# **Assessing the intra-crystalline approach to amino acid geochronology of *Neogloboquadrina pachyderma* (sinistral)**

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### Highlights:

* Isolation of intra-crystalline proteins in foraminifera
* Aspartic acid, glutamic acid and alanine D/L show good relationship with age
* Bleaching reduces variability of D/L values for younger material
* Conversion of A/I values to D/L values is successful for several amino acids

# Abstract

While amino acid (AA) geochronology has been widely applied to foraminiferal biomineral proteins, there has been limited assessment of the potential of isolating an ‘intra-crystalline’ fraction of proteins to improve the reliability of AA geochronology for foraminifera. In this study, bleaching experiments were carried out on the foraminifer *Neogloboquadrina pachyderma* (sinistral) from an independently dated core from the south eastern Norwegian Sea spanning the last 120 ka. Results show that this species contains a bleach-resistant fraction of biomineral proteins, and that this intra-crystalline fraction may behave as a closed system. Racemisation in both the intra-crystalline and whole-protein fractions was found to systematically increase with time.

Isoleucine epimerisation showed reliable trends with depth when analysed by ion exchange chromatography (IEC); analysis by reverse-phase liquid chromatography (RP-HPLC) resulted in reliable age-D/L relationships for aspartic acid, glutamic acid and alanine.

Isolating the intra-crystalline fraction with a 48 hour bleach treatment reduces the influence of contamination in younger material. Therefore this paper recommends that the intra-crystalline approach is used when analysing *Neogloboquadrina pachyderma* (sinistral) for AA geochronology, especially for younger samples. Material-specific regression equations developed in this study successfully convert IEC A/I to RP-HPLC D/L values for aspartic acid, glutamic acid and alanine, enabling integration of geochronologies developed using different methods for this class of biomineral.

# 1. Introduction

Amino acid (AA) geochronology relies on the retention of biomineral proteins in a closed system for the duration of diagenesis (Brooks et al., 1990; Collins and Riley, 2000). In certain biominerals, the majority of biomineral protein exists as an *inter*-crystalline “mesh” between inorganic crystallites (Towe, 1980; Gries et al., 2009). As this inter-crystalline protein is exposed to the external environment, it is susceptible to leaching, contamination or fluctuating environmental conditions, which can influence the rate of protein diagenesis and confound the interpretation of AA geochronology (Sykes et al., 1995).

These inter-crystalline proteins are vulnerable to oxidation, which can be exploited experimentally by exposure to an oxidising agent such as NaOCl (bleaching) (Sykes et al., 1995; Penkman et al., 2008). Conversely, *intra*-crystalline proteins form part of the crystal matrix (Towe, 1980; Berman et al., 1988; Miller et al., 2000) and therefore may be better protected from external influences than inter-crystalline protein. Any residual protein remaining after oxidation is operationally defined as intra-crystalline (Sykes et al., 1995), although depending on the biomineral structure this fraction may also contain well-protected inter-crystalline protein (e.g. chitin in ostracods (Bright and Kaufman, 2011a)). The adherence of a biomineral’s intra-crystalline protein to a closed system depends on the biomineral’s structure and relationship to the organic fraction. Investigations of a range of biominerals have found that this intra-crystalline protein diagenesis (IcPD) approach provides more reliable chronological information than the whole-shell approach in various mollusc shells (Penkman et al., 2008; Demarchi et al., 2013b; Ortiz et al., 2015, 2019) coral (Hendy et al., 2012; Tomiak et al., 2013), ostrich eggshell (Brooks et al., 1990; Crisp et al., 2013) and enamel (Dickinson et al., 2019), while some other biominerals have shown little or no improvement with the IcPD approach (Penkman et al., 2008; Bright and Kaufman, 2011b; Ortiz et al., 2017).

Foraminifera are single-celled organisms found worldwide mainly in marine and estuarine sediments, with widespread applications in Quaternary palaeo-environmental reconstructions (Boudagher-Fadel, 2015). Due to the abundance of foraminiferal shells (tests) in marine environments, amino acid racemisation of foraminifera has been identified as a technique potentially well suited to dating marine sediments (Sejrup et al., 1984; Kaufman, 2006). AA geochronology was first applied to foraminifera in the early 1970s (Wehmiller and Hare, 1971; Bada and Schroeder, 1972; Bada et al., 1973), and has since been applied to a wide range of species of foraminifera (see Kaufman et al., 2013).

