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Bulk vs. amino acid stable N isotope estimations of metabolic status and contributions of nitrogen fixation to size-fractionated zooplankton biomass in the subtropical N Atlantic

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ABSTRACT

A comparative analysis of natural abundance of stable N isotopes ($\delta^{15}N$) in individual amino acids and bulk organic matter of size-fractionated plankton revealed the differential impact of nitrogen fixation through the food web in a transect across the subtropical North Atlantic. All $\delta^{15}N$ measurements showed low values in the central region, followed by the western zone, while maximum $\delta^{15}N$ values were found in the eastern zone. These results were consistent with the prevalence of nitrogen fixation in the central and western zones, and the influence of the west Africa upwelling in the eastern zone. Use of compound-specific amino acid isotope data (CSI-AA) revealed relatively low variability in the impact of diazotrophic nitrogen within the different plankton size fractions, while $\delta^{15}N$ of bulk organic matter showed high variability with size. Explicit CSI-AA trophic position estimates showed a small increase with mean plankton size class and varied in a relatively narrow range 1.8–2.5), with the lowest values in the central zone. High correlations between bulk plankton $\delta^{15}N$ and individual amino acids (in particular Phe and Thr), as well as reconstructed total protein $\delta^{15}N$ values, suggest a set of new relationships that may be important to tracing direct plankton contributions to nitrogen recycling in the ocean, including detrital organic nitrogen pools. Overall, these new results represent the most detailed investigation of CSI-AA data in plankton size classes to date, and indicated a greater importance of diazotrophic N than suggested by concurrent measurements of bulk $\delta^{15}N$, abundance of large nitrogen fixing organisms or nitrogen fixation rates.

1. Introduction

Large regions of the surface Atlantic Ocean are characterized by nutrient deficient waters, where stratification of the surface reduce the input of nutrients from deep waters and the winds induce downwelling over subtropical gyres, to the north and south of the equator (Reynolds et al., 2007). In these conditions, inputs of nitrogen from the atmosphere may represent one of the main drivers of primary production (Montoya et al., 2007; Mouriño-Carballido et al., 2011). Stable isotopes can trace N_2 inputs because atmospheric nitrogen is relatively depleted in heavy (^{15}N) isotopes compared with marine nitrate (Montoya et al., 2002; Wannicke et al., 2010). Assimilation of the light N_2 by diazotrophs produces organic matter with a characteristic isotopic signature ($\delta^{15}N$, the excess in ^{15}N relative to atmospheric N) that can be traced along the food web. Therefore nitrogen isotope values in seston reflect the relative contributions of nitrate coupled with degree of uptake of atmospheric N_2 by cyanobacteria (Montoya et al., 2002, 2004; Fernández et al., 2014), while those in zooplankton reflect both trophic transfer and degree assimilation of organic matter initially produced by diazotrophs (McClelland et al., 2003).

Previous studies in the oligotrophic North Atlantic (Montoya et al., 2004; Landrum et al., 2011; Mompeán et al., 2013; Fernández et al., 2014) showed in general higher plankton $\delta^{15}N$ in eastern compared with western and central zones, consistent with the variable influence of deep water advection vs. atmospheric nitrogen fixation (Montoya et al., 2007; Benavides et al., 2013; Fernández et al., 2013). When analyzed by size fractions (Landrum et al., 2011; Mompeán et al., 2013; Fernández et al., 2014), different plankton size classes showed in general similar geographic change in $\delta^{15}N$, consistent with the propagation of the source N up the food web. However, the low $\delta^{15}N$ values measured in these regions may result either from atmospheric N_2 fixation or from a major use of regenerated nitrogen forms (mainly ammonium). As heterotrophic plankton preferentially excrete isotopically light nitrogen, meso- and macrozooplankton are expected to become

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more enriched than subsurface nitrate in the absence of significant N_2 fixation (Montoya et al., 2002), while phytoplankton (and seston) will be have lower $\delta^{15}N$ values because of the uptake of light dissolved nitrogen. Further processing of organic matter up the food web would affect the $\delta^{15}N$ of consumers depending on the fraction of their diet including N directly derived from diazotrophy vs. other sources.

Taking into account that pelagic food webs are strongly size-structured, as consumers are generally much larger than their prey, and that life span and mobility also depend on organism size, $\delta^{15}N$ signals of producers and consumers may become uncoupled (Jennings et al., 2008). This uncoupling has been observed several times in the subtropical North Atlantic in the form of lower $\delta^{15}N$ for large sized compared to small plankton (Landrum et al., 2011; Mompeán et al., 2013; Fernández et al., 2014), and implies a differential impact of N fixation across the food web. For instance, Mompeán et al. (2013) estimated a mean contribution of N from biological fixation of 43% for the 200–500 μm plankton and 54% for plankton >2000 μm in the central region of the subtropical N Atlantic, while Landrum et al. (2011) estimated contributions of $>60\%$ of N fixation for >4000 μm plankton in the western basin. These contributions were estimated by comparing bulk $\delta^{15}N$ of plankton samples between areas of presumably different nitrogen sources for primary producers. However, both the transient nature of blooms of the most conspicuous diazotrophic organisms (filament-forming *Trichodesmium*), and the persistence of low rates of fixation by small non-colonial cyanobacteria (Montoya et al., 2004) make the characterization of nitrogen sources actually contributing to local primary production difficult. Therefore more precise estimations of the contribution of different nitrogen sources, in particular N fixation, and impacts for the different organisms of the food web are required to understand main factors limiting the productivity of oceanic ecosystems.

Empirical observations have demonstrated that the $\delta^{15}N$ values in consumers increase with each trophic transfer (trophic enrichment factor, TEF), with an commonly applied average change of approximately ~3.4‰ (Post, 2002). The trophic position (TP) of consumer organisms has therefore been commonly estimated as the difference between the values of $\delta^{15}N$ of the consumer and that of primary producers scaled by the average increase between trophic transfers. Generally, whole individuals or tissues have been employed for $\delta^{15}N$ determinations ($\delta^{15}N_{bulk}$) because this analysis requires relatively small sample sizes and simple analytical preparation (e.g., Mompeán et al., 2013).

