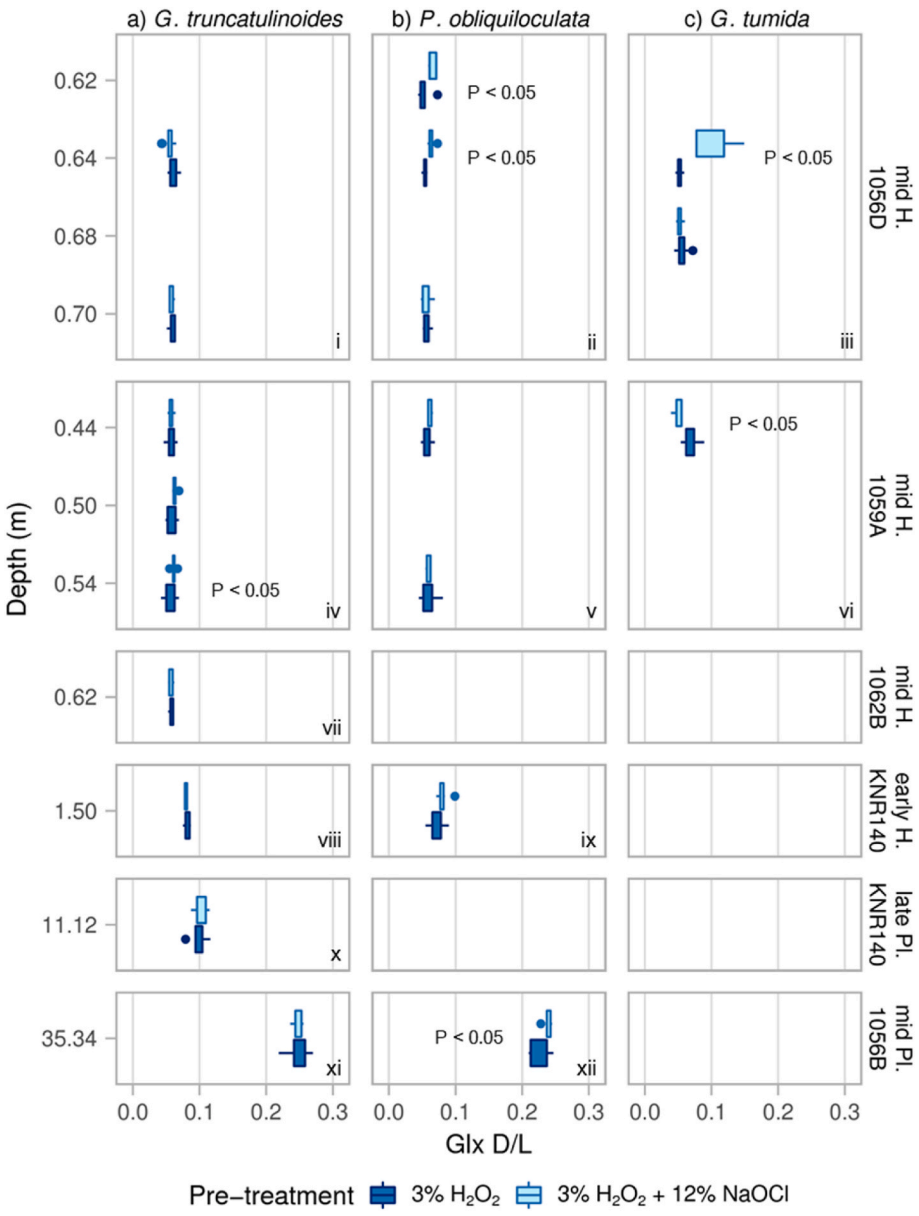

Fig. 8. Glx D/L values from ODP Sites 1056D, 1059A, 1062B, KNR140 JPC-37, and 1056B comparing the effects of bleaching on sample mean and standard deviation within a) *G.* truncatulinoides, b) P. *obliquiloculata*, and c) *G. tumida*. Samples are dated mid Holocene (H) to mid Pleistocene (Pl). The box is plotted from the first quartile to the third quartile with whiskers extending the full range and circular data points representing subsample values outside of the range by > 1.5 times the interquartile range. Pre-treatment comparisons with statistically significant differences are noted with P-values < 0.05.

analyses are limited. While there are 14 mid Holocene aged samples for comparison, there are only 2 early Holocene, 1 late Pleistocene, and 2 mid Pleistocene-aged samples for comparison. We must therefore be careful when interpreting the statistics for the older samples (n = 5).

Overall, the results show that bleaching can reduce the subsample variability, although not consistently. For example, in 14 out of the 19 samples analyzed (74%), the mean D/L for Asx and Glx show an improved CV with the bleaching method (negative values in Table 5, Fig. 10). However, the improvement for Asx is small, on average −3.4% ± 3.1%, with most samples falling within ±0.5% of the 1:1 regression line, suggesting that there is minimal difference between the CV of the two treatments. More specifically, we note that mid Holocene-aged samples, for which we have the largest amount of data (n = 14), show the largest variations in CV with the additon of bleach in comparison to the older samples (Table 5; Fig. 10). Less than half of these samples show noticeable reductions in their CV with bleaching (ΔCV < −4%; Fig. 10). For Asx, the late Pleistocene-aged sample shows a notable increase in its CV after bleaching (ΔCV = 6%), while the early Holocene and mid Pleistocene-aged samples are on or to the right of the 1:1 line (ΔCV = 0 to −4.6%). For Glx, all samples older than mid Holocene fall to the right of the 1:1 line (ΔCV = −2.8 to −5.6%), suggesting that bleach may more consistently reduce the inter-sample variability of Glx.