While targeted bleaching treatments have been used to remove suspected contamination during preparation of foraminiferal tests for AAR (Hearty et al., 2004; Nicholas, 2012; Kaufman et al., 2013), in-depth studies of the IcPD approach are limited (Stathoplos and Hare, 1993; Watson, 2019). In this study we compare the whole-protein and IcPD approaches to AAR on *Neogloboquadrina pachyderma* (sinistral) (hereafter Nps), a species of planktonic foraminifer ubiquitous in the polar waters of both hemispheres (Spindler, 1996). Nps has been the subject of detailed investigations of its racemisation properties (Sejrup et al., 1984; Macko and Aksu, 1986; Sejrup and Haugen, 1992; Kaufman et al., 2008, 2013; Adler et al., 2009; Kosnik et al., 2013; West et al., 2019). Early work on Nps used only sonication to clean tests (Sejrup et al., 1984; Macko and Aksu, 1986; Sejrup and Haugen, 1992), while more recent protocols have added a pre-treatment with a weak oxidising agent to remove labile and/or exogenous amino acids (Kaufman et al., 2008, 2013; Adler et al., 2009; West et al., 2019). However the IcPD approach has not yet been fully applied to this species of foraminifer. This study therefore aims to test whether an intra-crystalline protein fraction can be isolated from Nps through bleaching, and whether the IcPD approach provides more a reliable chronology than the whole-shell approach for this biomineral.

Early AA geochronology carried out on Nps focused on isoleucine epimerisation (Sejrup et al., 1984; Macko and Aksu, 1986; Sejrup and Haugen, 1992) as the two epimers, L-Ile and D-Aile, can be separated using ion-exchange liquid chromatography (IEC) without the use of chiral columns or derivatising agents (Schroeder and Bada, 1976). However, the development of reverse-phase liquid chromatographic (RP-HPLC) techniques have enabled the enantiomeric separation of a wider range of amino acids (Kaufman and Manley, 1998; Powell et al., 2013); more recent studies on Nps using RP-HPLC have focused on the racemisation of Asx and Glx (e.g. Kaufman et al., 2008, 2013). For simplicity the term “racemisation” will be used to refer collectively to racemisation or epimerisation of amino acids in this paper, as both involve inter-conversion at the α-C of an amino acid.

Whitacre et al. (2017) developed regression equations to convert isoleucine epimerisation (A/I) results from IEC to equivalent RP-HPLC D/L values for five amino acids (Asx, Glx, Ala, Val, Ile) for bivalve, eggshell, gastropod and whole-rock samples. This enables IEC data to be compared with data obtained by RP-HPLC for these biominerals. Pooled equations were developed for application to biominerals not included in the original study. As both separation techniques were used in the analysis of material in this study, in addition we evaluate the methods developed by Whitacre et al. to extend them to these foraminifer tests.

This study therefore seeks to answer three key research questions: 1. Does Nps have an intra-crystalline fraction of biomineral protein that can be isolated by exposure to NaOCl, and if so, what is the ideal duration of this treatment (Section 3.1)?; 2. Does analysis of the intra-crystalline fraction of amino acids show an improvement in the dating performance of these amino acids, compared with the whole-protein fraction of amino acids (Section 3.2)?; 3. Can A/I values measured by IEC be confidently converted to D/L values for other amino acids for Nps (Section 3.3)?

# 2. Materials and Methods

## 2.1. Location

MD99-2288 and MD99-2289 are two giant piston cores taken from the same location (64°39 N 04°12 E) in the Nyegga region of the Vøring Plateau, in the mid-Norwegian continental margin (Fig. 1), raised during the 1999 IMAGES V cruise (Hjelstuen et al., 2010). The cores are 32.21 m and 23.69 m long respectively. They were taken at a water depth of 1262 m on the northern escarpment of the Storegga Slide, a site of semi-regular submarine landslides taking place over the last 2.5 Ma (Bryn et al., 2005). Sedimentation on the Vøring Plateau is largely controlled by the advance and retreat of the Scandinavian Ice Sheet and its impact on regional ocean currents (Dahlgren and Vorren, 2003). Current bottom-water temperatures in the Norwegian Sea measure approximately -0.5°C to -1°C (Hovland et al., 2005). Palaeotemperature estimates for the Norwegian Sea suggest that bottom-water temperatures have fluctuated by up to 2-4°C between glacial and interglacial periods (Sejrup and Haugen, 1992; Bauch et al., 2000; Dwyer et al., 2000) due to changes in North Atlantic deep water formation in the northern Norwegian Sea (Sarnthein and Altenbach, 1995). In general there is a reduction in racemisation rates in glacial as compared to interglacial periods (e.g. Miller et al., 1997; Penkman et al., 2011; Ortiz et al., 2015), but as the purpose of this study is to compare protein fractions and amino acids between samples with the same diagenetic history, these temperature fluctuations will not impact the interpretation of these results.

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Fig. 1 – Location of MD99-2288 and MD99-2289 IMAGES cores in the Norwegian Sea. Isobath depths in metres; bathymetry data from NOAA.