However, there are several important drawbacks to TP estimation based only $\delta^{15}N_{bulk}$. First, accumulating evidence in recent years has clearly demonstrated that the commonly used ^{15}N-enrichment factor of ~3.4‰ is far from universal, but instead can vary substantially with different species, physiology and trophic ecology. For example McCutchan et al. (2003) reported that the ^{15}N-enrichment factor varied over a range of almost 8‰ (from −2.1‰ to 5.4‰) for insects and fish. Second, the classical TP estimation requires an integrated $\delta^{15}N$ value for primary producers in a given system (often referred to as baseline $\delta^{15}N$ value). One single representative baseline $\delta^{15}N$ value typically cannot be directly measured, and is rather assumed from literature values for most common primary producers in a given system. However, primarily producers in marine ecosystems (mainly cyanobacteria and algae) can themselves show very high spatial and temporal variability in $\delta^{15}N$, due to the assimilation of various nitrogen sources (i.e., N_2, NO_3^-, NH_4^+, urea) and to their short life span (Varela et al., 2005; Fernández et al., 2013). Consequently, variability in $\delta^{15}N_{bulk}$ of marine particles (as a proxy for marine phytoplankton) have been shown to be over a factor of three greater than assumed ^{15}N-trophic enrichment factor (Hannides et al., 2009). Finally, higher TP consumers themselves are often highly mobile. Therefore, they may feed at multiple depths or across wide geographic locations, potentially having substantially different baseline $\delta^{15}N$ values that are very difficult to determine.

Amino acids (AA) are key components of organic matter participating in most metabolic and growth process. Compound specific isotope analysis of individual amino acids (CSI-AA) is a rapidly growing technique that can address many limitations of bulk $\delta^{15}N$ analysis (McClelland and Montoya, 2002; McClelland et al., 2003; McCarthy et al., 2007; Chikaraishi et al., 2009). The first published CSI-AA studies in marine plankton (McClelland and Montoya, 2002) showed that $\delta^{15}N$ values of individual amino acids ($\delta^{15}N_{AA}$) have strongly variable isotopic enrichment with trophic transfer. Trophic enrichment factors (TEF) measured for individual AA reveal that at the molecular level, they fall into two broadly different groupings. One group (now commonly termed the "trophic" AA, Popp et al., 2007) include those AA which are rapidly transaminated, and so isotopically closely linked to an organisms central N pool (e.g., McCarthy et al., 2013). Trophic AA therefore have high $\delta^{15}N_{AA}$ TEF values, typically much greater than the canonical 3.4‰ value for $\delta^{15}N_{bulk}$. In contrast, a second group (commonly termed "source" AA) have far lower $\delta^{15}N_{AA}$ TEF values. For several AA, in particular Phenlyalanine (Phe) and Methionine (Met), $\delta^{15}N$ values have been shown to remain essentially unchanged through multiple trophic transfers (McClelland and Montoya, 2002; Chikaraishi et al., 2009; Germain et al., 2013). While the precise TEF values in different organisms remains under active investigation (e.g., Germain et al., 2013; Bradley et al., 2014; McMahon et al., 2015a, 2015b), a wide variety of studies have now confirmed this fundamental behavior of the source vs. trophic AA groups (McMahon et al., 2013). In addition, recent studies have pointed out the differential TEF observed for Threonine (Thr) which may be linked to specific metabolic processing (Germain et al., 2013; McMahon et al., 2015a; Bradley et al., 2014).

The unique behavior of trophic and source AA allows key issues with interpretation of bulk $\delta^{15}N$ TP estimation to be addressed using CSI-AA. First, an organism's TP can now be directly estimated using the offset in $\delta^{15}N$ values between selected source vs. trophic AA (McClelland and Montoya, 2002; Chikaraishi et al., 2009). Importantly, this CSI-AA approach to TP estimation is "internally normalized," meaning it does not require independent characterization of the $\delta^{15}N$ values of nitrate or primary producers in a system. At the same time, the baseline $\delta^{15}N$ value can also be directly estimated by measuring source AA. Together, these aspects mean that CSI-AA can for the first time de-couple the major individual factors underlying changes in $\delta^{15}N_{bulk}$. Further, relatively little sample is required (nanomolar amounts of nitrogen) for the nitrogen isotope analysis of AA by using gas chromatography combustion/isotope ratio mass spectrometry (GC/C/IRMS). Thus CSI-AA is now becoming widely used for more precise estimation of both TP and baseline $\delta^{15}N$ values, greatly extending the understanding of the actual structures of food webs, and also nitrogen source and flow in natural environments (Chikaraishi et al., 2014; Vokhshoori and McCarthy, 2014; Sherwood et al., 2014). Some examples of recent ecological studies using this method to clarify TP of marine plankton include studies in the central Pacific (McCarthy et al., 2007), and near to Hawaii (Hannides et al., 2009). The majority of studies to date have used Phe as the best indicator of the baseline $\delta^{15}N$ value, and glutamic acid (Glu) as the most consistent indicator of trophic transfer (Chikaraishi et al., 2009).

Because CSI-AA can simultaneously supply information about TP, while also identifying the underlying N sources at the base of a food web, it is particularly well suited investigating N fixation in ecosystems, including the propagation of newly fixed N to higher trophic levels (McClelland et al., 2003; Sherwood et al., 2014). CSI-AA therefore offers a new approach to track inputs from atmospheric N to ecosystems, simultaneously corrected for change in

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TP. However, studies using planktonic CSI-AA in the subtropical N Atlantic were limited to a few samples from a single (smallest) zooplankton size class, and did not explicitly investigate CSI-AA derived TP, nor the potential to track relative diazotrophic contributions into multiple plankton size classes (McClelland et al., 2003).

