Determination of picomolar dissolved free amino acids along a South Atlantic transect using reversed-phase high-performance liquid chromatography

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Manuscript version: 22 May 2017

Abstract

Dissolved free amino acids (DFAA) in seawater are a form of nitrogen (N) available for marine microbes. In oligotrophic environments where N-containing nutrients are the limiting factor for microbial growth, N nutrition from DFAA could be crucial, but as yet it is poorly resolved. Measurements of individual DFAA are challenging as concentrations are typically in the low nmol L^{-1} range. Here we report modifications to methodology using o-phthaldialdehyde (OPA) derivatization and reversed phase high performance liquid chromatography (HPLC) that provide a 30-fold improvement in sensitivity enabling the detection of 15 amino acids in seawater with a limit of detection as low as 10 pmol L^{-1} with accuracy and precision of better than 10 %. This analytical methodology is now suitable for the challenging quantitation of DFAA in oligotrophic seawaters. The method was successfully applied to a suite of seawater samples collected on a cruise crossing the South Atlantic Ocean, where concentrations of DFAAs were generally low (sub nmol L^{-1}), revealing basin-scale features in the oceanographic distributions of DFAA. This unique dataset implies that DFAAs are an important component of the N cycle in both near-coastal and open oceans. Further calculations suggest that the proportions of organic N originating from DFAA sources were significant, contributing between 0.2–200 % that of NH_4^+ and up to 77 % that of total inorganic nitrogen in the upper 400 m in some regions of the transect.

1. Introduction

Phytoplankton account for around half of primary production on earth, fueling marine food webs and contributing to the biogeochemical cycling of elements (Falkowski et al. 1998). Biologically accessible nitrogen (N) limits phytoplankton growth in most regions of the global ocean (Moore et al. 2013). Dissolved N in seawater is found both in inorganic (NH_4^+, NO_3^-, NO_2^-) and labile organic forms, including urea, dissolved free amino acids (DFAAs), peptides and proteins. Biological utilisation of inorganic nutrients and urea is well established (Lund and Blackburn 1989, Rondell et al. 2000) and the characterisation and uptake of peptides has been thoroughly described in Mulholland and Lee (2009); however, the role of DFAAs in the ocean N cycle, in particular their contribution to supporting primary productivity, remains unclear.

DFAAs represent a small but important fraction of the dissolved organic nitrogen (DON) pool (1.2–12.5 %, Carlson and Hansell (2015)). They mainly originate from microbial cells via exudation during cell senescence (extracellular release; Rosenstock and Simon (2001)), but can also be released by N fixing bacteria (e.g. *Trichodesmium* sp), or from zooplankton excretion/inadvertent loss whilst feeding on phytoplankton or other forms of organic matter (Webb and Johannes 1967, Carlucci et al. 1984, Rosenstock and Simon 2001). Collectively, DFAAs represent up to 30–50 % of fixed N (Glibert and Bronk 1994) and constitute an important source of N for the marine system. DFAA are reported to have a fast turnover (minutes) as they are rapidly consumed by bacteria, which in turn can lead to their transformation into inorganic nutrients, thereby making them available for phytoplankton uptake (Fuhrman 1987, Kirchman 1994). Some microalgae also appear capable of taking up DFAA directly when inorganic N is low (Kaiser and Benner 2008).

The South Subtropical Convergence (SSTC) is an oceanographic feature encircling the globe at around 40 ^{o}S: it represents the intersection point of the high nitrate upwelling waters of the Southern Ocean and the nitrate-impoverished oligotrophic gyres (Ito et al. 2005). Satellite images suggest productivity here is typically elevated relative to the waters north and south of the convergence (Longhurst 1998, Browning et al. 2014). A cruise transect penetrating both the high nitrate, iron-limited Antarctic Circumpolar Current (ACC) waters to the south, and the low nitrate waters to the north of the convergence offered a unique opportunity to study the contribution of DFAA to N utilisation in two contrasting biogeochemical regimes (Browning et al. 2014).

DFAA research to date has mainly focussed on studying cycling using laboratory experiments (Andersson et al. 1985, Linares 2006, Sarmento et al. 2013), lakes (Rosenstock and Simon 2001) or in coastal waters (Tada et al. 1998, Kiel and Kirchman 1999, Lu et al. 2014), where concentrations of DFAA are high. Very little is known about the role of DFAA in the oligotrophic ocean, where extremely low concentrations of inorganic N are likely to be the primary factor limiting phytoplankton growth (Moore et al. 2013). A major challenge is that the concentrations of DFAAs are

also extremely low in these regions (pmol L^{-1}), making their quantification difficult as available techniques have detection limits at the nmol L^{-1} level. This lack of sensitivity in analytical techniques has hindered accurate assessment of the size of this N reservoir, determination of the relative roles of phytoplankton and bacteria on marine DFAA concentrations, evaluating potential environmental conditions that might influence DFAA cycling, and resolving the possible influence of DFAA concentrations on oceanic microbial nutrient limitations.

Here we report the improvement and validation of existing HPLC methodologies so as to enable the quantification of DFAA at the concentrations typically encountered in the oligotrophic ocean. The two principle aims of the study were to (1) improve the analytical approach to enable the determination of 15 individual DFAA in seawater at sub nmol L^{-1} concentrations, and (2) to study the spatial and depth variations in DFAA dynamics in meso- and oligotrophic waters in a cross-basin oceanographic section. We focus on the dominant individual DFAA shown to be important in phytoplankton release and bacterial consumption (Sarmento et al. (2013)), specifically: serine (Ser), aspartic acid (Asp), glutamic acid (Glu), histidine (His), valine (Val), phenylalanine (Phe), and methionine (Met). We relate the individual DFAA concentrations to NH_4^+ and other N-containing inorganic nutrients along the transec, leading us to hypothesise that the DFAA contribution to primary productivity in oligotrophic ocean systems is potentially more important than previously thought.