4. Discussion

This study shows the effects of oxidative pre-treatments on biomineral amino acids within foraminiferal tests by combining the results of two studies: 1) sequential oxidation experiments involving peroxide versus bleach conducted on sub-modern benthic *Ammonia* spp. and *H. germanica*, and 2) experiments testing the effects of bleach on peroxide pre-treated mid Holocene through mid Pleistocene planktic species (*P. obliquiloculata*, *G. truncatulinoides*, and *G. tumida*). Our integrated results suggest that bleach more effectively reduces sample [THAA] in comparison to sonication-only treatments (Fig. 2), while peroxide moderately reduces [THAA] (Figs. 2 and 3). These results demonstrate that bleach pre-treatment is effective in removing exogenous and/or inter-crystalline proteins in accordance with previous studies (e.g., Crisp et al., 2013; Demarchi et al., 2013a, 2013b; Hendy et al., 2012; Ortiz et al., 2015, 2018; Penkman et al., 2008, 2011). This is important because contamination by surface amino acids is of particular

Table 2
Mean D/L values for planktic foraminifera from the BBOR and P-values for statistical significance between the pre-treatments.

Site	Sediment Depth (m)	[a]Species	Asx D/L			Glx D/L		
			H_2O_2	[b]NaOCl	[c]P-value	H_2O_2	[b]NaOCl	[c]P-value
Mid Holocene (6.0-7.1 ka)								
1056D	0.62	*P. obliq*	0.110	0.129	0.0093*	0.053	0.064	0.0434*
1056D	0.64	*P. obliq*	0.121	0.134	0.0002*	0.054	0.063	0.0005*
1056D	0.70	*P. obliq*	0.122	0.120	0.3131	0.057	0.056	0.9175
1056D	0.64	*G. trun*	0.143	0.123	0.0004*	0.060	0.054	0.0715
1056D	0.70	*G. trun*	0.143	0.129	0.0014*	0.060	0.058	0.4209
1056D	0.64	*G. tum*	0.124	0.143	0.0004*	0.052	0.100	0.0002*
1056D	0.68	*G. tum*	0.136	0.124	0.0334*	0.057	0.052	0.2189
1059A	0.44	*P. obliq*	0.124	0.126	0.5682	0.057	0.062	0.1603
1059A	0.54	*P. obliq*	0.129	0.123	0.1310	0.060	0.060	0.9817
1059A	0.44	*G. trun*	0.134	0.132	0.4346	0.057	0.057	0.9568
1059A	0.50	*G. trun*	0.135	0.132	0.6323	0.058	0.063	0.0983
1059A	0.54	*G. trun*	0.136	0.136	0.9809	0.056	0.061	0.0057*
1059A	0.44	*G. tum*	0.152	0.130	0.0009*	0.069	0.051	0.0007*
1062B	0.62	*G. trun*	0.154	0.133	0.0134*	0.058	0.057	0.7570
Early Holocene (10.5 ka)								
KNR140	1.50	*P. obliq*	0.178	0.181	0.6082	0.072	0.081	0.1233
KNR140	1.50	*G. trun*	0.207	0.193	0.0042*	0.081	0.080	0.3287
Late Pleistocene (51.5 ka)								
KNR140	11.12	*G. trun*	0.286	0.280	0.4878	0.098	0.101	0.6806
Mid Pleistocene (410.0 ka)								
1056B	35.34	*P. obliq*	0.432	0.437	0.6963	0.225	0.239	0.0386*
1056B	35.34	*G. trun*	0.463	0.469	0.4254	0.249	0.247	0.8414

[a] *P. obliq* = *Pulleniatina obliquiloculata*, *G. trun* = *Globorotalia truncatulinoides*, *G. tum* = *Globorotalia tumida*.
[b] NaOCl stands for samples treated with 3% H_2O_2 plus 12% NaOCl.
[c] P-values from independent t-tests for the difference of means. Samples with $P < 0.05$ (asterisk) show a significant difference in the mean D/L of the treatments.

Table 3
P-values from three-way ANOVA models used to determine whether the pre-treatments, planktic foraminifera species, and ODP sites (i.e., water depth) significantly impacted the mean D/L of Asx and Glx.

Sample Age (ka)	Asx P-values[a]			Glx P-values[a]		
	Treatment	Species	Site	Treatment	Species	Site
6.0–10.5[b]	0.0096*	0.0000*	0.0000*	0.0081*	0.3296	0.0000*
51.5–410.0	0.7753	0.0044*	0.0000*	0.2647	0.0004*	0.0000*
6.0–410.0[b]	0.1868	0.0000*	0.0000*	0.0260*	0.0219*	0.000*

[a] $P < 0.05$ (asterisk) show a significant difference in the mean D/L between groups.
[b] The Shapiro-Wilks test for normality showed that the Glx data for samples aged 6.0–10.5 ka and the whole dataset for samples aged 6.0–410.0 ka were non-normally distributed. Therefore, individual Kruskal-Wallis tests were used.

Table 4
Results of Welch's independent t-tests to evaluate species differences in the D/L values of Asx and Glx among two pre-treatments (3% H_2O_2 only versus 3% H_2O_2 plus 12% NaOCl).

Site	Sediment Depth (m)	Species Comparison[a]	Asx P-values[b]		Glx P-values[b]	
			H_2O_2	NaOCl[c]	H_2O_2	NaOCl[c]
Mid Holocene (6.0-7.1 ka)						
1056D	0.64	*P. obliq* vs *G. trun*	0.0001*	0.0068*	0.0298*	0.0058*
1056D	0.64	*P. obliq* vs *G. tum*	0.3591	0.0348*	0.2565	0.0014*
1056D	0.64	*G. trun* vs *G. tum*	0.0005*	0.0004*	0.0057*	0.0002*
1056D	0.70	*P. obliq* vs *G. trun*	0.0000*	0.0014*	0.2992	0.6660
1059A	0.44	*P. obliq* vs *G. trun*	0.0009*	0.0081*	0.9325	0.0208*
1059A	0.44	*P. obliq* vs *G. tum*	0.0001*	0.1150	0.0180*	0.0026*
1059A	0.44	*G. trun* vs *G. tum*	0.0028*	0.4641	0.0112*	0.0301*
1059A	0.54	*P. obliq* vs *G. trun*	0.1224	0.0024*	0.2867	0.3451
Early Holocene (10.5 ka)						
KNR140	1.50	*P. obliq* vs *G. trun*	0.0003*	0.0022*	0.0692	0.7268
Late Pleistocene (51.5 ka)						
1056B	35.34	*P. obliq* vs *G. trun*	0.0017*	0.0336*	0.0050*	0.1108