## 2.2 Age model

MD99-2288 and MD99-2289 were matched by magnetic susceptibility and spliced at 2303.5 cm (MD99-2288)/2270.0 cm (MD99-2289) (Brendryen, 2011); therefore the established chronology for MD99-2289 was applied to MD99-2288 with a 33.5 cm correction. Chronological control for the core MD99-2289 is provided by AMS radiocarbon dating between 120 and 1300 cm (Berstad, 2003; Becker et al., 2018), correlation between the NGRIP δ18O record and XRF core scanning results for Ca and Ti/K from 1441 to 1863 cm (Brendryen et al., 2010; Brendryen, personal communication 2019), and one identified tephra zone, the Faroe Marine Ash Zone II, at 1064 cm and 26.69 ± 0.39 ka BP (Nilsen, 2014). An age depth model was constructed for the 120-1863 cm range of the spliced core using the Bayesian age modelling R package “Bacon” (v. 2.3.7) (Blaauw and Christeny, 2011) using the Marine13 calibration curve (Reimer et al., 2013) and a 405 year reservoir correction for the 14C ages as used by Becker et al. (2018). As no uncertainties were provided for the NGRIP tie points, a 1.2 ka uncertainty was applied to the model based on calculated uncertainties for NGRIP chronology (Svensson et al., 2008). While this may result in an underestimation of the age uncertainty in the latter half of the core, it is sufficient to explore the racemisation trends for Nps protein in this study. The output of the model (Fig. 2) shows a dramatic increase in sedimentation rate at ca. 1250 cm ( ~ 30 ka). The radiocarbon ages used and modelled ages for each sampling depth can be found in the supplementary information.

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**Fig. 2** – Bayesian age depth model (Blaauw and Christeny, 2011) for 120-1863 cm of core MD99-2289. Radiocarbon ages are shown in blue; NGRIP tie points and tephra zones are shown in teal. The dark blue line shows the model’s weighted mean age. Dashed lines represent the model’s 95% probability intervals.

## 2.3 Preparation of foraminifera

Foraminifera were collected at regular intervals from the >150 μm fraction of both cores. While records of the core from which each sample was collected have unfortunately been lost, it is assumed for this study that samples between 10-2200 cm were taken from MD99-2289 and samples between 2300-2985 cm were from MD99-2288 (Brendryen, personal communication 2019). A full record of samples taken can be found in the supplementary information. For IEC analysis, 100 to 150 individuals were pooled for each analytical sample, while 30 to 50 individuals were pooled for RP-HPLC. All samples were cleaned by sonication according to Miller et al., (1983).

### 2.3.1. Isolation of the intra-crystalline fraction

To initially determine the stability of the intra-crystalline fraction of Nps biomineral proteins during prolonged oxidation, foraminiferal samples were taken from three depths (250 cm, 500 cm and 1450 cm) for extended bleaching experiments. Oxidation treatments using NaOCl (bleach) were carried out on Nps between 24 hours and 336 hours (1450 cm) or 360 hours (250 cm and 500 cm).

30-50 μL NaOCl (12% w/v) was added to each sample; samples were shaken every 24 hours to aid penetration of the bleach into the biomineral. At the end of the bleaching period the bleach was pipetted off, the sample rinsed six times with ultrapure water and once with HPLC-grade methanol, then air dried prior to demineralisation. Samples analysed by IEC were dried for 15 minutes at 60°C; samples analysed by RP-HPLC were dried for 5-10 minutes at 110°C. This difference in procedure reflects the standard protocols of the different laboratories in which the analyses were carried out: IEC at the University of Bergen and RP-HPLC at the University of York.

### 2.3.2. Analysis of racemisation trends in Nps

Based on the results of the extended oxidation experiments (Sec 2.3.1), a 48 hour NaOCl exposure was chosen to prepare the rest of the “bleached” samples in this study. To assess differences between the diagenesis of the whole-protein and intra-crystalline protein fractions of Nps, unbleached and bleached samples from a range of depths throughout the cores were analysed. Depending on the amount of material available at each depth, 1-3 replicate samples were prepared for each treatment.

Procedural blanks were collected and analysed for each experiment to quantify background levels of amino acid contamination; due to the sample sizes used, corrections for contaminants identified in the blanks were not necessary.

## 2.4 Amino acid analysis

Only the total hydrolysable amino acid (free and bound amino acids; THAA) fraction was analysed to conserve material. Samples were demineralised with 8 μL 7 M HCl, with addition of a further 8 μL HCl if necessary to complete demineralisation. Samples were hydrolysed by heating at 110°C for 22 hours in nitrogen-flushed vials, then dried at 80°C for 15 minutes prior to rehydration for analysis by IEC, or dried overnight under vacuum prior to rehydration for analysis by RP-HPLC.

Separation of the hydrolysed amino acids was carried out either using IEC (at the University of Bergen) or RP-HPLC (at the University of York). During hydrolysis asparagine and glutamine are irreversibly hydrolysed to aspartic acid and glutamic acid respectively; therefore these amino acids are analytically indistinguishable and reported as Asx and Glx (Hill, 1965). The fluorescent derivatising agent *o*-phthaldialdehyde (OPA), used in both methods, does not allow for the detection of proline or hydroxyproline (Miller and Brigham-Grette, 1989), while the OPA derivative of lysine is prone to degradation during separation (Brückner et al., 1994); therefore these amino acids are not reported for either method.