2. Materials and Procedures

2.1. Study site and sampling procedures

Seawater for quantification of DFAA concentrations were collected during the UK-GEOTRACES GA10 South Atlantic research cruise on the RRS Discovery (http://www.ukgeotraces.com/), in mid-austral summer (January 2012). Fig 1a presents the whole cruise transect with the 24 sampling stations (St). St 1–3 were located in low N Agulhas current (AC) waters, St 4–13 were located in high N ACC waters, St 14–19 in low N SSTC waters, St 20–24 in the low N Brazilian current (BC), with St 23–24 in close proximity to the Rio Plata outflow. Seawater was collected for DFAA concentrations at 14 stations: 1, 3, 5, 6, 8, 12, 14, 15, 16, 17, 18, 19, 20 and 21. Water was collected from the CTD-rosette into clean Nalgene® bottles using clean handling techniques, in particular taking care to avoid ammonia contamination. Seawater samples were gently filtered through 0.2 µm filters (Millex® syringe filter) immediately after collection and an aliquot of 2 ml of filtrate was frozen (–18 ^{o}C) for DFAA analysis.

2.2. Nutrient concentrations and phytoplankton biomass and community structure

Nutrients – The water column concentrations of nitrate (NO_3^-) and nitrite (NO_2^-) were determined at sea (Woodward and Rees 2001) using a Bran and Luebbe segmented-flow colorimetric auto-analyser. Around 50 mL of water was taken at all sampled depths from the CTD Rosette, employing GO-SHIP repeat hydrography protocols (Hydes et al. 2010). Ammonium (NH_4^+) analysis was by a nano-molar analyser using pH differential gas diffusion across a Teflon membrane, followed by fluorescence analysis (Jones 1991). Quality control for micromolar nutrients was undertaken using KANSO certified nutrient reference materials. The detection limit of nitrite+nitrate was 0.02 µmol L^{-1} during the cruise, and the accuracy was between 1 % and 4 % (1 SD) as determined from analysis of the KANSO reference materials.

Biomass and community structure – Phytoplankton community structure was characterised using HPLC pigment analysis and flow cytometry cell counts. Full methods are reported in Browning et al. (2014); briefly, total chlorophyll-a and accessory pigments were determined from filtered material (0.2–2 L; 0.7 µm Whatman GF/F; –80 ^{o}C stored) using a Thermo HPLC system following the method described in Gibb et al. (2000). Flow cytometry analysis quantified cell counts of nanophytoplankton, picophytoplankton, *Synechococcus*, *Prochlorochoccus*, and total bacteria: samples (2 mL; preserved in 1% paraformaldehyde at –80 ^{o}C) were analysed using a FACSort flow cytometer (Becton Dickinson) according to methods described in Davey et al. (2008).

2.3. DFAA measurements using HPLC method

The method described here was adapted from that orignially developed by Lindroth and Mopper (1979) and subsequently improved upon by Kuznetsova et al. (2004). In this method o-phthaldialdehyde (OPA) derivatives of DFAA are measured using high-performance liquid chromatography (HPLC) with fluorescence detection. Here we further modify the method in order to improve its sensitivity for samples with a seawater matrix.

Instrumentation – An Agilent 1100 series HPLC system comprising an online degasser (G1379), a quaternary pump (G1379), thermostated autosampler (G1329A), and a thermostated column compartment (G1316A) was used. Detection of fluorescent amino acid derivatives was carried out using an Agilent G1321A fluorescence detector equipped with an 8 µL flow cell. Separations were performed using a 250 x 4.6 mm C18 column (Phenomenex Luna 5µ C18(2) 100 Å), and a guard column containing the same phase (Phenomenex SecurityGuard™ cartridges kit), both maintained at 40 ^{o}C during analysis.

Reagents and solvents – All solutions were prepared from analytical-grade chemicals (Fisher Scientific), dissolved in Milli-Q water (MQ, 18.2 MΩ cm^{-1} at 25 °C) and HPLC-grade solvents (Fisher Scientific). The OPA was purchased from Sigma-Aldrich, UK. An amino acid standard (Standard H, Thermo Scientific Pierce), which was a quantitative mixture of 18 amino acids (individual AA concenrations: 2.5 µmol mL^{-1} in 0.1 N HCl), was used as a high-purity calibration standard for HPLC analysis and was kept at -18 ^{o}C, in the dark, to prevent degradation of the amino acids. Working standards were diluted in MQ and used within a week.

Amino acid standard solutions – Amino acid standards were prepared by diluting the appropriate amount of Standard H solution with MQ. Calibration solutions used for method development were prepared in the range 0 – 20 nmol L^{-1}. The standard solutions used for DFAA analysis from an open ocean sampling site in the South Atlantic were 0 – 12.5 nmol L^{-1}, encompassing the concentration range expected for these waters.

OPA working solution – The fluorogenic reagent consisted of an OPA solution fixed at pH 9.5 using a borate buffer. This pH has previously been shown to be optimal for the derivatisation reaction (Kuznetsova et al. 2004). A saturated OPA solution was prepared in absolute ethanol (1 g in 20 mL EtOH), stored at 4 ^{o}C, and was used for one month before being discarded. The pH 9.5 borate buffer solution was prepared using a 0.4 M boric acid solution, and pH adjusted with 1 M NaOH; both solutions were made up on a weekly basis. The working reagent solution was made of 2.7 mL OPA solution, 10.8 mL of borate buffer and 66 µL of 2-Mercaptoethanol (2-MEt). This mixture was placed in an amber HPLC vial and stored at 4 ^{o}C for 24 h to prevent the fluorogen degrading when exposed to light. This time period allowed decay of the background fluorescence. For maximum efficiency and reliability, the working reagent solution was used at the optimum age of between 24 h and 48 h. In

153 other studies (Lindroth and Mopper 1979, Godel et al. 1984) the fluorogenic reagent was reused by
154 adding a few µL of thiol (2-MEt) every 3-4 days. In this study, to ensure maximum precision, the
155 buffered OPA reagent was prepared daily.