Finally, CSI-AA has recently evolved very rapidly into multiple new applications, crossing ecology, paleoceanography and biogeochemical cycle research. There is growing interest in using CSI-AA parameters in different archives to reconstruct past planktonic ecosystem structure, as well as N fixation. Recent work has begun to address these questions in paleoarchives such as deep-sea corals (Sherwood et al., 2011, 2014), archived sperm whale tissues (Ruiz-Cooley et al., 2014), and ocean sediments (Batista et al., 2014). Such work fundamentally depends, however, on understanding how CSI-AA parameters may be altered in primary vs. secondary export production, However, to date, such data is extremely limited; the most detailed examination of planktonic CSI-AA data has been mostly confined to primary producers (Chikaraishi et al., 2009; McCarthy et al., 2013) while relatively scarce data exists for natural zooplankton populations.

We present here a first examination of CSI-AA values and derived ecosystem parameters in multiple plankton size fractions, across a strong ocean-basin scale gradient in nitrogen fixation and associated plankton $\delta^{15}N_{bulk}$ values in the subtropical N Atlantic. The objectives are, first to compare estimations of trophic position in size-fractions of plankton along the transect, based on $\delta^{15}N_{AA}$, and second to use CSI-AA to estimate diazotrophic N contribution to different size fractions of plankton, and compare these results with more common $\delta^{15}N_{bulk}$ approaches. To our knowledge, the relative importance of diazotrophic nitrogen input measured together with more precise CSI-AA trophic transfer values through a planktonic food web has never been directly evaluated, as in previous studies only one plankton size fraction was considered (McClelland et al., 2003). Finally, we use this unique data set of $\delta^{15}N_{AA}$ values to examine key CSI-AA parameters in diverse plankton sources. These new data should be invaluable to shape future hypotheses regarding CSI-AA parameter interpretation in both ecological and paleoceanographic studies.

2. Materials and methods

2.1. Sampling and bulk stable isotope analysis

Plankton samples were obtained during Leg 8 of the Malaspina-2010 expedition on R/V Sarmiento de Gamboa (January–March 2011), on a transect predominantly along 24°N, between the Canary Island and Florida (Fig. 1). This cruise transect and detailed sample collection protocols has been described previously (Mompeán et al., 2013). Briefly, plankton samples were collected by vertical tows of a microplankton net (40 μm mesh size) and a mesoplankton net (200 μm mesh size) through the upper 200 m of the water column. Sampling was between 10:00 and 16:00 h GMT. Plankton was separated into five size fractions (40–200, 200–500, 500–1000, 1000–2000 and 2000 μm) by gentle filtration of the samples by a graded series of nylon sieves (2000, 1000, 500, 200 and 40 μm). Large gelatinous organisms were removed before filtration. Aliquots for each size fraction were collected on pre-weighed glass-fiber filters, dried (60 °C, 48 h) and stored in a desiccator before determination of biomass (dry weight), carbon and nitrogen content and natural abundance of stable carbon and nitrogen isotopes ashore. Nominal values of the individual size of organisms in each size fraction were estimated as the geometric mean of the values defining each size interval and expressed as carbon content (μg C) in a logarithmic scale (Rodriguez and Mullin, 1986).

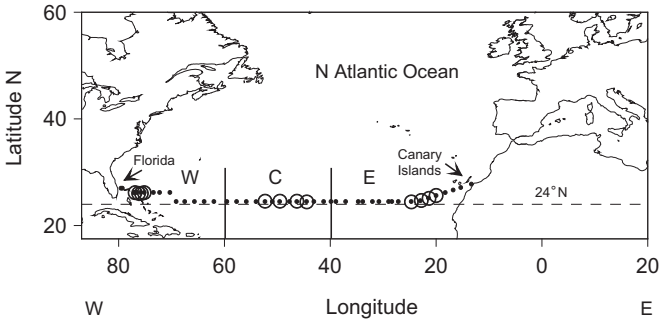

Fig. 1. Location of plankton sampling stations during cruise Leg 8 of Malaspina-2010 expedition along 24°N crossing the Atlantic basin. Open circles indicate location of samples selected for compound-specific amino acid stable nitrogen isotope analysis in western (W), central (C) and eastern (E) zones.

After determination of dry weight, finely ground aliquots of each size fraction were packed in tin capsules for elemental and stable isotope analysis by conversion into CO_2 and N_2 in an elemental analyzer (Carlo Erba CHNSO 1108) coupled to an isotope-ratio mass-spectrometer (Finnigan Mat Delta Plus). These measurements were reported as $\delta^{15}N_{bulk}$ and were already analyzed and discussed in Mompeán et al. (2013).

2.2. Compound-specific amino acid $\delta^{15}N$ analysis

Samples for CSI-AA were selected to span gradients in $\delta^{15}N_{bulk}$ values. We chose plankton from four sampling stations in each of the three zones (eastern, central and western regions). Individual samples were then pooled (quantitatively, so that each subsample was represented equally in the final composite) to have enough material in each size fraction for CSI-AA (Fig. 1). In total 15 samples in the transect were chosen for CSI-AA. Approximately 1 mg of total dry plankton material was then hydrolyzed for subsequent analysis.