### 2.4.1. Separation by ion exchange liquid chromatography (IEC)

For analysis using IEC, the internal standard norleucine was added with the 7 M HCl used for demineralisation. Samples were rehydrated with 100 μL pH 2 buffer before being separated on an automatic amino acid analyser with post-column OPA derivatisation and fluorometric detection according to the methods of Sejrup and Haugen (1992). Due to the poor separation of Ser and Thr by IEC, these two amino acids are reported together for this technique. The only amino acid for which chiral information is available using IEC is Ile; this is reported as A/I.

### 2.4.2. Separation by reverse-phase high performance liquid chromatography (RP-HPLC)

For analysis using RP-HPLC, samples were rehydrated with 8-10 μL rehydration fluid (2.38 M HCl, 0.171 mM NaN3, 9.97 mM internal standard L-*homo*-arginine). The rehydrated samples underwent pre-column derivatisation with OPA and the chiral thiol *N*-isobutyryl-L-cysteine (IBLC), followed by chiral separation and fluorometric detection following a modified method of Kaufman and Manley (1998) using an Agilent 1100 Series HPLC fitted with a C18 HyperSil BDS column. The D-Arg peak co-eluted with another compound in all RP-HPLC chromatograms; therefore Arg D/L values are not reported and D-Arg has been excluded from total amino acid concentrations as in other studies using RP-HPLC (Powell et al., 2013).

### 2.4.3 Data screening

A range of diagnostic criteria can be used to assess the integrity of AAR data, such as Ser, Thr or Gly abundance (e.g. Miller and Brigham-Grette, 1989; Sejrup and Haugen, 1992; Kaufman, 2000), expected depth or age trajectories (Wehmiller et al., 1976; Kosnik and Kaufman, 2008), covariance between concentrations or D/L values for different amino acids (e.g. Kaufman, 2000; Preece and Penkman, 2005), and the variability of replicate samples (Kosnik and Kaufman, 2008). Cut-offs for sample exclusion are determined empirically, and are therefore potentially somewhat subjective, but it is important that exclusion criteria, once determined, are applied uniformly to the data set to minimise systematic bias (Kaufman, 2006). D/L values lying far above or below the general trend could be indicative of a compromised sample, but as some natural variability due to reworking or intrusion is inevitable (McCarroll, 2002) only extreme outliers were excluded from this study.

The following screening criteria were chosen:

1. High concentrations of Gly (>50%), which indicate contamination resulting from human handling (Sejrup and Haugen, 1992). High Ser and Thr concentrations were also considered, but poor separation of Ser and Thr using IEC precluded using these amino acids as criteria.
2. Deviation of increasing trend of A/I values for IEC, and D/L values of Asx, Glx and Ala (the three amino acids with the best chromatographic separation) for RP-HPLC.

Outliers were identified and excluded from the dataset according to the criteria listed in Table 1. 0 A full list of the samples excluded and the criteria used to exclude each sample is available in the supplementary information.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | IEC | | RP-HPLC | |
| Unbleached (U) or bleached (B) | **U** | **B** | **U** | **B** |
| 1. > 50% Gly | 0 | 0 | 2\* | 2\* |
| 3. Deviation of D/L from depth trend | 5 | 4 | 3 | 3 |
| Total samples analysed | 69 | 75 | 53 | 51 |
| % Removed | **7.2** | **5.1** | **5.7** | **5.9** |

**Table 1** – Screening criteria used to identify outliers and total number of samples excluded by each criterion for each analytical method. \*Only one of two analytical replicates found to have >50% Gly, indicating issues with chromatographic separation rather than contamination.

## 2.5 IEC A/I to RP-HPLC D/L conversion

Whitacre et al. (2017) developed linear regressions to convert IEC A/I data into D/L values for five amino acids (Asx, Glx, Ala, Val, Ile) equivalent to those measured by RP-HPLC. Biomineral-specific equations were found to minimise errors associated with the conversion by accounting for variations in diagenetic patterns and chromatographic separation between biominerals; however “pooled” regressions were also developed for use with sample types not represented in the original study:

As foraminifera were not included in the original study, the pooled regressions were used to convert IEC A/I values into predicted Asx, Glx, Ala, Val D/L and RP-HPLC equivalent Ile A/I values, and compared with the values actually measured by RP-HPLC, in order to test the efficacy of the pooled equations for Nps.

New linear regressions were then carried out on log2-transformed Nps data. Unlike the data used by Whitacre et al. (2017), analyses carried out in this study were not paired, as this comparison had not been the original intention of the study. Therefore average D/L values for each depth and bleaching treatment were used to develop the regressions. This means that the errors associated with the derived conversions are likely to be higher in this study. No significant differences between separate regressions run on bleached and unbleached material were found, so pairs of IEC and RP-HPLC data where the depth and bleaching treatment were the same were combined to increase the sample size for the models. Between 30 and 32 averaged D/L values were used for each regression. Following Whitacre et al. (2017) the following criteria were used to assess the quality of each model: the adjusted R2 value, 1 and 2σ prediction uncertainties, and the Akaike information criterion (AICc), a measure of model quality which penalises for overparameterisation. All statistical analysis was carried out using the statistical programming language “R” version 3.6.2. Analytical scripts are included as supplementary information.