[a] P. obliq = Pulleniatina obliquiloculata, G. trun = Globorotalia truncatulinoides, G. tum = Globorotalia tumida.
[b] P-values from independent t-tests for the difference of means. Samples with $P < 0.05$ (asterisk) show a significant difference in the mean D/L between species.
[c] NaOCl stands for samples treated first with H_2O_2 and additionally with NaOCl.

concern in foraminifera due to the high surface area-to-mass ratio related to test porosity (Hearty et al., 2004; Stathoplos and Hare, 1993).

While oxidative pre-treatments reduce [THAA] of foraminifera (Figs. 2 and 3), neither hydrogen peroxide nor bleach substantially affect the proportion of individual amino acids in a sample (Figs. 4 and 5), with the exception of *Ammonia* spp. For this benthic species, increasing the

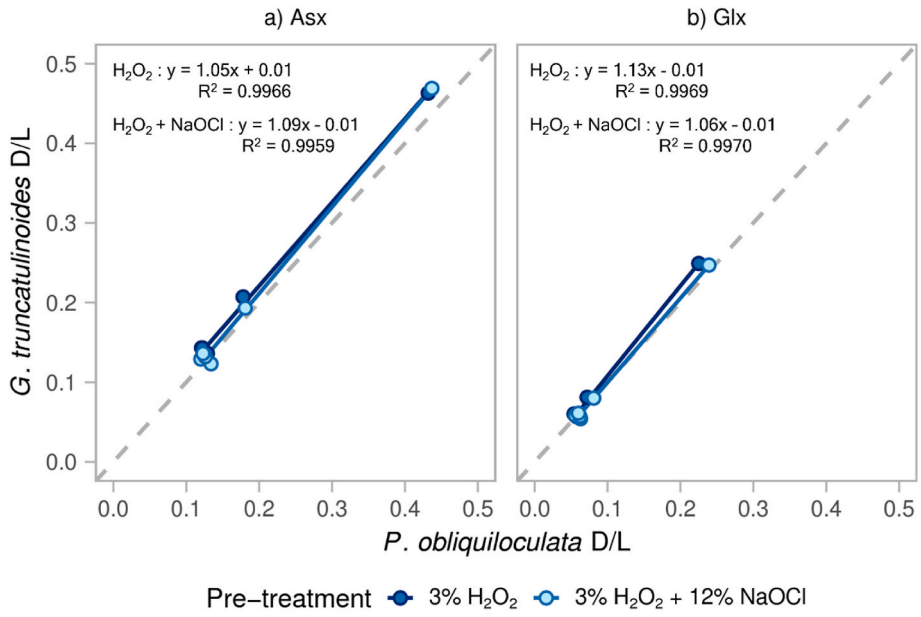

Fig. 9. Extent of racemization (D/L) in a) Asx and b) Glx measured in six coeval intervals of *P. obliquiloculata* and *G. truncatulinoides* (Table 2). The slope indicates the relative racemization rates for the species where i) slope = 1 indicates that both species racemize at the same rate, ii) slope >1 indicates that *G. truncatulinoides* racemizes faster than *P. obliquiloculata*, and iii) slope <1 indicates that *P. obliquiloculata* racemizes faster than *G. truncatulinoides*.

Table 5
Average intra-sample variability in D/L values as described by the coefficient of variation (CV) compared between the 3% H_2O_2-only and 12% NaOCl-addition pre-treatments of planktic foraminiferal tests.

Site	Sediment Depth (m)	Species[a]	Asx CV (%)			Glx CV (%)		
			H_2O_2	NaOCl[b]	ΔCV[c]	H_2O_2	NaOCl[b]	ΔCV[c]
Mid Holocene (6.0-7.1 ka)								
1056D	0.62	*P. obliq*	10.0	3.0	−7.0	20.0	8.0	−12.0
1056D	0.64	*P. obliq*	3.7	3.9	0.2*	5.5	7.7	2.2*
1056D	0.70	*P. obliq*	2.7	3.3	0.6*	9.7	13.8	4.8*
1056D	0.64	*G. trun*	7.9	6.7	−1.2	11.0	13.1	2.1*
1056D	0.70	*G. trun*	6.1	2.3	−3.8	8.2	5.9	−2.3
1056D	0.64	*G. tum*	5.6	7.8	2.2*	6.9	25.7	18.8*
1056D	0.68	*G. tum*	9.5	6.6	−2.9	16.6	7.8	−8.8
1059A	0.44	*P. obliq*	4.5	3.2	−1.3	12.3	5.1	−7.2
1059A	0.54	*P. obliq*	6.7	5.1	−1.6	17.9	5.6	−12.3
1059A	0.44	*G. trun*	5.1	4.6	−0.5	10.1	6.3	−3.8
1059A	0.50	*G. trun*	15.1	2.9	−12.2	13.0	4.8	−8.2
1059A	0.54	*G. trun*	10.2	7.3	−2.9	13.4	4.4	−9.0
1059A	0.44	*G. tum*	8.5	4.6	−3.9	15.3	12.4	−2.9
1062B	0.62	*G. trun*	8.8	6.9	−1.9	5.5	7.2	1.7*
Early Holocene (10.5 ka)								
KNR140	1.50	*P. obliq*	7.6	3.0	−4.6	16.6	11.0	−5.6
KNR140	1.50	*G. trun*	5.4	3.3	−2.1	6.9	2.7	−4.2
Late Pleistocene (51.5 ka)								
KNR140	11.12	*G. trun*	2.1	8.1	6.0*	13.8	10.1	−2.8
Mid Pleistocene (410.0 ka)								
1056B	35.34	*P. obliq*	4.2	4.2	0.0	6.1	3.3	−2.8
1056B	35.34	*G. trun*	3.4	2.0	−1.4	6.2	3.0	−3.2