The $\delta^{15}N$ values of individual AAs were measured via GC-IRMS, after 6N HCl acid hydrolysis and the formation of TFA ester derivatives following previously published methods (e.g., McCarthy et al., 2013; Germain et al., 2013). We determined $\delta^{15}N$ values for 12 AAs: glutamic acid + glutamine (Glx), aspartic acid + asparagine (Asx), alanine (Ala), isoleucine (Ile), leucine (Leu), proline (Pro), valine (Val), glycine (Gly), serine (Ser), lysine (Lys), phenylalanine (Phe), and threonine (Thr). Each AA was run four times on the GC-IRMS. Based on previous studies (e.g., McClelland and Montoya et al., 2002; Chikaraishi et al., 2009; Germain et al., 2013), AA values were categorized and presented in 3 groups, based on their relative $\delta^{15}N$ values and changes with trophic transfer: the source AAs (Gly, Ser, Lys, Phe), the trophic AAs (Glx, Asx, Ala, Ile, Leu, Pro and Val), and one "metabolic" AA (Thr).

2.3. Trophic position and related variables

To calculate CSI-AA based TP of plankton we used the most widely used current equation and TEF value, based on the isotopic offset between Glx and Phe (Chikaraishi et al., 2009):

$$TP = \left[\left(\delta^{15}N_{Glx} - \delta^{15}N_{Phe} - 3.4 \right) / 7.6 \right] + 1$$

where $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$ are measured values, +3.4‰ is the assumed isotopic difference between the Glx and Phe in primary producers, and +7.6‰ is the assumed ^{15}N enrichment in Glx relative to Phe with each trophic transfer from food source to consumer (TEF value). The standard errors in the estimation of TP, computed by propagation of analytical error in the individual AA

determinations, did not exceed 0.1 TP.

The $\delta^{15}N$ value of total hydrolysable AAs ($\delta^{15}N_{THAA}$) is used as a proxy for total protein $\delta^{15}N$ value (e.g., McCarthy et al., 2007, 2013), and was estimated as the molar-weighted average of individual $\delta^{15}N$ values:

$$\delta^{15}N_{THAA} = \Sigma\left(\delta^{15}N_{AA} mol\%_{AA}\right)$$

where $\delta^{15}N_{AA}$ is the $\delta^{15}N$ value of each individual AA measured and $mol\%_{AA}$ is the molar percentage contribution of each AA. In our study we used the $\delta^{15}N$ value of each individual AA and $mol\%_{AA}$ were obtained from Lehman (2009).

The degradation index (ΣV), proposed by McCarthy et al. (2007) as a measure of the relative resynthesis of the original autotrophic AA pool in detritus or different organisms (plankton size fractions, in our case), was computed for each size individual fraction sample as the mean deviation of $\delta^{15}N$ of individual trophic amino acid, from their average:

$$\sum V = \sum \left| AA_i - Avg_{trp} \right| / n$$

where AA_i were individual $\delta^{15}N$ amino acid values, Avg_{trp} the average value and n the total number of trophic amino acids.

2.4. Diazotrophic N contribution

The impact of N fixation ($\%N_{fix}$) was estimated for each size-fraction using either measured $\delta^{15}N_{bulk}$ and/or $\delta^{15}N_{Phe}$, where the latter was applied as an alternate indicator of the baseline $\delta^{15}N$ values (source of N for primary producers; McCarthy et al., 2013). In all cases, final calculations were based on the model of Montoya et al. (2002):

$$\%N_{fix} = 100\ (\delta^{15}N_{bulk} - \delta^{15}N_{ref}) / (\delta^{15}N_{diazo} - \delta^{15}N_{ref})$$

where $\delta^{15}N_{diazo} = -2‰$ is the value determined for N-fixers in the N Atlantic (Montoya et al., 2002) and $\delta^{15}N_{ref}$ is the value measured in reference material in areas without significant influence of diazotrophy (Montoya et al., 2002; Landrum et al., 2011, Mompeán et al., 2013).

For all samples on which we measured CSI-AA, two alternate calculations were made: one based on the measured $\delta^{15}N_{bulk}$, and a second based on CSI-AA values for $\delta^{15}N_{Phe}$. In the first case, $\delta^{15}N_{bulk}$ was set equal to average $\delta^{15}N_{bulk}$ values previously reported in this same region (Mompeán et al., 2013) and $\delta^{15}N_{ref}$ values for each size class were those reported in Landrum et al. (2011) for regions without diazotrophy. For the CSI-AA based calculation of $\%N_{fix}$, the corrected $\delta^{15}N_{bulk}$ term was estimated from the measured $\delta^{15}N_{Phe}$ values as:

$$\delta^{15}N_{bulk} = \delta^{15}N_{Phe} + \beta_{Phe}$$

where $\delta^{15}N_{Phe}$ is the measured value in each size fraction, and β_{Phe} is the average offset between phytoplankton bulk $\delta^{15}N$ and Phe values ($\delta^{15}N_{bulk} - \delta^{15}N_{Phe}$), following the convention of Chikaraishi et al. (2009). Reported β_{Phe} values ranged between ~2‰ (Chikaraishi et al., 2009) and 3.4‰ (McCarthy et al., 2013). Because these values are relatively similar, we therefore used the average β_{Phe} value from both of these studies (+2.7‰) as the best estimate currently available. In the case of CSI-AA based estimates $\delta^{15}N_{ref}$ was set to the average value reported by Montoya et al. (2002) for subsurface nitrate (mean ± se = +4.5 ± 0.3‰).

The values of $\%N_{fix}$ were finally compared with several different proxies which should be related to the input of fixed atmospheric N: $\delta^{15}N_{Phe}$ (as an estimate of the isotopic signature of the source inorganic nitrogen for primary producers), N*(stoichiometric excess of nitrogen due to remineralization, Gruber and Sarmiento, 1997) and previously reported abundance of the colonial cyanobacterium *Trichodesmium* in our sampling regions. The index N* was computed as:

$$N^* = \left[(N - 16\,P) + 2.9\right] 0.87$$

where N and P were the subnitracline (down to 300 m depth) concentrations of nitrate and phosphate, respectively. As the values of N* are arbitrary, relatively high values indicate potential areas for N fixation where nitrogen rich organic matter is remineralized while low N* values are expected in areas where denitrification prevails (Gruber and Sarmiento, 1997). Values for N, P and *Trichodesmium* abundance for the same stations where $\delta^{15}N_{AA}$ was determined were obtained from data reported in Mompeán et al. (2013).