# 3. Results and discussion

## 3.1 Testing oxidation times

### 3.1.1. The effect of bleaching on amino acid concentration

If Nps biomineral contains an intra-crystalline protein fraction resistant to oxidation, then bleaching via exposure to NaOCl should cause an initial decrease in the total hydrolysable amino acid concentration ([THAA]), followed by a plateau once the intra-crystalline fraction has been isolated.

The presence of amino acids after the longest bleaching duration shows that Nps contains a bleach-resistant intra-crystalline protein fraction (Fig. 3), as has been observed in other species of foraminifera (Watson, 2019). While the variability of [THAA] in the extended Nps bleaching experiments is high, the similarity between unbleached and bleached [THAA] suggests that the majority of the inter-crystalline protein fraction has been lost by 18 ka, as has been observed in other biominerals (Sykes et al., 1995; Penkman et al., 2008; Ortiz et al., 2015, 2017).

The samples with much higher than average [THAA] (>0.9 nmol / foram) at 250 cm (and seen in some unbleached samples at other depths, see Section 3.2.1) may be due to some between-shell variability in the survival of inter-crystalline protein (as seen in gastropods (Penkman et al., 2008), although given that each sample in this study consists of 100 individual tests this interpretation is unlikely. An alternate explanation may be due to modern laboratory contamination; however if this was the case these samples would have a correspondingly low A/I value, which is not seen (Fig. 5). This variability is therefore most likely due to the challenges in accurately measuring amino acid concentration, especially at very small sample sizes (Powell, 2012).

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**Fig. 3** – Trends in concentration of total hydrolysable amino acids ([THAA]) with increasing bleach time for Nps at three core depths: a) 250 cm, b) 500 cm and c) 1450 cm. Dots show individual replicates; bars show means of all replicates at each time and depth point. Where data are absent, this is because experiments of this duration were not carried out.

### 3.1.2. The effect of bleaching on amino acid composition

The biomineral protein of Npsis dominated by Asx and Gly, with smaller contributions from Ser + Thr, Ala, Glx, Val, Leu and Ile (Fig. 4). Phe, His, Arg and Tyr were not resolved using IEC, but observed in small amounts (total contribution from all four amino acids 11 ± 3 %) in samples analysed by RP-HPLC (see supplementary information). This composition is consistent with other foraminifera (King and Hare, 1972; Weiner and Erez, 1984; Robbins and Brew, 1990). The high abundance of Asx indicates the presence of acidic proteins involved in biomineral formation (Weiner, 1979), while Gly and Ala likely represent silk-like proteins found in other biominerals (Pereira-Mouriès et al., 2002; Gotliv et al., 2003).

Differences in the composition of amino acids between the whole-shell and intra-crystalline fractions have been found in some mollusc shells (Penkman et al., 2008; Demarchi et al., 2013b; Ortiz et al., 2015; Pierini et al., 2016), indicating the presence of different proteins in different fractions of those biominerals. Other biominerals do not have significant differences between the two fractions (e.g. ostracods (Ortiz et al., 2013), ostrich eggshell (Crisp et al., 2013)). The composition of amino acids is quite variable for Nps, especially for Ser + Thr and Gly, confounding a clear assessment of the effect of bleaching on composition, with no clear patterns emerging from the extended bleaching treatment (Fig. 4). The apparent stability of both the concentration and composition of the intra-crystalline amino acid pool over extended exposure to bleach suggests that this fraction is oxidation resistant.

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**Fig. 4** – The effect of increasing exposure to bleach on total hydrolysable amino acid (THAA) composition of Nps tests at three core depths (analysed using IEC). While there is some variability in the THAA composition in the first 48 hours of bleach exposure, no clear compositional changes are caused by oxidation. Due to poor separation of Ser from Thr using IEC, these amino acids are reported together. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.1.3. The effect of bleaching on A/I values

Differences in A/I or D/L values between the inter- and intra-crystalline fractions of biominerals may be due to the loss of more highly racemised short-chain peptides and free amino acids from an open-system inter-crystalline fraction (Penkman et al., 2008). Free amino acids in a biomineral are more highly racemised than peptide-bound amino acids due to the accelerated rate of racemisation at the terminal positions of peptides (Crisp et al., 2013; Tomiak et al., 2013). Therefore, if this fraction is more susceptible to leaching (diffusive loss) from inter-crystalline sites than intra-crystalline sites, unbleached material will show “suppressed” A/I or D/L values compared with bleached material. This pattern has also been observed in some molluscs (e.g. Penkman et al., 2008; Demarchi et al., 2013b; Ortiz et al., 2015; Pierini et al., 2016) and ostrich eggshell (Crisp et al., 2013).