[a] *P. obliq* = *Pulleniatina obliquiloculata*, *G. trun* = *Globorotalia truncatulinoides*, *G. tum* = *Globorotalia tumida*.
[b] NaOCl stands for samples treated first with H_2O_2 and additionally with NaOCl.
[c] The values with an asterisk show an increase in variance with the NaOCl bleaching pre-treatment where $\Delta CV = CV_{NaOCl} - CV_{H_2O_2}$.

strength of the oxidizing agent causes the proportion of Asx, Glx, and Ala to increase and the proportion of Gly to decrease, most noticeably in the 12% NaOCl treatment. In contrast to *Ammonia* spp., the compositional differences between the pre-treatments for *H. germanica* and the planktic species are less pronounced. This has also been observed in naturally aged *N. pachyderma* (Wheeler et al., 2021), and suggests that in general, the inter- and intra-crystalline fractions of foraminifera have similar amino acid compositions. The different pattern seen in *Ammonia* spp. could be due to the sonication and weak oxidizing treatments being less effective at removing surface organic material for this species than the others investigated, although the variability of the composition data precludes a firm conclusion.

Effective removal of surface contaminants by bleach could also partly explain the overall reduction in the subsample variability in D/L values of bleach-treated samples with respect to those treated with peroxide only. Replicate analyses of the BBOR samples suggests that

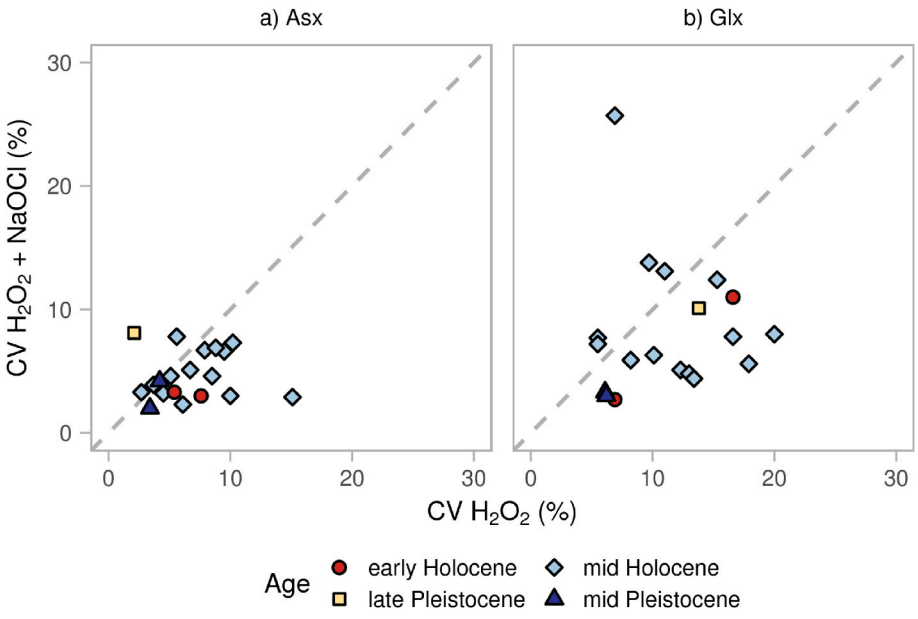

Fig. 10. Relationship between the coefficient of variation (CV) pertaining to the two pre-treatment methods in planktic foraminifera for a) Asx and b) Glx. The diagonal dashed line indicates the 1:1 relationship on which the points should fall if additional treatment with bleach does not produce a change in the CV. Points to the left of the line indicate that additional bleaching increases the CV with respect to peroxide treatment only; points to the right of the line indicate that the CV is lower after bleaching with respect to peroxide treatment only.

bleaching tends to reduce the variability within samples, albeit with differences in the extent depending on the amino acid. For Asx D/L, the reduction in CV is minimal overall, not very consistent, and not apparent in older samples. For Glx D/L, bleaching more effectively reduces the CV at all ages, although the difference in variability between bleached and peroxide-only samples is inconsistent. This slight reduction in D/L variability in bleached samples, especially younger samples, is consistent with bleaching experiments carried out on *N.pachyderma* (sinistral) (Wheeler et al., 2021). Although the bleach pre-treatment appears to isolate the intra-crystalline fraction of foraminiferal amino acids, the small reduction in subsample variability does not warrant the additional preparation time and larger sample size that the bleach pre-treatment requires. Instead, the hydrogen peroxide pre-treatment is sufficient at removing surface contaminants from foraminiferal tests, resulting in D/L values that are not statistically different between the pre-treatments.

Neither set of experiments demonstrates a consistent effect of oxidation on Asx and Glx D/L values that warrant extensive chemical pre-treatment. For the sub-modern benthic foraminifer species, there is no observable difference in D/L values between tests that were H_2O sonicated, peroxide-treated, or bleach-treated (Fig. 6). Bleaching does cause statistically significant differences in the D/L values of mid Holocene-aged planktic foraminifera tests, although the differences are small and inconsistent in their direction and magnitude (Table 2; Figs. 7 and 8). In early Holocene through mid Pleistocene-aged samples, additional bleaching of peroxide-treated foraminifer tests does not consistently affect the overall D/L values. These irregularities suggest that most of the surface contaminants are removed before the oxidizing pre-treatments are applied, an interpretation that is supported by the observation that neither pre-treatment method consistently affects the D/L values of a sample.