156 *Derivatisation and HPLC procedure* – The binary mobile phase comprised 5%
157 tetrahydrofuran (THF) in 0.05 M sodium acetate trihydrate (6.08 g in 1 L MQ) (A) and methanol (B)
158 used at a flow rate of 1 mL min^{-1}. The gradient was modified from Kuznetsova et al. (2004) as
159 follows: starting composition 95% A, 5% B, changing to 80% A, 20% B in 12 min, then to 35% A,
160 65% B over 35 min, then to 100% B in 4 min and held at 100% B for 2 min. A 2-step post-run
161 gradient of 7 + 5 min returns to starting solvent composition (Supp. 2). For derivatisation, the
162 autosampler was programmed to mix 10 µL of OPA reagent with two 45 µL aliquots of sample, and
163 the resulting 100 µL mixture was injected after a reaction time of 2 min. The derivatised amino acids
164 were detected by fluorescence (excitation 342 nm, and emission 452 nm), as described by Parsons et
165 al. (1984).

166 *Performance parameters* – The degree of agreement among a series of measurements of the
167 same sample is typically reported as the coefficient of variation: *%CV = (SD/mean)*100* (Eq. 1). The
168 resolution between critical pairs of amino acids from the chromatogram was calculated using
169 equation: $R_s = 2*(T_{R2}-T_{R1})/(W_2+W_1)$ (Eq. 2), where T_R is the retention time of peaks 1 and 2
170 respectively and W is their respective peak width at the tangents' baseline. The limit of detection
171 (LoD), by definition, is the lowest analyte concentration able to be reliably distinguished from the
172 blank. The value of LoD was calculated using equation: *LoD = (3.3*residual SD)/S* (Eq. 3), where
173 residual SD is the residual standard deviation of the blank measurement, and S is the slope of the
174 calibration curve. Reliable LoQ can be assumed using: *LoQ = (10*residual SD)/S* (Eq. 4).

175 2.4. Statistical analysis and N content calculations

176 Data are presented as the mean (± SD). Linear regression analysis was used to explore the
177 relationships between DFAA and various determinants. A Pearson's Correlation Coefficient I was
178 computed as a measure of the strength of association between variables. A P-value of ≤ 0.05 was
179 considered statistically significant. Data analysis was performed using R (version 3.1.3).

180 The weighted amount of N (m_N) contributed by each N-containing inorganic nutrient or
181 individual DFAA, was calculated based on one litre of seawater: $m_N = C*M_N*n$ (Eq. 5), where C is
182 the concentration of the inorganic nutrient or DFAA, M_N the atomic weight of N, equal to 14.01 g
183 mol^{-1} and *n* the number of N per molecule.

3. Results

3.1. Method optimisation and validation

Optimisation of the derivatisation reaction – The derivatisation reaction was carried out using different OPA:buffer (v/v) ratios on a 20 nmol L^{-1} amino acid standard solution. The highest fluorescence intensity was obtained using an OPA:buffer ratio of 1:4 ratio (v/v), instead of 1:9 (v/v) used in previously published methods (Roth 1971, Godel et al. 1984, Roach and Harmony 1987, Fisher et al. 2001, Kuznetsova et al. 2004).

Having established the optimum OPA:buffer composition, we then carried out a series of derivatisations with different ratios of OPA:sample to optimise that variable. The concentration of the buffered OPA working solution was increased, from 11 mL OPA in 100 ml buffered solution to 25 mL OPA in 100 mL buffered solution, in order to improve the limit of detection (LoD). The highest fluorescence was produced when OPA and sample seawater were combined in a 1:9 (v/v) ratio. The objective was to increase the proportion of seawater in the chemical reaction in order to maximise the sensitivity of the method. With an increased ratio of seawater to reagent, the following care has to be taken with the HPLC column as the contact with seawater salts can degrade the stationary phase of the column. We included a 2-step conditioning procedure (12 min) at the end of the analysis run, incorporating a 5 min gradient using a highly aqueous mobile phase composition, to flush salts from the column. It is also recommended that a full system cleaning step is completed after each sampling station (i.e. after approximately 24 samples) this consisting of flushing the column with 10 × column volume with a MQ:MeOH (1:1, v/v, 5 min at 1 ml min^{-1}). Precautions should be taken when preparing the mobile phase as the signal-to-noise ratio is affected by impurities in the buffer solutions used for the analysis (Benson and Hare 1975). It is therefore recommended that buffers should be freshly prepared every day prior to any new batch of samples and filtered through 0.7 μm filters (Whatman GF/F).

Reproducibility – Retention time reproducibility (Supp. 3), and the resolution between critical pairs of amino acids was assessed. The average precision of retention times of amino acids in the standard solution and seawater, defined by the coefficient of variance (%CV) calculated using Equation 1, were 0.41 % and 0.65 %, respectively. The small %CV values demonstrate the high reproducibility of the method. The maximum difference (%Δ) of retention times between the standard solution and seawater samples was < 2 %, and the mean difference was 0.67 %. A difference within 3 % is considered acceptable (Reason 2003). The resolution R_s between critical pairs of amino acids, calculated from Equation 2, was 0.87 and 0.99 for Ser/His (peaks 3/4, Fig 2) in the standard solution and seawater respectively, and 0.66 and 0.89 for Arg/Thr (peaks 6/7, Fig 2). If Rs is >1, the two peaks

are generally considered resolved (Snyder et al. 1997). Hence, the resolution was considered reasonable for Arg/Thr and for Ser/His (see Supp. 1 for abbreviations of DFAA names).