3. Results

3.1. CSI-AA patterns across plankton size fractions

Across all size fractions, AA from in the samples from the central zone showed the lowest $\delta^{15}N$ values while those in the samples from the eastern zone showed the highest values (Fig. 2). Mean values of pooled (averaged) trophic and source AA were significantly correlated across all three zones ($r = 0.635$, $P < 0.05$, $n = 15$), with a slope of 0.85. This finding is consistent with changes in the source of nitrogen (baseline $\delta^{15}N$ values) driving most of the variability in the sample set, given the relatively similar ranges of trophic positions in samples from the different zones (Table 1).

The mean values of $\delta^{15}N_{Glx}$ (used as an indicator of TP) increased from the smallest to the largest size fractions in all zones (Table 1). Individual AA, however, displayed variability with size in the different zones. For instance, $\delta^{15}N$ of Leu, Pro, and Asp, showed small variation in their mean values within the western and central zones but $\delta^{15}N$ values clearly increased in larger size fractions in the eastern zone, following the general pattern for trophic AA. In contrast, the variability for source AA was relatively small within zones, but there were a few cases of significantly high or low values. Phe was the source AA with the lowest variability in $\delta^{15}N$ between size fractions within zones, consistent with assumptions that this AA is the best indicator of baseline $\delta^{15}N$ values. One exception was a single anomalous low value determined for the largest size fraction in the western zone. The $\delta^{15}N$ values of Thr consistently decreased with size fraction in all zones, consistent with prior indications that this unique "metabolic" AA has negative TEF values with trophic transfer (McClelland and Montoya, 2002; Germain et al., 2013). Values for $\delta^{15}N_{bulk}$ generally increased with size class (Table 1).

3.2. Trophic position estimates

Irrespective of the zone, the $\delta^{15}N$ of the average trophic AA increased significantly with organism size ($r = 0.547$, $P < 0.05$, $n = 15$), while in contrast the average $\delta^{15}N$ values for source AA were not related to size (Fig. 3a). The relative change in trophic vs. source AA values, utilizing averages of the source and trophic AA

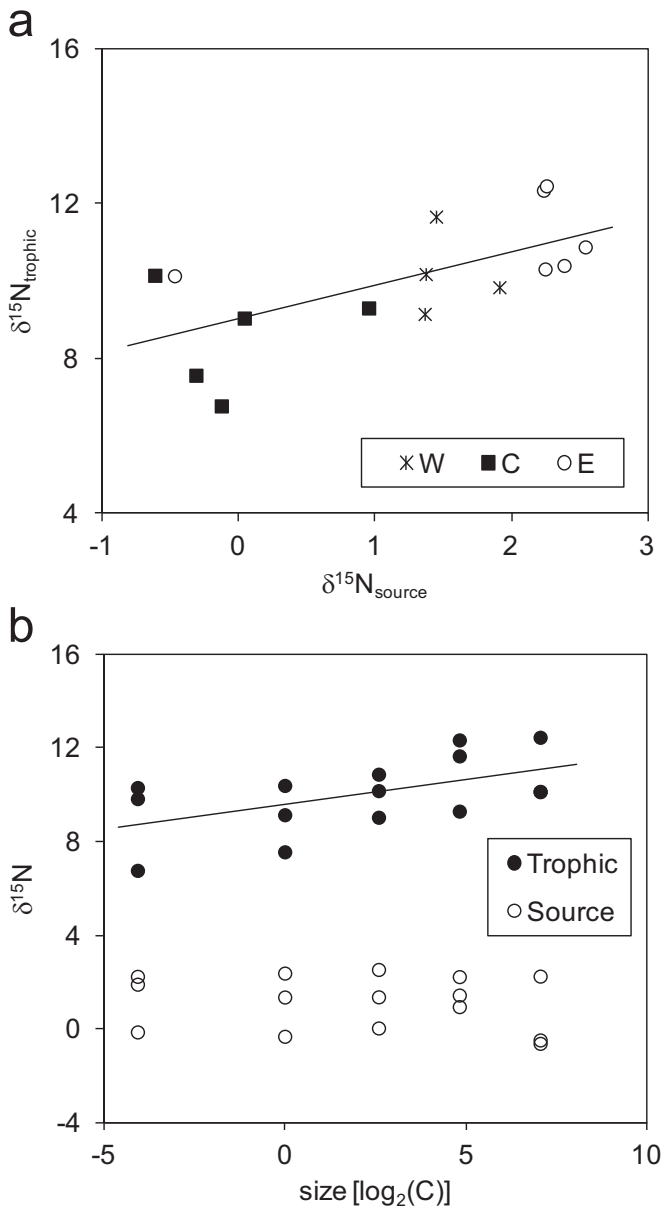

Fig. 2. Relationship between average trophic vs. average source amino acid $\delta^{15}N$ (‰), averaged across all size classes sampled in west (W), central (C), and eastern (E) Atlantic. Regression line is significant ($P<0.05$, with corresponding Pearson correlation coefficient r=0.635.

groupings, represents the broadest measure of relative stability vs. change in trophic position (Popp et al., 2007; Sherwood et al., 2014). The increasing relationship of trophic AA values vs. the constant relationship of source AA with size class therefore indicates a consistent overall increase in TP with plankton size in all zones, expressed at the molecular level in individual AA $\delta^{15}N$ values.