Species-specific differences in D/L values are evident in several samples. By comparing down-core changes in D/L for coeval species, we can see how the pre-treatments affect apparent species-specific racemization rates (Fig. 9). The down-core changes in D/L values for *G. truncatulinoides* and *P. obliquiloculata* are similar, but not the same, suggesting small differences in racemization rates. The results show that for Asx, *G. truncatulinoides* has a higher racemization rate than *P. obliquiloculata*, and the pre-treatment has minimal effect on the rate (e.g., *G. truncatulinoides* racemizes 5% and 9% faster than *P. obliquiloculata* for H_2O_2 and NaOCl treated samples, respectively). Conversely, the bleach pre-treatment reduces the species differences for Glx D/L, where *G. truncatulinoides* racemizes 13% and 6% faster than *P. obliquiloculata* for H_2O_2 and bleach treated samples, respectively.

The bleach pre-treatment does not affect the overall trend of planktic foraminiferal D/L values over time (Fig. 11), meaning D/L increases with age, reflecting the increased proportion of D-Asx and D-Glx due to the increased extent of racemization in older fossils. While the overall trend is consistent, the pre-treatments induce a relatively small effect on the D/L values of Glx in *G. truncatulinoides* (Fig. 11a), but not in *P. obiquiloculata* (Fig. 11b). Thus, in this study, bleaching of foraminifer tests does not improve the replicability of D/L values of planktic foraminiferal species. While analyzing only the bleach-isolated intra-crystalline fraction of amino acids can reduce the variability of D/L values in some biominerals, this study suggests that this is not the case for foraminifera. This behavior is also seen in ostracod valves (Bright and Kaufman, 2011) and some aragonitic mollusks (e.g., Orem and Kaufman, 2011; Demarchi et al., 2015; Ortiz et al., 2017).

The most likely explanation for why the older (mid Holocene through mid Pleistocene) foraminiferal tests do not show differences in D/L values between the pre-treatments could be because some of the more easily accessed free amino acids and matrix proteins from the inter-crystalline fraction have already been removed over time, possibly due to leaching (diffusive loss) and/or microbial influences (Demarchi and Collins, 2015; Ortiz et al., 2015, 2017; Penkman et al., 2008). Therefore, the amino acids remaining in these older samples would be likely dominated by the intra-crystalline fraction, which is resistant to exposure to chemical oxidation (Penkman et al., 2008; Stathoplos and Hare, 1993; Wheeler et al., 2021). For peroxide treated mid Holocene-aged samples (~6 ka), a larger proportion of inter-crystalline proteins would remain due to the younger age of these samples.

5. Conclusions

If foraminifer tests contain a closed-system intra-crystalline protein fraction, bleaching should isolate this fraction and reduce post-depositional environmental influences on the preservation of amino acids (i.e., contamination by exogenous amino acids, microbial decomposition, and leaching), thus improving the analytical variability (Penkman et al., 2008). We evaluate the effects of oxidative pre-treatments on benthic and planktic foraminiferal species through sequential oxidation experiments on sub-modern benthic foraminifer tests (0–5 cm sample depth) and through comparative oxidation experiments using peroxide and peroxide plus bleach on three species of

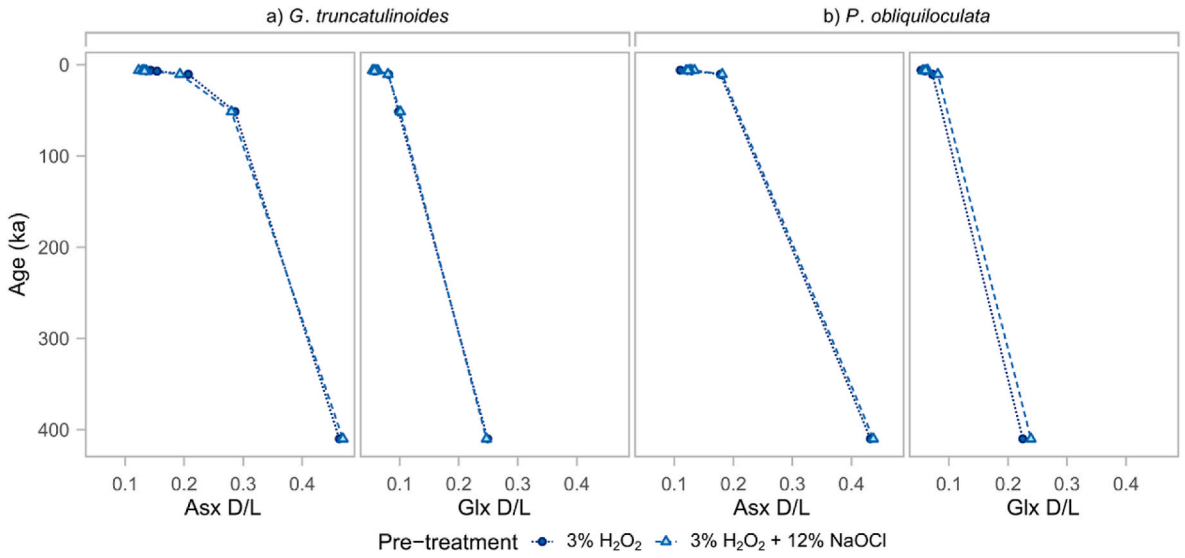

Fig. 11. Early Holocene through mid Pleistocene (10.5–410.0 ka) changes in a) *G. truncatulinoides* b) and *P. obliquiloculata* D/L Asx (and D/L Glx in c and d for the two species respectively) for peroxide treated (dark blue circles) and bleached (light blue triangles).

planktic foraminiferal tests (~6–410 ka). We investigate how bleach pre-treatment affects the variability in foraminiferal D/L values in three foraminiferal species of different ages. The results indicate that bleaching for 24 h reduces amino acid concentrations but does not completely remove amino acids within the foraminifer tests. This decrease in amino acid concentration reflects the removal of exogenous and/or easily accessed matrix proteins and the subsequent isolation of bleach resistant intra-crystalline amino acids.