Linearity of response – The detector response was found to be linear over the concentration range used in this study (0 nmol L^{-1}, 0.15 nmol L^{-1}, 0.30 nmol L^{-1}, 0.60 nmol L^{-1}, 1.25 nmol L^{-1}, 2.50 nmol L^{-1}, 5 nmol L^{-1}, 10 nmol L^{-1} and 20 nmol L^{-1}). The correlation coefficient I squared for the calibration with amino acid standards was $\geqslant$ 0.99 (*n = 15*), hence demonstrating the linearity of the method. The average residual of the calibration curve was 0.33 %. As part of the method validation, standard solutions were run in triplicate.

Precision – To demonstrate repeatability of the method, a series of standards of different concentrations (0 nmol L^{-1}, 0.15 nmol L^{-1}, 0.30 nmol L^{-1}, 0.60 nmol L^{-1}, 1.25 nmol L^{-1}, 2.50 nmol L^{-1}, 5 nmol L^{-1}, 10 nmol L^{-1} and 20 nmol L^{-1}) were measured. Seawater samples were also measured in triplicate. The relative standard deviation (RSD) for the seawater measurements was <6% and had an average of 3%. Thus the proposed method demonstrated an acceptable level of precision.

Limits of detection and quantification – The LoD and LoQ for the 15 amino acids targeted in this study ranged between 9 to 163 pmol L^{-1} and 27 to 490 pmol L^{-1}, respectively (Supp. 3).

3.2. Oceanographic context for seawater DFAA samples

3.2.1. Nutrient and phytoplankton distributions

This study focused on surface waters less than 200 m depth, where NO_3^- was generally < 25 µmol L^{-1} (Fig 3a). The cross-Atlantic transect could clearly be divided in two parts during cruise occupation; the Eastern and the Western basins. From South Africa to 28 ^oW (Eastern basin, St1 to St 15, Fig 1a), the water column was well-mixed with elevated NO_3^- (Fig 3a) and chlorophyll concentrations (Fig 1a). In the Eastern basin, NH_4^+ and NO_2^- were both present between 50 – 150 m depth (0 to 0.90 µmol L^{-1} and 0 to 0.78 µmol L^{-1} respectively). To the west of 28 ^oW, in the Western basin, NO_3^- and chlorophyll concentrations were highly depleted (below detection limit) in surface waters with an established nutricline below the mixed layer. NH_4^+ and NO_2^- were also depleted (0 to 0.17 µmol L^{-1} and 0 to 0.65 µmol L^{-1} respectively). In the Western basin, NO_2^- and to a lesser extent NH_4^+ exhibit a sharp maximum around the depth of the thermocline (Fig. 3b), coincident with enhanced biological activity there (Fig. 1c). An upwelling eddy was identified as driving NO_3^- upwelling around 45 ^oW (Browning et al., 2014). In comparison with the rest of the western basin the concentrations of NO_2^- and NH_4^+ in the eddy were also considerably higher than elsewhere in the transect (Fig 3b and c).

Chlorophyll-a concentrations were low (< 0.2 mg m^{-3}, Fig 1c) in surface waters with distinct sub-surface maxima (up to 1.46 mg m^{-3}) in the Agulhas Current and throughout most of the western

basin (St 1-3; 20–22). Conversely, concentrations were elevated (0.2 to 0.7 mg m^{-3}) and uniform throughout the well-mixed surface layer of the ACC waters on the southern edge of the SSTC (St 8, 15). Close to the coast of Uruguay, enhanced chlorophyll-a (up to 0.9 mg m^{-3}) was observed near waters strongly influenced by the Rio Plata outflow (St 23–24). Similar surface patterns were also generally apparent in composite images of ocean colour-derived chlorophyll-a from NASA MODIS satellite images (Browning et al. 2014).

3.2.2. Spatial and depth variations of DFAA concentrations

Concentration profiles of selected DFAA are presented in the supplementary information (Supp. 4 and 5). DFAA concentrations ranged from undetectable (see LoD, Supp. 3) to 53.1 ± 0.5 nmol L^{-1} for Asp; 12.9 ± 0.5 nmol L^{-1} for Glu; 62.5 ± 0.5 nmol L^{-1} for Ser; 14.1 ± 0.5 nmol L^{-1} for His; 1.2 ± 0.5 nmol L^{-1} for Met; 8.5 ± 0.5 nmol L^{-1} for Phe and 10.8 ± 0.5 nmol L^{-1} for Val. On average, across the whole transect, DFAA were present in the following order from high to low concentration: Ser, Asp, Glu, His, Val, Phe and Met. Ser had a relatively high concentration in the Agulhas current (10 to 30 nmol L^{-1}) and relatively lower concentration in the rest of the transect (< 5 nmol L^{-1}). Concentrations of Asp were also elevated in the Agulhas current (20 to 40 nmol L^{-1}), compared to the ACC waters (~10 nmol L^{-1}), and were very low in the SSTC waters. Concentrations of Asp increased closer to the Uruguayan coast (10 to 20 nmol L^{-1}), most likely due to the Brazil Current mixed with the Rio Plata inputs. His, like the other DFAA, also had its highest concentrations in the Agulhas current region (5 to 15 nmol L^{-1}), as well as between 0 and 30 ^{o}W (2 to 5 nmol L^{-1}), suggesting inputs from the Uruguayan coastal waters in the West. Val was mainly present in coastal waters, with relatively high concentrations in the Aghulas current (3 to 10 nmol L^{-1}) and in waters strongly influenced by Rio Plata inputs (up to 3 nmol L^{-1}). Phe was relatively abundant in the Aghulas current (2 to 3 nmol L^{-1}), but then decreased in the ACC waters (1 to 2 nmol L^{-1}). Phe concentrations were low in the Eastern basin, especially in the uppermost layer of surface waters, and relatively low concentrations were also present in close proximity to the Rio Plata. Met concentrations were relatively low (up to 1.25 nmol L^{-1}) compared to the other AAs presented in this study. The Met profile presented in Supp. 5 indicated that the coastal waters were relatively rich in Met, compared to the open ocean stations with the concentrations in the Brazil Current among the highest of the whole transect (> 1 nmol L^{-1}). There was a clear division at ~50 m corresponding to changes in Glu concentrations, where in the upper layer of the surface waters Glu was present, while below this depth Glu concentrations were undetectable. Similarly to Asp, Glu concentrations were elevated in the Eastern basin and lower in the Western Basin. The Agulhas Current showed high concentrations of Glu in comparison to the rest of the transect (5 to 12.5 nmol L^{-1}) and on the other side of the basin, the continental shelf of the Uruguayan coast contributed between 5 to 7.5 nmol L^{-1} of Glu.