As the extended bleaching experiments for Nps were analysed by IEC only, the only amino acid for which racemisation data are available is Ile. At 250 cm the A/I value increases slightly between 24 and 48 hours and then plateaus (Fig. 5a), indicating that bleach-induced racemisation is not taking place. Due to the variability of the data there is no clear effect of bleaching onA/I at 500 and 1450 cm (Fig. 5b, c); however, lower A/I values in unbleached Nps are also seen in the down-core trend for ages greater than ~60 ka (Fig. 8). This suggests that the remaining inter-crystalline protein may behave as an open system even after the initial loss of protein during early stages of diagenesis; this may be due to the progressive generation of highly racemised free amino acids via hydrolysis.

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**Fig. 5** – Trends in A/I value with increasing bleach time for Nps at a) 250 cm, b) 500 cm and c) 1450 cm. Dots show individual replicates; bars show means of all replicates at each time and depth point. Where data are absent, this is because experiments of this duration were not carried out, with the exception of 360 hours at 500 cm, where none of the samples had resolved A-Ile peaks. An increase in A/I is observed between 24-48 hours at 250 cm, while bleaching has no clear effect on A/I at 500 cm and 1450 cm.

Based on the results of these extended bleaching experiments, a 48 hour NaOCl exposure was chosen for the rest of the samples in the study, as this treatment is sufficient to isolate the intra-crystalline fraction of amino acids in Nps.

## 3.2 Comparison of down-core diagenetic trends in racemisation between unbleached and bleached Nps

Samples of Nps were taken for analysis by IEC and RP-HPLC at regular intervals down the cores to enable a more detailed investigation of any amino acid trends and to compare the behaviour of the whole-protein and intra-crystalline fractions for this class of biomineral. A full list of intervals sampled can be found in the supplementary information.

### 3.2.1. Trends in concentration and composition

There is substantial variability in both the [THAA] and composition of amino acids for both unbleached and bleached samples through the cores (Fig. 6, Fig. 7). In general, the concentration of bleached samples is slightly lower than concentration of unbleached samples (Fig. 6), indicating that the majority of protein in naturally-aged Nps is in the intra-crystalline fraction. With the exception of a few relatively young samples having higher than average [THAA], the concentration of amino acids appears to be relatively stable over the timescale represented by this core.

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**Fig. 6** – Down core trends in total hydrolysable amino acid concentration of bleached and unbleached Nps tests (analysed using RP-HPLC)

As was seen in the extended bleaching experiments, there is no clear difference between the composition of whole-protein and intra-crystalline protein fractions of Nps (Fig. 7). Neither fraction shows a systematic change in amino acid composition down core. This indicates that both the whole-shell and intra-crystalline fractions remain relatively stable during diagenesis. While some work has found differential loss of acidic proteins from a range of planktonic species within ~300 ka (Robbins and Brew, 1990), this trend is not seen here over the 120 ka studied.

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### **Fig. 7** – Down-core compositions of unbleached and bleached (48 h) Nps tests analysed by IEC, showing a relatively stable composition of both the whole and intra-crystalline fractions of amino acids. Due to poor separation of Ser from Thr using IEC, these amino acids are reported together. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)3.2.2. Trends in racemisation

As determined by IEC, the A/I value of Nps increases with age, with the rate of racemisation slowing over time in both bleached and unbleached samples (Fig. 8). Due to the change in sedimentation rate at ~ 30 ka in the cores (see Fig. 2), plots of D/L against age and depth are included to show more clearly the trends in racemisation during the younger section of the core (Fig. 8, 9, 10). In the first ~ 60 ka (1550 cm) of the core, the extent of racemisation for bleached and unbleached Nps are similar, with bleached Nps having a slightly higher degree of racemisation (Fig. 10, presented separately for clarity). Older than ~60 ka, the levels of racemisation are lower for unbleached Nps than bleached Nps. This may be due to the loss of free amino acids from the unbleached biomineral, effectively “suppressing” the rate of racemisation (see section 3.1.3).

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**Fig. 8** – Downcore trends in A/I for Nps analysed by IEC: a) A/I against depth showing samples taken between 10-2951.5 cm (corrected depth); b) A/I against age for samples taken between 120-1863 cm, the range of the age model for the core (see Fig. 2). Horizontal lines show 1σ age error from age model. Bleached samples were subjected to 48 h NaOCl exposure. A locally weighted smoothing (loess) function has been applied (solid line) to highlight the differences between the racemisation trends for bleached and unbleached Nps.

Of the amino acids analysed using RP-HPLC, Asx and Glx showed the clearest racemisation trends (Fig. 9i, ii). While previous studies of amino acid racemisation in Nps using RP-HPLC have focused on Asx and Glx only (Kaufman et al., 2008, 2013), Ala also showed clear trends in racemisation in this core after ~25 ka (Fig. 9iv), suggesting that Ala could be used to provide additional chronological control for Nps in older material.