The effective removal of surface contaminants and labile inter-crystalline matrix proteins by bleach can explain the overall reduction in subsample variability and change in some D/L values observed in our mid Holocene foraminifer tests. The older (i.e., early Holocene to mid Pleistocene) foraminiferal tests do not show differences in D/L values across the various pre-treatments and the reduction in subsample variability in D/L values is less apparent, especially for Asx. This could be due to the removal of easily accessed free amino acids and matrix proteins over time due to leaching and/or microbial influences, leaving behind a greater proportion of intra-crystalline amino acids.

In conclusion, due to the lack of consistent improvement in foraminiferal D/L variability between oxidation by hydrogen peroxide and bleach, the short oxidation procedure using hydrogen peroxide appears to be sufficient for AAR studies involving foraminifera. Analyzing the bleach-resistant fraction of amino acids requires a larger sample size and additional preparation time and does not yield results that are substantially better than results derived from the less time-intensive peroxide pre-treatment. This study shows that the inconsistent reduction in variability produced by the IcPD bleach pre-treatment does not outweigh the additional analyst time and larger sample size required, and therefore the short (2 h) 3% H_2O_2 oxidation protocol is recommended.

Data availability

All data will be published in the NOAA data archive.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.quageo.2022.101401.

References

Bada, J.L., Schroeder, R.A., 1975. Amino acid racemization reactions and their geochemical implications. Naturwissenchaften 62, 71–79. https://doi.org/10.1007/BF00592179.

Billups, K., Chaisson, W., Worsnopp, M., Thunell, R., 2004. Millennial-scale fluctuations in subtropical northwestern Atlantic surface ocean hydrography during the mid-Pleistocene. Paleoceanography 19, PA2017. https://doi.org/10.1029/2003PA000990.

Blakemore, A.G., 2014. Middle Pleistocene to Holocene Sea-Level Changes and Coastal Evolution on the Mount Gambier Coastal Plain, Southern Australia (PhD Thesis). University of Wollongong.

Bright, J., Kaufman, D.S., 2011. Amino acid racemization in lacustrine ostracodes, part I: effect of oxidizing pre-treatments on amino acid composition. Quat. Geochronol. 6, 154–173. https://doi.org/10.1016/j.quageo.2010.11.006.

Bush, S.L., Santos, G.M., Xu, X., Southon, J.R., Thiagarajan, N., Hines, S.K., Adkins, J.F., 2013. Simple, rapid, and cost effective: a screening method for ^{14}C analysis of small carbonate samples. Radiocarbon 55, 631–640. https://doi.org/10.1017/S0033822200057787.

Crisp, M., Demarchi, B., Collins, M., Morgan-Williams, M., Pilgrim, E., Penkman, K., 2013. Isolation of the intra-crystalline proteins and kinetic studies in *Struthio camelus*