Concentrations of DFAA at some depths were lower than the limit of detection (e.g. Met), however, a number of correlations were found between individual DFAA and biological or physical variables. Across the entire 40 ^{o}S transect, concentration of all DFAAs are strongly correlated ($p < 0.05$ with Pearson correlation). From St 1 to 6, individual DFAA were significantly correlated with each other ($p < 0.05$ with Pearson correlation). Ser was never correlated to a specific DFAA, and Met, as stated above, was often too close to the limit of detection to allow for a robust correlation analysis. At all stations, concentrations of Glu peaked at the chlorophyll maximum. In the open ocean region i.e. from St 6 to St 18, Glu was highly correlated with all phytoplankton biomass indices as well as NH_4^+ ($p < 0.05$ with Pearson correlation).

Within the Agulhas current (St 1 and 3) the contribution of DFAA N relative to the total amount of inorganic N calculated using Eq. 5 ranged from undetectable to up to 77 % at around 20 m depth. Glu was again an interesting DFAA, contributing between 0.2–200 % that of NH_4^+ across the transect, in the top 400 m.

4. Discussion

4.1. Optimisation of HPLC method

A total of 15 amino acids were targeted for this study (Supp. 1). Three modifications to the analytical technique were made. First, increasing the polarity of the mobile phase starting composition compared to Kuznetsova (2004) held the amino acids on the column for longer, thus improving separation. The polarity index (P') was calculated as P' = 9.4 for a starting composition of 5 % MeOH and P' = 8.9 for a composition of 20 % MeOH (Kuznetsova, 2004). Secondly, setting the column at 40 ^{o}C rather than room temperature (~25 ^{o}C) gave a marked improvement in peak sharpness and resolution between critical pairs, as has been observed when column temperatures were increased for other analytes (Van Heukelem and Thomas 2001). OPA does not react with secondary amines; hence, amino acids like proline or hydroxyproline were not detectable. However, it is possible to analyse DFAA with secondary amines or short-chained polyamines (e.g. putrescine, spermidine or spermine) by adding FMOC as a derivatising reagent (Lu et al. 2014). Cysteine and cystine, which both show low fluorescence with OPA/2-ME, have to be firstly converted to cysteic acid or to S-3 sulfopropylcysteine before being detectable by the method, hence these DFAA were not included in the present study (Lee and Drescher 1979). Thirdly, closely tuning the fluorescence excitation and emission wavelengths (excitation 342 nm, and emission 452 nm) led to an increase of the sensitivity, allowing lower concentrations of DFAA to be measured.

The OPA/2-MEt analytical method presented here offers a number of advantages that suit a future ship-board system for DFAA analysis. Only 90 µL of seawater is required for each analysis, therefore, sample volume requirements are very low, even if duplicates or triplicates are necessary. Online filtration directly from the Niskin bottles through a 0.22 µm filter minimizes risk of contamination during sampling. Previously published procedures for amino acids analysis described desalting of the sample (Siegel and Degens 1966, Pocklington 1972, Dawson and Gocke 1977, Dawson and Pritchard 1978, Dawson and Liebezeit 1981). Desalting is not required for our revised method; whole seawater samples can thus be injected onto the column without amendment. Without desalting, sample handling steps that cause contamination or loss are avoided and considerable analytical time is saved.

The mean LoD was 68 pmol L^{-1}, representing a 30 fold improvement compared to that obtained by Kuznetsova et al. (2004) and is comparable to Lu et al. (2014) who reached a LoD of 10 to 100 pmol L^{-1}.

4.2. DFAA along the 40 ^{o}S transect

The five dominant DFAA in order of abundance across the 40 ^{o}S transect were: Ser, Asp, Glu, His, Val; which is in accordance with the findings of previous seawater and estuarine studies (Table 1). Table 1 compiles total concentrations of DFAA from different global oceans and estuaries, and now adds observations from the South Atlantic Ocean to this dataset. Concentrations of individual DFAA are predictably higher in studies conducted in higher-biomass near-coastal waters, but our results are in a comparable range to other open ocean sites: 4 to 134 nmol L^{-1} in this study compared to 3 to 132 nmol L^{-1} in the North Atlantic Ocean (Kuznetsova et al. 2004) and 3 to 9 nmol L^{-1} in the highly oligotrophic Sargasso Sea (Kiel and Kirchman 1999).