Explicit CSI-AA based estimates of TP varied within a relatively narrow range, between 1.8 and 2.5 (Table 1). Within zones, TP increased significantly with size, except for within the eastern zone Considering all data, there was a significant trend of increasing in mean TP with size class across all zones (r=0.675, $P<0.01$, n=15, Fig. 3b). The agreement of the broader trends in average sources and average trophic AA values (Fig. 3a) with this trend in TP values strongly supports the validity of our TP estimates, despite being derived from $\delta^{15}N$ values of two specific diagnostic AA (Phe and Glu). Further, the standard errors in the estimation of TP were quite small, not exceeding 0.1 TP (Fig. 3b), indicating quite high precision in the ability of CSI-AA to resolve TP values between plankton size classes, despite the fairly narrow range in overall TP values.

3.3. Composition and degradation indices

The CSI-AA resynthesis index (ΣV) was low in all size fractions, with an average of 1.4 (range 1.1–1.8, sd=0.7; Table 1). These values are similar to ΣV ranges previously reported for sinking particles, but are somewhat elevated vs. cultured phytoplankton (McCarthy et al., 2007), indicating the expected heterotrophic resynthesis with trophic transfers in zooplankton. In turn, $\delta^{15}N_{bulk}$ values were strongly correlated with $\delta^{15}N_{THAA}$ (r=0.965, $P<0.001$, n=14), with a slope ~1 and an intercept ~3.4‰ (Fig. 4a). There was also a significant correlation between $\delta^{15}N_{bulk}$ and the main source AA ($\delta^{15}N_{Phe}$) across zones (Fig. 4b), however with a much lower slope (~0.5).

Threonine displayed unique behavior vs. all the other AA, with a strong negative relationship between $\delta^{15}N_{Thr}$ values and all proxies for trophic transfer. When normalized for changes in the isotopic signature of the nitrogen source, $\delta^{15}N_{Thr}$ values displayed linear decreases with TP (Fig. 5). While this same correlation obtained in all zones, there were also clear differences between the regressions lines found in different zones. For example, the central and eastern zones had offset regression lines for ($\delta^{15}N_{Thr}-\delta^{15}N_{Phe}$) vs. TP, having very similar slopes (−14.928, −18.908 respectively), but significantly different intercepts (ANCOVA, $P<0.05$). The regression line for the western zone (excluding the outlier) had a lower slope than the other zones, also was not statistically significant at the 95% confidence level.

3.4. Impact of diazotrophy

The estimation of contribution of nitrogen fixation to zooplankton ($\%N_{fix}$) based on the measured $\delta^{15}N_{bulk}$ values indicated generally similar contributions from diazotrophy for all size classes in the central and western zones, where $\delta^{15}N$ enrichments were relatively low (Fig. 6a). In contrast, the eastern zone displayed a marked increase of $\%N_{fix}$ with size, consistent with values reported in Mompeán et al. (2013). The CSI-AA derived estimates of nitrogen fixation, based on corrections for baseline values using $\delta^{15}N_{Phe}$ (see Section 2), produced slightly different results (Fig. 6b). While in both bulk and CSI-AA based data $\%N_{fix}$ reached almost 50% in the central zone, $\%N_{fix}$ from the CSI-AA in the eastern and western zones were higher than $\%N_{fix}$ values derived from $\delta^{15}N_{bulk}$, reaching between 20% and 30%. Moreover the overall pattern of $\%N_{Fix}$ values vs. size class was different between the two calculations, particularly for eastern and western zones. Estimates using CSI-AA indicated approximately equal contributions of diazotrophy in all size classes for all zones, while the bulk approach showed increases of %Nfix with size only in the eastern and western zones. We note that the single very high value for the largest size fraction in the western zone (indicated by a circle in Fig. 6b) is likely an artifact, generated by a single anomalously low value for $\delta^{15}N_{Phe}$ noted above (Table 1).

The zonal variation of the new CSI-AA based estimates for $\%N_{fix}$ across all size fractions were also strongly consistent with the relative patterns in a number of ancillary measurements that would be expected to correlate with diazotrophy (Fig. 7). The $\delta^{15}N_{Phe}$ varied inversely with $\%N_{fix}$ in both the CSI-AA estimate (Fig. 7a), and the fully independent estimate derived from $\%N_{bulk}$ (not shown). Similarly, the relative offsets in N^* and the abundance of *Trichodesmium*, varied inversely with values of $\delta^{15}N_{Phe}$. These comparisons indicate that the highest impact of diazotrophy was actually in the central zone, consistent with the largest potential

Table 1
Mean $\delta^{15}N$ (‰) of individual amino acids and bulk organic matter for five plankton size fractions analyzed in the western (W), central (C) and eastern zones (E). Significant differences between fractions for each zone (ANOVA and Dunnet-C post-hoc tests, $P<0.05$) are indicated with different letters (a, b, c). Cases with no letters (e.g. Phe for C and E zones) indicate no significant differences within zones. Amino acids were grouped as trophic, source and metabolic (e.g. Germain et al., 2013). Trophic positions (TP) were computed using the $\delta^{15}N$ values of Phe and Glx (Chikaraishi et al., 2009) and the degradation index (ΣV,‰) as the average deviation from the mean $\delta^{15}N$ for trophic amino acids (McCarthy et al., 2007).