(ostrich) eggshell for amino acid geochronology. Quat. Geochronol. 16, 110–128. https://doi.org/10.1016/j.quageo.2012.09.002.
Demarchi, B., Clements, E., Coltorti, M., van de Locht, R., Kröger, R., Penkman, K., Rose, J., 2015. Testing the effect of bleaching on the bivalve *Glycymeris*: a case study of amino acid geochronology on key Mediterranean raised beach deposits. Quat. Geochronol. 25, 49–65. https://doi.org/10.1016/j.quageo.2014.09.003.
Demarchi, B., Collins, M., 2015. Amino acid racemization dating. In: Encyclopedia of Scientific Dating Methods, pp. 13–26. Encyclopedia of Earth Sciences Series.
Demarchi, B., Collins, M.J., Tomiak, P.J., Davies, B.J., Penkman, K.E.H., 2013a. Intra-crystalline protein diagenesis (IcPD) in *Patella vulgata*. Part II: breakdown and temperature sensitivity. Quat. Geochronol. 16, 158–172. https://doi.org/10.1016/j.quageo.2012.08.001.
Demarchi, B., Rogers, K., Fa, D.A., Finlayson, C.J., Milner, N., Penkman, K.E.H., 2013b. Intra-crystalline protein diagenesis (IcPD) in *Patella vulgata*. Part I: isolation and testing of the closed system. Quat. Geochronol. 16, 144–157. https://doi.org/10.1016/j.quageo.2012.03.016.
Dickinson, M.R., Lister, A.M., Penkman, K.E.H., 2019. A new method for enamel amino acid racemization dating: a closed system approach. Quat. Geochronol. 50, 29–46. https://doi.org/10.1016/j.quageo.2018.11.005.
Dowling, L.A., Sejrup, H.P., Coxon, P., Heijnis, H., 1998. Palynology, aminostratigraphy and U-series dating of marine gortian interglacial sediments in cork harbour, Southern Ireland. Quat. Sci. Rev. 17, 945–962. https://doi.org/10.1016/S0277-3791(98)00027-4.
Dungworth, G., 1975. Optical configuration and the racemization of amino acids in sediments and in fossils – a review. Chem. Geol. 17, 135–153. https://doi.org/10.1016/0009-2541(76)90027-9.
Figueira, B., Hayward, B.W., 2014. Impact of reworked foraminifera from an eroding salt marsh on sea-level studies, New Zealand. N. Z. J. Geol. Geophys. 57, 378–389. https://doi.org/10.1080/00288306.2014.924971.
Goodfriend, G.A., 1989. Complementary use of amino-acid epimerization and radiocarbon analysis for dating of mixed-age fossil assemblages. Radiocarbon 31, 1041–1047. https://doi.org/10.1017/S0033822200012698.
Goodfriend, D.A., Brigham-Grette, J., Miller, G.H., 1996. Enhanced age resolution of the marine Quaternary record in the Arctic using aspartic acid racemization dating of bivalve shells. Quat. Res. 45, 176–187. https://doi.org/10.1006/qres.1996.0018.
Grützner, J., Giosan, L., Franz, S.O., Tiedemann, R., Cortijo, E., Chaisson, W.P., Flood, R. D., Hagen, S., Keigwin, L.D., Poli, S., Rio, D., Williams, T., 2002. Astronomical age models for Pleistocene drift sediments from the western North Atlantic (ODP Sites 1055–1063). Mar. Geol. 189, 5–23. https://doi.org/10.1016/S0025-3227(02)00320-1.
Hagen, S., Keigwin, L.D., 2002. Sea-surface temperature variability and deep water reorganisation in the subtropical North Atlantic during Isotope Stage 2–4. Mar. Geol. 189, 145–162. https://doi.org/10.1016/S0025-3227(02)00327-4.
Hayward, B.W., Holzmann, M., Grenfell, H.R., Pawlowski, J., Triggs, C.M., 2004. Morphological distinction of molecular types in *Ammonia* – towards a taxonomic revision of the world's most commonly misidentified foraminifera. Mar. Micropaleontol. 50, 237–271. https://doi.org/10.1016/S0377-8398(03)00074-4.
Hearty, P.J., O'Leary, M.J., Kaufman, D.S., Page, M.C., Bright, J., 2004. Amino acid geochronology of individual foraminifer (*Pulleniatina obliquiloculata*) tests, north Queensland margin, Australia: a new approach to correlating and dating Quaternary tropical marine sediment cores. Paleoceanography 19, PA4022. https://doi.org/10.1029/2004PA001059.
Hendy, E.J., Tomiak, P.J., Collins, M.J., Hellstrom, J., Tudhope, A.W., Lough, J.M., Penkman, K.E.H., 2012. Assessing amino acid racemization variability in coral intra-crystalline protein for geochronological applications. Geochem. Cosmochim. Acta 86, 338–353. https://doi.org/10.1016/j.gca.2012.02.020.
Hemleben, C.H., Spindler, M., Anderson, O.R., 1989. Modern Planktonic Foraminifera. Springer Verlag, p. 363.
Hill, R.L., 1965. Hydrolysis of proteins. In: Advances in Protein Chemistry. Elsevier, pp. 37–107. https://doi.org/10.1016/S0065-3233(08)60388-5.
Kaufman, D.S., 2000. Amino acid racemization in ostracodes. In: Goodfriend, G., Collins, M., Fogel, M., Macko, S., Wehmiller, J. (Eds.), Perspectives in amino acid and protein geochemistry. Oxford University Press, New York, NY, pp. 145–160.
Kaufman, D., 2003. Amino acid paleothermometry of quaternary ostracodes from the Bonneville basin, Utah. Quat. Sci. Rev. 22, 899–914. https://doi.org/10.1016/S0277-3791(03)00006-4.
Kaufman, D., 2006. Temperature sensitivity of aspartic and glutamic acid racemization in the foraminifera *Pulleniatina*. Quat. Geochronol. 1, 188–207. https://doi.org/10.1016/j.quageo.2006.06.008.
Kaufman, D.S., Polyak, L., Adler, R., Channell, J.E.T., Xuan, C., 2008. Dating late Quaternary plaktonic foraminifer *Neogloboquadrina pachyderma* from the Arctic Ocean using amino acid racemization. Paleoceanography 23, PA3224. https://doi.org/10.1029/2008PA001618.
Kaufman, D.S., Cooper, K., Behl, R., Billups, K., Bright, J., Gardner, K., Hearty, P., Jakobsson, M., Mendes, I., O'Leary, M., Polyak, L., Rasmussen, T., Rosa, F., Schmidt, M., 2013. Amino acid racemization in mono-specific foraminifera from Quaternary deep-sea sediments. Quat. Geochronol. 16, 50–61. https://doi.org/10.1016/j.quageo.2012.07.006.
Kaufman, D.S., Manley, W.F., 1998. A new procedure for determining enantiomeric (D/L) amino acid ratios in fossils using reverse phase liquid chromatography. Quat. Sci. Rev. 17, 987–1000. https://doi.org/10.1016/S0277-3791(97)00086-3.
Kaufman, D.S., Miller, G.H., 1992. Overview of amino acid geochronology. Comp. Biochem. Physiol. Part B: Comparative Biochemistry 102, 199–204. https://doi.org/10.1016/0305-0491(92)90110-D.
King, K., Neville, C., 1977. Isoleucine epimerization for dating marine sediments: importance of analyzing monospecific foraminiferal samples. Science 195, 1333–1335. https://doi.org/10.1126/science.195.4284.1333.