Glutamic acid (Glu) was present across the entire transect (Fig 4) and was often found to be correlated with other DFAAs and chlorophyll-a biomass. This prevelance and close correspondance to concentrations of other biologically-derived substances is likely a result of Glu's central role as the dominant metabolic N compound, acting as the main N shuttle for protein synthesis, donating and receiving amine groups through transamination reactions (McCarthy et al. 2013). Glu is also important in the N-assimilation pathway of bacteria and phytoplankton (Suttle et al. 1991), and can be released by certain phytoplankton (Sarmento et al. 2013), with concentrations previously shown to parallel the daily pattern of photosynthesis (Capone et al. (1994).

Overall, changes in the Glu concentrations appeared related to the different water masses, and their distinct microbial communities, that were encountered during the cruise. The Agulhas current had the highest concentrations (50 to 134 nmol L^{-1} total DFAA concentrations) of the whole transect, centred around the subsurface chlorophyll-a maximum (Fig. 1). The Agulhas current comprises warm water derived from the Indian Ocean *via* close association with the East African coast. The Agulhas current water is low in NO_3^- (< 10 µmol L^{-1}) and other N-containing inorganic nutrients (Browning et al. 2014). DFAA could therefore play an important role in N-nutrition in these waters, particularly Glu, which we calculated to represent up to 17 % of the bioavailable N present in the Agulhas current (Fig 4). Although chlorophyll-a was low in surface waters, concentrations were elevated in the sub-surface maximum, implying a biological source for the observed elevated DFAA. Other biological sources could include pre-cruise surface layer spring bloom production (see Fig. 3 of Browning et al., 2014 highlighting pre-bloom conditions in these waters). Indeed, enhanced total bacterial counts observed throughout these waters (Browning et al., 2014) could be interpreted to represent a post-bloom condition where phytoplankton-derived organic matter had, or was, being consumed, possibly leading to enhanced levels of DFAA release.

Further to the West (St 4–12) pigment-derived estimates of dinoflagellates coincided with surface waters with relatively high concentrations of NO_3^-; also corresponding to an area with relatively high concentrations of Glu, other DFAA, and bacterial cell counts (Fig 4, Supp. 4 and 5, Browning et al. (2014)). Dinoflagellates have been reported to preferably utilise, in order of preference: NH_4^+, followed by DFAA and Urea, rather than NO_3^- (Fan et al. 2003). Therefore elevated DFAA concentrations are potentially a contributing factor for their enhanced concentrations. Linear correlations for these waters (St 6 to 18) showed Glu was correlated with NO_3^-, both nano- and pico-eukaryotic phytoplankton, and total bacteria concentrations. However, the generally lower concentrations of DFAA compared with the lower chlorophyll-a Agulhas current region is difficult to reconcile with direct production by the phytoplankton community and, again, therefore potentially suggests complexity of sources/sinks linked to phytoplankton-grazer bloom and decline phases that are difficult to resolve with transient standing stock concentrations.

Where there was an increased contribution of diatoms to the total chlorophyll biomass (i.e. in the eddy, or in the Brazil Current, St 23 and 24, Browning et al. (2014)), coupled with a sufficient inorganic N availability (i.e., high NO_3^-), there is a decrease in the abundance of Glu and other DFAA. This is logical, as DFAA are consumed by diatoms (Admiraall et al. 1984); and more specifically, diatoms tend to utilize Asp, Glu and Arg down to levels lower than 10 nmol L^{-1} in early stages of growth but do not consume other AAs (Admiraall et al. 1984).

The comparison of DFAA concentrations, especially Glu, with other biochemical observations highlights potential new insights into the origin and fate of DFAA in the water column, and controls on the distribution of microbial populations, with our optimised analytical method. (Jørgensen et al. (2014). However, even with very precise measurement techniques, the potentially rapid turnover rate of DFAA in seawater complicates attempts to trace their origin. One possible way of overcoming this in future studies could be to apply stable isotope analyses of DFAA, however concentrating sufficient DFAA for such analyses represents a significant challenge.

Acknowledgements and funding

This work was generously supported by the NERC funded UK-GEOTRACES Consortium [grant number: NE/H006095/1] and A.J.M.S would like to warmly thank Prof. Gideon Henderson for the opportunity to join the UK-GEOTRACES cruise to collect the seawater samples. Our deepest appreciation is also extended to the officers, technician, crew and scientists on-board the R.V. James Cook. A.J.M.S also acknowledges the University of Otago, New Zealand, and the Plymouth Marine Laboratory, UK, for practical and financial support.

References

Admiraall, W., R. W. P. M. Laane and H. Peletier (1984). "Participation of diatoms in the amino acid cycle of coastal waters; uptake and excretion in cultures." Marine Ecology Progress Series 15: 303-306.

Andersson, A., C. Lee, F. Azam and H. Hagstrom (1985). "Release of amino acids and inorganic nutrients by heterotrophic marine microflagellates " Marine Ecology Progress Series 23: 99-106.

Benson, J. R. and P. E. Hare (1975). "O-phthalaldehyde: fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrin." Proceedings of the National Academy of Sciences 72(2): 619-622.

Browning, T. J., H. A. Bouman, C. M. Moore, C. Schlosser, G. A. Tarran, E. M. S. Woodward and G. M. Henderson (2014). "Nutrient regimes control phytoplankton ecophysiology in the South Atlantic." Biogeosciences 11(2): 463-479.

Capone, D. G., M. D. Ferrier and E. J. Carpenter (1994). "Amino Acid Cycling in Colonies of the Planktonic Marine Cyanobacterium Trichodesmium thiebautii." Applied and Environmental Microbiology 60(11): 3989-3995.

Carlson, C. A. and D. A. Hansell (2015). Chapter 3 - DOM Sources, Sinks, Reactivity, and Budgets. Biogeochemistry of Marine Dissolved Organic Matter (Second Edition). Boston, Academic Press: 65-126.

Carlucci, A. F., D. B. Craven and S. M. Henrichs (1984). "Diel production and microheterotrophic utilization of dissolved free amino acids in waters off Southern California." Applied and Environmental Microbiology 48(1): 165-170.