Zone	Size fraction	$\delta^{15}N$ (‰)														
		Trophic							Source				Metab.		TP	ΣV (‰)
	(μm)	Glx	Asx	Ala	Ile	Leu	Pro	Val	Gly	Ser	Lys	Phe	Thr	Bulk		
W	40–200	11.9^{a}	8.7^{b}	12.3^{b}	8.7^{a}	8.5	8.6	10.2^{a}	3.4^{c}	2.7^{b}	1.8^{b}	−0.2^{b}	−10.5^{c}	2.6^{a}	2.2^{a}	1.4
	200–500	11.7^{a}	7.8^{a}	11.4^{a}	7.9^{a}	7.4	8.4	9.5^{a}	2.3^{b}	2.6^{b}	1.3^{b}	−0.8^{b}	−10.8^{c}	2.7^{a}	2.2^{a}	1.1
	500–1000	13.2^{b}	9.0^{b}	12.5^{b}	8.5^{a}	8.5	9.8	9.7^{a}	2.9^{c}	1.3^{a}	1.0^{b}	0.3^{b}	−11.6^{b}	2.8^{a}	2.2^{a}	1.4
	1000–2000	14.7^{c}	9.9^{c}	14.8^{c}	11.9^{c}	8.5	10.2	11.7^{b}	2.1^{b}	3.2^{b}	1.0^{b}	−0.4^{b}	−13.9^{a}	3.0^{a}	2.5^{b}	1.2
	>2000	12.4^{a}	8.6^{b}	12.0^{b}	9.9^{b}	8.9	9.3	9.9^{a}	−0.8^{a}	0.4^{a}	−0.2^{a}	−1.8^{a}	−14.5^{a}	4.3^{b}	2.4^{b}	1.6
C	40–200	8.6^{a}	6.0	8.8^{a}	5.7^{a}	5.8	5.9^{a}	6.6^{a}	1.4^{d}	−0.8^{a}	0.0	−1.1	−9.9^{d}	0.4^{a}	1.8^{a}	1.8
	200–500	10.3^{b}	6.7	9.7^{b}	6.0^{a}	5.9	6.7^{b}	7.6^{b}	0.4^{b}	0.8^{b}	−0.8	−1.6	−12.9^{c}	1.1^{a}	2.1^{b}	1.5
	500–1000	11.0^{c}	7.7	11.8^{c}	7.8^{b}	7.7	8.2^{c}	9.1^{c}	0.6^{b,c}	1.2^{b}	0.1	−1.8	−15.5^{b}	0.9^{a}	2.2^{b}	1.5
	1000–2000	11.6^{d}	6.4	12.6^{d}	8.2^{b}	4.3	8.6^{c}	8.7^{d}	0.8^{c}	1.5^{b}	2.3	−0.8	−15.1^{b}	1.3^{a}	2.2^{b}	1.4
	>2000	11.9^{d}	8.4	13.4^{e}	8.7^{b}	8.7	9.9^{d}	10.0^{e}	−1.4^{a}	0.7^{b}	−0.2	−0.9	−16.7^{a}	1.9^{b}	2.2^{b}	1.6
E	40–200	13.0^{a}	9.2^{a}	12.2^{a}	8.9^{a}	8.7^{a}	9.3^{a}	10.8^{b}	3.9^{c}	2.6	2.3^{a}	0.1	−9.8^{b}	3.1	2.2	1.4
	200–500	12.2^{a}	9.8^{b}	13.2^{b}	9.0^{a}	8.5^{a}	10.0^{a}	10.1^{a}	4.0^{c}	3.2	2.7^{a}	−0.3	−10.0^{b}	3.5	2.2	1.4
	500–1000	12.7^{a}	10.0^{b}	13.7^{c}	8.8^{a}	9.2^{b}	10.9^{b}	11.0^{b}	3.9^{c}	4.0	2.2^{a}	0.0	−10.2^{b}	3.8	2.2	1.4
	1000–2000	13.9^{b}	11.1^{c}	15.1^{d}	10.8^{b}	10.6^{c}	10.9^{b}	11.6^{b}	1.7^{a}	2.9	4.2^{b}	0.7	−12.3^{a}	4.1	2.4	1.3
	>2000	14.1^{b}	10.7^{c}	15.4^{d}	11.4^{b}	11.6^{d}	11.3^{b}	12.6^{c}	3.0^{b}	3.2	2.6^{a}	0.2	−12.6^{a}	3.9	2.4	1.1

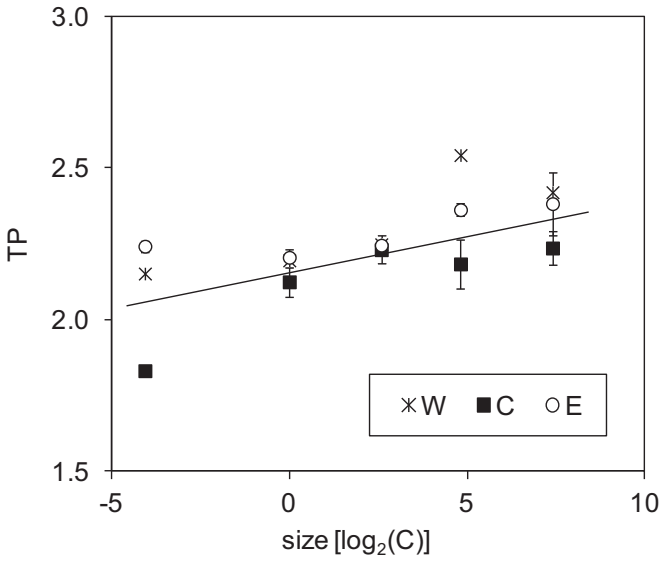

Fig. 3. The relationship between compound-specific amino acid derived indications of trophic position, and plankton size. (a) averaged trophic and source amino acid $\delta^{15}N$ (‰) values vs. plankton size class [$\log_2$(C), μg C indiv^{-1}]; the regression for only the trophic AA group is significant ($P<0.05$; Pearson r=0.547). (b) Relationship between mean (±se) CSI-AA derived trophic position (TP) and plankton size class [$\log_2$(C), μg C indiv^{-1}] in samples from western (W), central (C) and eastern (E) zones. The overall regression line is significant ($P<0.01$; Pearson r=0.675). Standard errors (se) of TP were estimated by error propagation using analytical variability for $\delta^{15}N_{Phe}$ and $\delta^{15}N_{Glx}$ (see Section 2).

for remineralization of nitrogen rich organic matter indicated by N*, along with the highest abundance of *Trichodesmium*. Indeed, pair wise differences in $\%N_{fix}$ and $\delta^{15}N_{Phe}$ between zones were not as clear as for the environmental variables associated with N fixation (ANOVA and Dunnet-C post-hoc tests, $P<0.05$).