For most amino acids separated by RP-HPLC, the D/L values were similar between bleached and unbleached Nps, with bleached material having slightly higher D/L values in older (> 60 ka) samples, suggesting some loss of highly-racemised free amino acids from the inter-crystalline fraction (Fig. 9). Ser has substantially higher D/L values (Fig. 9, 10iii) in the bleached samples. This may be due a result of Ser having a higher abundance in the free amino acid pool as a result of the lability of Ser-X peptide bonds (Hill, 1965) results in a greater “suppression” of Ser racemisation than other amino acids in the whole-protein fraction. As L-Ser is a common laboratory contaminant, the increase in Ser D/L on bleaching may also be in part due to the removal of exogenous amino acids.

As also seen in studies of the kinetics of amino acid racemisation in other biominerals (Penkman et al., 2008; Demarchi et al., 2011, 2015), Ser racemises the most quickly of all the amino acids due to its ability to racemise in-chain (Demarchi et al., 2013a), plateauing at ~ 30 ka at a D/L of around 0.3 for bleached samples (Fig. 9 and 10 iii). This plateau may be due to the comparative instability of Ser (Bada et al., 1978) resulting in apparent “steady-state” behaviour of racemisation as the rates of Ser racemisation and decomposition converge (Schroeder and Bada, 1977). Asx, Glx and Ala show a similar trend to Ile, with rapid initial racemisation in the first ~30 ka followed by a slowing of the observed rate of racemisation (Fig. 9 and 10 i, ii, iii). This “two-stage” racemisation pattern is consistent with observations of racemisation kinetics in foraminifera (Wehmiller and Hare, 1971; Harada and Handa, 1995; Kaufman, 2006), and with other biominerals (see Clarke and Murray-Wallace, 2006).

Chart, scatter chart

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**Fig. 9** – Trends in racemisation for four amino acids (i) Asx, ii) Glx, iii) Ser, iv) Ile) analysed by RP-HPLC. a) D/L against depth showing samples taken between 10-2951.5 cm (corrected depth); b) D/L against age for samples taken between 120-1863 cm, the range of the age model for the core (see Fig. 1). Horizontal lines show 1σ age error from age model. All four amino acids have higher D/L values for bleached than unbleached Nps in older samples. Bleached samples were subjected to 48 h NaOCl exposure. A locally weighted smoothing (loess) function has been applied to highlight the differences between the racemisation trends for bleached and unbleached Nps.

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**Fig. 10** – Trends in racemisation for four amino acids analysed by RP-HPLC for the youngest part of the core, (10-60 ka, 10-1500 cm) of the core: a) D/L against depth; b) D/L against age. Horizontal lines show 1σ age error from age model. During this early stage of racemisation D/L values are similar between bleached and unbleached Nps for Asx, Glx and Ala. Bleached samples were subjected to 48 h bleach exposure. A locally weighted smoothing (loess) function has been applied to highlight the differences between the racemisation trends for bleached and unbleached Nps.

Of these three amino acids, Asx racemises the most quickly in Nps, reaching a D/L of ~ 0.3 by 120 ka, while Glx racemises more slowly, reaching a D/L of ~ 0.15 over the same period. The apparent racemisation rate of Ala is intermediate, reaching a D/L of ~ 0.2 by 120 ka. This order of apparent racemisation rate (Ser > Asx > Ala > Glx ~ Ile) is consistent with the results of high-temperature decomposition experiments carried out on other biominerals (e.g. Kimber and Griffin, 1987; Ortiz et al., 2017; Dickinson et al., 2019). Studies of Asx and Glx racemisation trends in heated and naturally-aged planktonic foraminifera have found that Asx racemises more quickly than Glx (Hearty et al., 2004; Kaufman, 2006; Kaufman et al., 2013); however a study of *P. obliquiloculata* investigating a wider range of amino acids found that Ile racemised more quickly than Glx, in contrast to the results of this study, which observed similar apparent racemisation rates between Ile and Glx (Harada and Handa, 1995).

If the intra-crystalline fraction better approximates a closed system than the whole-protein fraction, then there should be fewer environmental influences on racemisation in the intra-crystalline fraction, resulting in lower variability between D/L values at any given horizon for bleached samples (Penkman et al., 2008). For Nps, bleaching results in a decrease in variability between similar-age samples during early stages of diagenesis (Fig. 11). This result is consistent with the reduced effect of bleaching on total amino acid concentration in older material (Section 3.1.1), suggesting that the majority of the inter-crystalline protein is leached from the biomineral during early stages of diagenesis. While these reductions in variability on bleaching are not seen in older material, the observed increase in D/L values in the intra-crystalline fraction suggest that the IcPD approach also limits the effects of leaching and contamination in older samples.

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**Fig. 11** – Covariance of Asx and Glx D/L for bleached and unbleached Nps analysed by RP-HPLC, showing decreased variability of D/L values on bleaching for young material (Asx D/L < 0.2). A locally weighted smoothing (loess) function has been applied to show the general trends of the data.