Coffin, R. B. (1989). "Bacterial uptake of dissolved free and combined amino-acids in estuarine waters." Limnology and Oceanography 34: 531-542.

Davey, M., G. A. Tarran, M. M. Mills, C. Ridame, R. J. Geider and J. LaRoche (2008). "Nutrient limitation of picophytoplankton photosynthesis and growth in the tropical North Atlantic." Limnology and Oceanography 53: 1722-1733.

Dawson, R. and K. Gocke (1977). "Heterotrophic activity in comparison to the free amino acid concentrations in Baltic Sea water samples." Oceanologica Acta 1(1).

Dawson, R. and G. Liebezeit (1981). Chapter 15 - The Analytical Methods for the Characterisation of Organics in Seawater. Elsevier Oceanography Series. E. K. Duursma and R. Dawson, Elsevier. 31: 445-496.

Dawson, R. and R. G. Pritchard (1978). "The determination of α-amino acids in seawater using a fluorimetric analyser." Marine Chemistry 6(1): 27-40.

Falkowski, P. G., R. T. Barber and V. Smetacek (1998). "Biogeochemical controls and feedbacks on ocean primary production." Science 281(5374): 200-206.

436 Fan, C., P. M. Glibert and J. M. Burkholder (2003). "Characterization of the affinity for
437 nitrogen, uptake kinetics, and environmental relationships for Prorocentrum minimum in
438 natural blooms and laboratory cultures." Harmful Algae 2(4): 283-299.
439 Fisher, G. H., I. Arias, I. Quesada, S. D'Aniello, F. Errico, M. M. Di Fiore and A. D'Aniello
440 (2001). "A fast and sensitive method for measuring picomole levels of total free amino acids
441 in very small amounts of biological tissues." Amino Acids 20(2): 163-173.
442 Fuhrman, J. (1987). "Close coupling between release and uptake of dissolved free amino
443 acids in seawater studied by an isotope dilution approach*." Marine Ecology Progress
444 Series 37: 45-52.
445 Gibb, S. W., R. G. Barlow, D. G. Cummings, N. W. Rees, C. C. Trees, P. Holligan and D.
446 Suggett (2000). "Surface phytoplankton pigment distributions in the Atlantic Ocean: an
447 assessment of basin scale variability between 50 N and 50 S." Progress in Oceanography
448 45(3): 339-368.
449 Glibert, P. M. and D. A. Bronk (1994). "Release of Dissolved Organic Nitrogen by Marine
450 Diazotrophic Cyanobacteria, Trichodesmium spp." Applied and Environmental Microbiology
451 60(11): 3996-4000.
452 Godel, H., T. Graser, P. Földi, P. Pfaender and P. Fürst (1984). "Measurement of free amino
453 acids in human biological fluids by high-performance liquid chromatography." Journal of
454 Chromatography A 297(0): 49-61.
455 Hammer, K. D. and G. Kattner (1986). "Dissolved free amino-acids in the marine-
456 environment-a carbon to nitrogen ratio shift during diatom blooms." Mar. Ecol. Prog. Ser. 31:
457 35-45.
458 Hubberten, U., R. J. Lara and G. Kattner (1995). "Refractory organic compounds in polar
459 waters: Relationship between humic substances and amino acids in the Arctic and Antarctic."
460 Journal of Marine Research 53: 137-149.
461 Hydes, D. J., Aoyama, M., Aminot, A., Bakker, K., Becker, S., Coverly,, D. S., A., Dickson,
462 A. G., Grosso, O., Kerouel, R., Van, J. Ooijen, Sato, K., Tanhua, T., Woodward, E. M. S., and
463 Zhang and J. Z. (2010). Determination of dissolved nutrients (N,P,Si) in seawater with high
464 precision and inter-comparability using gas-segmented continuous flow analysers The GO-
465 SHIP Repeat Hyrography Manual: a collection of expert reports and guidelines, IOCCP
466 report No. 14, ICPO publication series No. 134, version 1.
467 Ito, T., P. Parekh, S. Dutkiewicz and M. J. Follows (2005). "The Antarctic Circumpolar
468 Productivity Belt." Geophysical Research Letters 32: L13604,
469 doi:13610.11029/12005GL023021.
470 Jones, R. D. (1991). "An improved fluorescence method for the determination of nanomolar
471 concentrations of ammonium in natural waters." Limnology and Oceanography 36 (4): 814–
472 819.