Kosnik, M.A., Kaufman, D.S., 2008. Identifying outliers and assessing the accuracy of amino acid racemization measurements for geochronology: II. Data screening. Quat. Geochronol. 3, 328–341. https://doi.org/10.1016/j.quageo.2008.04.001.
Kosnik, M.A., Kaufman, D.S., Hua, Q., 2013. Radiocarbon-calibrated multiple amino acid geochronology of Holocene molluscs from Bramble and Rib Reefs (Great Barrier Reef, Australia). Quat. Geochronol. 16, 73–86. https://doi.org/10.1016/j.quageo.2012.04.024.
Long, A.J., Innes, J.B., Kirby, J.R., Lloyd, J.M., Rutherford, M.M., Shennan, I., Tooley, M. J., 1998. Holocene sea-level change and coastal evolution in the Humber estuary, eastern England: an assessment of rapid coastal change. Holocene 8, 229–247. https://doi.org/10.1191/095968398677984183.
Macko, S.A., Aksu, A.E., 1986. Amino acid epimerization in planktonic foraminifera suggests slow sedimentation rates for Alpha Ridge, Arctic Ocean. Nature 322, 730–732. https://doi.org/10.1038/322730a0.
McCoy, W.D., 1987. The precision of amino acid geochronology and paleothermometry. Quat. Sci. Rev. 6, 43–54. https://doi.org/10.1016/0277-3791(87)90016-3.
Miller, G.H., Hollin, J.T., Andrews, J.T., 1979. Aminostratigraphy of UK Pleistocene deposits. Nature 281, 539–543. https://doi.org/10.1038/281539a0.
Miller, G.H., Kaufman, D.S., Clarke, S.J., 2013. Amino acid dating. In: Encyclopedia of Quaternary Science. Elsevier, pp. 37–48.
Miller, G.H., Mangerud, J., 1985. Aminostratigraphy of European marine interglacial deposits. Quat. Sci. Rev. 4, 215–278. https://doi.org/10.1016/0277-3791(85)90002-2.
Nicholas, W.A., 2012. Aminostratigraphy of Semi-enclosed Basins (PhD Thesis). University of Wollongong.
Orem, C.A., Kaufman, D.S., 2011. Effects of basic pH on amino acid racemization and leaching in freshwater mollusk shell. Quat. Geochronol. 6, 233–245. https://doi.org/10.1016/j.quageo.2010.11.005.
Ortiz, J.E., Gutiérrez-Zugasti, I., Torres, T., González-Morales, M., Sánchez-Palencia, Y., 2015. Protein diagenesis in Patella shells: implications for amino acid racemisation dating. Quat. Geochronol. 27, 105–118. https://doi.org/10.1016/j.quageo.2015.02.008.
Ortiz, J.E., Torres, T., Sánchez-Palencia, Y., Ferrer, M., 2017. Inter- and intra-crystalline protein diagenesis in *Glycymeris* shells: implications for amino acid geochronology. Quat. Geochronol. 41, 37–50. https://doi.org/10.1016/j.quageo.2017.05.007.
Ortiz, J.E., Sánchez-Palencia, Y., Gutiérrez-Zugasti, I., Torres, T., González-Morales, M., 2018. Protein diagenesis in archaeological gastropod shells and the suitability of this material for amino acid racemisation dating: *Phorcus lineatus* (da Costa, 1778). Quat. Geochronol. 46, 16–27. https://doi.org/10.1016/j.quageo.2018.02.002.
Penkman, K.E.H., Kaufman, D.S., Maddy, D., Collins, M.J., 2008. Closed-system behaviour of the intra-crystalline fraction of amino acids in mollusc shells. Quat. Geochronol. 3, 2–25. https://doi.org/10.1016/j.quageo.2007.07.001.
Penkman, K.E.H., Preece, R.C., Bridgland, D.R., Keen, D.H., Meijer, T., Parfitt, S.A., White, T.S., Collins, M.J., 2011. A chronological framework for the British Quaternary based on *Bithynia* opercula. Nature 476, 446–449. https://doi.org/10.1038/nature10305.
Penkman, K.E.H., Preece, R.C., Bridgland, D.R., Keen, D.H., Meijer, T., Parfitt, S.A., White, T.S., Collins, M.J., 2013. An aminostratigraphy for the British Quaternary based on *Bithynia* opercula. Quat. Sci. Rev. 61, 111–134. https://doi.org/10.1016/j.quascirev.2012.10.046.
Scourse, J.D., Austin, W.E.N., Sejrup, H.P., Ansari, M.H., 1999. Foraminiferal isoleucine epimerization determinations from the nar valley Clay, Norfolk, UK: implications for quaternary correlations in the southern north sea basin. Geol. Mag. 136, 543–560. https://doi.org/10.1017/S0016756899002812.
Sejrup, H.P., Rokoengen, K., Miller, G.H., 1984. Isoleucine epimerization in Quaternary benthonic foraminifera from the Norwegian Continental Shelf: a pilot study. Mar. Geol. 56, 227–239. https://doi.org/10.1016/0025-3227(84)90015-X.
Stathoplos, L., Hare, P.E., 1993. Bleach removes labile amino acids from deep sea planktonic foraminiferal shells. J. Foraminifer. Res. 23, 102–107. https://doi.org/10.2113/gsjfr.23.2.102.
Stuiver, M., Polach, H., 1977. Discussion reporting of 14C data. Radiocarbon 19 (3), 355–363. https://doi.org/10.1017/S0033822200003672.
Sykes, G.A., Collins, M.J., Walton, D.I., 1995. The significance of a geochemically isolated intracrystalline organic fraction within biominerals. Org. Geochem. 23, 1059–1065. https://doi.org/10.1016/0146-6380(95)00086-0.
Towe, Kenneth M., 1980. Preserved organic ultrastructure: an unreliable indicator for Paleozoic amino acid biogeochemistry. In: Biogeochemistry of Amino Acids. John Wiley and Sons, pp. 65–74.
Walker, M.J.C., 2005. Quaternary Dating Methods. John Wiley and Sons, pp. 184–195.
Watson, E., 2019. Amino Acid Racemization of Planktonic Foraminifera: Pretreatment Effects and Temperature Reconstruction (Masters Thesis). University of Delaware.
Wehmiller, J.F., 1977. Amino acid studies of the Del Mar, California, midden site: apparent rate constants, ground temperature models, and chronological implications. Earth Planet Sci. Lett. 37, 184–196. https://doi.org/10.1016/0012-821X(77)90163-7.
Wehmiller, J.F., 2013. United States Quaternary coastal sequences and molluscan racemization geochronology – what have they meant for each other over the past 45 years? Quat. Geochronol. 16, 3–20. https://doi.org/10.1016/j.quageo.2012.05.008.
Wehmiller, J.F., Hall, F.R., 1997. Amino acid racemization geochronological studies of selected Leg 155 samples (Scientific Results No. 155). In: Proceedings of the Ocean Drilling Program. Ocean Drilling Program, College Station, TX, pp. 375–378.

Wehmiller, J.F., Hare, P.E., 1971. Racemization of amino acids in marine sediments. Science 173, 907–911. https://doi.org/10.1126/science.173.4000.907.
Wheeler, L.J., Penkman, K.E.H., Sejrup, H.P., 2021. Assessing the intra-crystalline approach to amino acid geochronology of *Neogloboquadrina pachyderma* (sinistral). Quat. Geochronol. 61, 101131 https://doi.org/10.1016/j.quageo.2020.101131.
Williams, K.M., Smith, G.G., 1977. A critical evaluation of the application of amino acid racemization to geochronology and geothermometry. Orig. Life Evol. Biosph. 8, 91–144. https://doi.org/10.1007/BF00927978.