## 3.3 IEC A/I to RP-HPLC D/L conversions

Conversion from IEC Ile A/I and equivalent RP-HPLC amino acid D/L was carried out using the pooled regressions developed by Whitacre et al. (2017), as well as Nps-specific regressions derived using the Nps data set. Due to the poor separation of L-Ile and D-Aile using RP-HPLC (Powell et al., 2013; Wehmiller, 2013), direct comparison between IEC Ile A/I and RP-HPLC Ile A/I was not possible using this data set.

When applied to the Nps data set, the 2σ prediction uncertainty for the pooled regressions were ±23 to 42% depending on the amino acid, similar to the ranges found by Whitacre et al. for other biominerals (Table 2). While Whitacre et al. (2017) found that creating material-specific models reduced the 2σ prediction uncertainty compared with pooled regressions, this has not been observed for Nps – perhaps due to the use of averaged rather than paired analyses. For Asx, Glx and Ala the 2σ prediction uncertainty was ±24-35% when using the Nps-specific regression. Conversely, Val and Ile had much higher 2σ prediction uncertainties for the Nps-specific regression, highlighting the poorer chiral separation of both Val and Ile by RP-HPLC for this biomineral (Fig. 12, Table 2). The pooled regressions did, however, slightly overestimate Asx, Glx and Ala D/L values (Fig. 12).

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**Fig. 12** – Relationship between IEC Ile A/I and RP-HPLC amino acid D/L values, modelled by log2-transformed linear regressions. Lines represent the Nps-specific and pooled linear regressions; shaded envelopes are 2σ prediction uncertainties using a “dummy” data set for the Nps-specific regressions (as used by Whitacre et al. (2017)) and the Nps data set for the pooled regressions.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Pooled regression | | | | Nps-specific regression | | | | | | | | |
|  | 1σ PU | 2σ PU | AICc | Success rate (%) | Slope | ± | Intercept | ± | R2 | 1σ PU | 2σ PU | AICc |
| Asx | 0.24 | 0.24 | -239 | 69 | 0.41 | 0.03 | -0.35 | 0.14 | 0.83 | 0.23 | 0.24 | -24 |
| Glx | 0.41 | 0.42 | 126 | 94 | 0.51 | 0.05 | -1.22 | 0.2 | 0.76 | 0.36 | 0.38 | 3 |
| Ala | 0.33 | 0.33 | -16 | 78 | 0.59 | 0.05 | -0.21 | 0.2 | 0.84 | 0.33 | 0.35 | -2 |
| Val | 0.39 | 0.40 | 43 | 31 | 0.51 | 0.10 | -1.13 | 0.4 | 0.44 | 0.77 | 0.82 | 48 |
| Ile | 0.41 | 0.42 | 59 | 27 | 0.49 | 0.13 | -0.82 | 0.6 | 0.31 | 0.98 | 1.05 | 57 |

**Table 2** – Results of linear regressions using log2 transformed A/I and D/L values. PU refers to the prediction uncertainty calculated using the Nps data set for the pooled regression, and a “dummy” data set for the Nps-specific regression (as used by Whitacre et al. (2017)). Success rate refers to the proportion of predicted ranges which included the D/L value measured by RP-HPLC.

The results of this analysis show that the Nps-specific regressions can confidently convert Nps IEC A/I values into equivalent D/L values for Asx, Glx and Ala over the ranges tested, with similar prediction uncertainty ranges as for the pooled regressions developed by Whitacre et al. (2017). Due to the poor separation of Val and Ile for Nps it was not possible to validate the regressions for these amino acids. Due to the overestimation of Asx, Glx and Ala D/L values using the pooled regressions and the similar prediction uncertainties between the Nps-specific and pooled regressions, we recommend that the Nps-specific regressions developed in this paper are used to convert Nps A/I values to D/L Asx, Glx and Ala values.

# 4. Conclusions

Bleaching experiments show that Nps tests contain an intra-crystalline fraction of amino acids which is resistant to extended exposure to bleach. A 48 hour treatment with 12% w/v NaOCl isolates the intra-crystalline fraction in Nps, with a greater proportion of amino acids being removed by bleaching in younger samples. Bleaching results in slightly increased Ile, Asx, Glx and Ala D/L values in older samples, indicating that the inter-crystalline fraction of amino acids behaves as an open system during diagenesis. Additionally the variability of D/L values is lower for bleached Nps than unbleached Nps in samples where Asx D/L < ca. 0.2. This suggests that the IcPD approach limits the influences of leaching on racemisation trends for younger Nps, while older material may also benefit from bleaching by minimising the potential impact of contamination.

Given this dataset, it is therefore recommended that a bleaching step is included during the preparation of Nps foraminifera for amino acid dating, if sufficient sample mass is present to compensate for the reduction in concentrations caused by oxidation of the inter-crystalline fraction.

IEC A/I values were successfully converted to RP-HPLC D/L values for Asx, Glx and Ala in Nps using material-specific regression equations with a ±24-38% 2σ prediction uncertainty depending on the amino acid.

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*Data Availability*: All amino acid data from this study will be made available through the NOAA repository upon publication: ftp://ftp.ncdc.noaa.gov/pub/data/paleo/aar/.

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