473 Jørgensen, L., O. J. Lechtenfeld, R. Benner, M. Middelboe and C. A. Stedmon (2014).
474 "Production and transformation of dissolved neutral sugars and amino acids by bacteria in
475 seawater." Biogeosciences 11(19): 5349-5363.
476 Jørgensen, N. O. G. (1982). "Heterotrophic assimilation and occurrence of dissolved free
477 amino-acids in a shallow estuary." Marine Ecology Progress Series 8: 145-159.
478 Kaiser, K. and R. Benner (2008). "Major bacterial contribution to the ocean reservoir of
479 detrital organic carbon and nitrogen." Limnology and Oceanography 53(1): 99-112.
480 Kiel, R. G. and D. L. Kirchman (1999). "Utilization of dissolved protein and amino acids in
481 the northern Sargasso Sea." Aquatic Microbial Ecology 18: 293–300.
482 Kirchman, D. L. (1994). "The uptake of inorganic nutrients by heterotrophic bacteria."
483 Microbial Ecology 2: 255-271.
484 Kuznetsova, M., C. Lee, J. Aller and F. Nelson (2004). "Enrichment of Amino Acids in the
485 Sea Surface Microlayer at Coastal and Open Ocean Sites in the North Atlantic Ocean."
486 Limnology and Oceanography 49(5): 1605-1619.
487 Lee, K. S. and D. G. Drescher (1979). "Derivatization of cysteine and cystine for fluorescence
488 amino acid analysis with the o-phthaldialdehyde/2-mercaptoethanol reagent." The Journal of
489 Biological Chemistry 254: 6248-6251.
490 Linares, F. (2006). "Effect of dissolved free amino acids (DFAA) on the biomass and
491 production of microphytobenthic communities." Journal of Experimental Marine Biology and
492 Ecology 330(2): 469-481.
493 Lindroth, P. and K. Mopper (1979). "High performance liquid chromatographic determination
494 of subpicomole amounts of amino acids by precolumn fluorescence derivatization with o-
495 phthaldialdehyde." Analytical Chemistry 51(11): 1667-1674.
496 Longhurst, A. R. (1998). Ecological Geography of the Sea. San Diego, Academic Press.
497 Lu, X., L. Zoub, C. Clevinger, Q. Liuc, J. T. Hollibaugh and X. Moua (2014). "Temporal
498 dynamics and depth variations of dissolved free amino acids and polyamines in coastal
499 seawater determined by high-performance liquid chromatography." Marine Chemistry 163:
500 36–44.
501 Lund, B. A. and H. T. Blackburn (1989). "Urea turnover in a coastal marine sediment
502 measured by a 14C-urea short-term incubation." Journal of Microbiological Methods 9(4):
503 297-308.
504 Maie, N., K. J. Parish, A. Watanabe, H. Knicker, R. Benner, T. Abe, K. Kaiser and R. Jaffé
505 (2006). "Chemical characteristics of dissolved organic nitrogen in an oligotrophic subtropical
506 coastal ecosystem." Geochimica et Cosmochimica Acta 70(17): 4491-4506.
507 McCarthy, M. D., J. Lehman and R. Kudela (2013). "Compound-specific amino acid δ15N
508 patterns in marine algae: Tracer potential for cyanobacterial vs. eukaryotic organic nitrogen
509 sources in the ocean." Geochimica et Cosmochimica Acta 103(0): 104-120.

Moore, C. M., M. M. Mills, K. R. Arrigo, I. Berman-Frank, L. Bopp, P. W. Boyd, E. D. Galbraith, R. J. Geider, C. Guieu, S. L. Jaccard and T. D. Jickells (2013). "Processes and patterns of oceanic nutrient limitation." Nature Geoscience 6(9): 701-710.

Mulholland, M. R. and C. Lee (2009). "Peptide hydrolysis and the uptake of dipeptides by phytoplankton." Limnology and Oceanography 54(3): 856-868.

Pocklington, R. (1972). "Determination of nanomolar quantities of free amino acids dissolved in North Atlantic ocean waters." Analytical Biochemistry 45(2): 403-421.

Reason, A. J. (2003). Validation of Amino Acid Analysis Methods. Protein Sequencing Protocols. B. Smith, Humana Press. 211: 181-194.

Roach, M. C. and M. D. Harmony (1987). "Determination of amino acids at subfemtomole levels by high-performance liquid chromatography with laser-induced fluorescence detection." Analytical Chemistry 59(3): 411-415.

Rondell, J. B., K. W. Finster and B. A. Lomstein (2000). "Urea and DON uptake by a Lyngbya gracilis dominated microbial mat: a controlled laboratory experiment." Aquatic Microbial Ecology 21: 169-175.

Rosenstock, B. and M. Simon (2001). "Sources and sinks of dissolved free amino acids and protein in a large and deep mesotrophic lake." Limnology and Oceanography 46(3): 644–654.

Roth, M. (1971). "Fluorescence reaction for amino acids." Analytical Chemistry 43(7): 880-882.

Sarmento, H., C. Romera-Castillo, M. V. Lindh, J. Pinhassi, M. M. Sala, J. M. Gasol, C. Marrasé and G. T. Taylor (2013). "Phytoplankton species-specific release of dissolved free amino acids and their selective consumption by bacteria." Limnology and Oceanography 58: 1125-1135.

Shi, D., G. Yang, Y. Sun and G. Wu (2016). "Spatiotemporal variation of dissolved carbohydrates and amino acids in Jiaozhou Bay, China." Chinese Journal of Oceanology and Limnology: 1-17.

Siegel, A. and E. T. Degens (1966). "Concentration of dissolved amino acids from saline waters by ligand-exchange chromatography." Science (New York, N.Y.) 151(3714): 1098-1101.

Suttle, C. A., A. M. Chan and J. A. Fuhrman (1991). "Dissolved free amino acids in the Sargasso Sea: Uptake and respiration rates, turnover times and concentrations. ." Marine Ecology Progress Series 70: 189-199.

Tada, K., M. Tada and Y. Maita (1998). "Dissolved free amino acids in coastal seawater using a modified fluorometric method." Journal of Oceanography 54(4): 313-321.

Tappin, A. D., G. E. Millward and M. F. Fitzsimons (2010). "Particle–water interactions of organic nitrogen in turbid estuaries." Marine Chemistry 122(1–4): 28-38.

546 Van Heukelem, L. and C. S. Thomas (2001). "Computer-assisted high-performance liquid
547 chromatography method development with applications to the isolation and analysis of
548 phytoplankton pigments." Journal of Chromatography A 910(1): 31-49.
549 Webb, K. L. and R. E. Johannes (1967). "Studies of the release of dissolved free amino acids
550 by marine zooplankton " Limnology and Oceanography 12(3): 376-382.
551 Woodward, E. M. S. and A. P. Rees (2001). "Nutrient distributions in an anticyclonic eddy in
552 the northeast Atlantic Ocean, with reference to nanomolar ammonium concentrations." Deep
553 Sea Research Part II: Topical Studies in Oceanography 48(4–5): 775-793.