4. Discussion

4.1. Plankton size and trophic position

Trophic positions in this study were computed with the most commonly applied CSI-AA model, based on Glu and Phe (e.g. Chikaraishi et al., 2009). The accuracy of TP estimates depends on two main factors: first, the assumed offsets between Glu and Phe in primary producers (as noted above, the β value), and second the assumed $\delta^{15}N$ change with trophic transfer (Trophic enrichment factor; TEF). Recent studies have shown that TEF values widely assumed for Glx (+7.6‰ increase with each trophic step) are likely not constant across all trophic levels, and so can result in underestimates of TP for higher level consumers. High TP consumers have now been widely reported as having low trophic enrichment factors (Popp et al., 2007; Germain et al., 2013; Bradley et al., 2014), and one recent controlled feeding study has indicated that change in TEF is biochemically predictable, linked largely to diet composition changes commonly associated with increasing trophic level (Chikaraishi et al., 2015; McMahon et al., 2015a). A recent meta-analysis of Nielsen et al. (2015) pooling data from many sources is consistent with this, and has suggested ranges of TEF values with increasing TP. Germain et al. (2013) first proposed that a multi-TEF equation was necessary for use in marine mammals, and more recent work has confirmed that a very similar multi-TEF formulation is likely needed for higher TP carnivores generally, testing it explicitly in fish and birds (McMahon et al., 2015a, 2015b; Nielsen et al., 2015).

However, for lower TP organisms, and for oceanic plankton in particular, the TEF issues documented for higher trophic level carnivores are less clear. Specifically, much of the early work which determined the "classic" TEF values for Glu and Phe were specifically conducted on plankton (e.g., McClelland and Montoya, 2002; Chikaraishi et al., 2009). Subsequent CSI-AA TP elaborations applying these TEF values to sinking marine particles and plankton tows have confirmed that they yield reasonable values (McCarthy et al., 2007; Batista et al., 2014), and these higher TEF values for the first steps of marine food webs are also supported by multi-TEF approaches (Germain et al., 2013). It is therefore not surprising that the use of the "classic" $TEF_{Glu\text{-}Phe}$ (7.6) yields reasonable TP estimates also for our plankton samples. Applying instead the meta-analysis values would result in an increase of ~0.3 TP overall, or a total TP range 2.1–2.8. Therefore, based on literature for lower TP organisms, we have elected to use here the earlier TEF values for these plankton samples as the spatial and size-related TP patterns would remain unchanged.

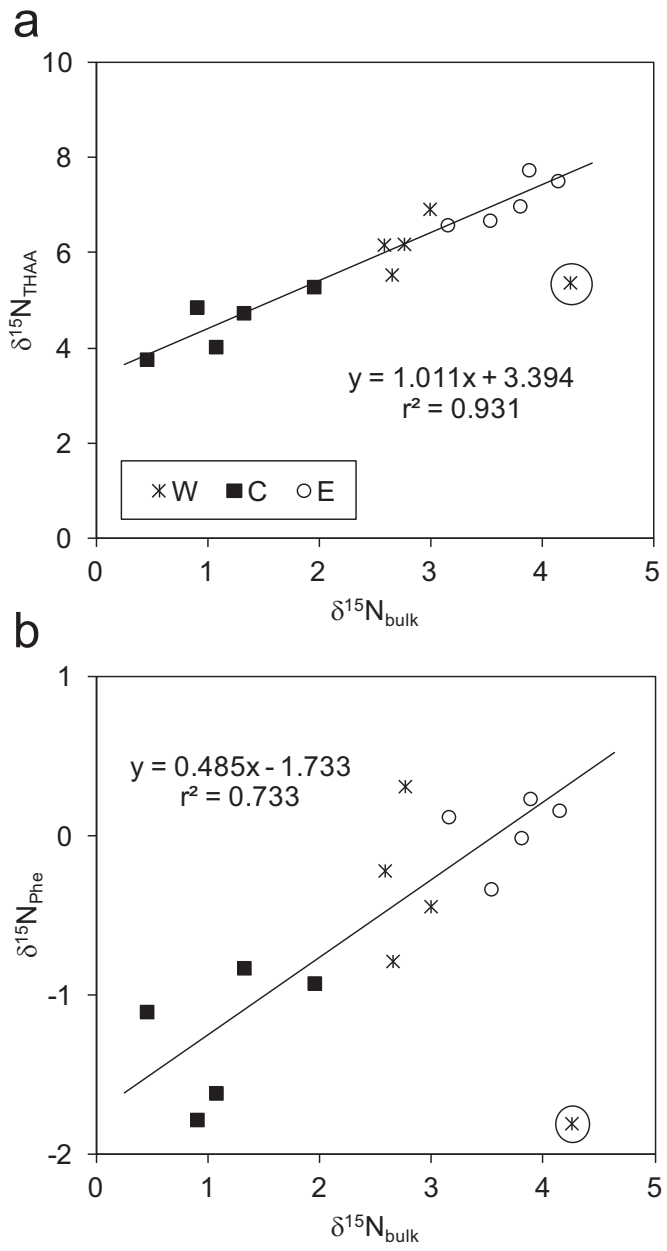

Fig. 4. Relationship between $\delta^{15}N$ in bulk plankton ($\delta^{15}N_{bulk}$) and a) total hydrolizable amino acids ($\delta^{15}N_{THAA}$), (a) proxy for total protein $\delta^{15}N$ value, and (b) Phe ($\delta^{15}N_{Phe}$) in samples from western (W), central (C) and eastern (E) zones. Both regression lines are significant ($P < 0.001$; Pearson r values are 0.964 and 0.856, for panel a and b respectively). An outlier, corresponding to the anomalously low value of $\delta^{15}N_{Phe}$ in the western zone (Table 1), was not used in the regression computations and is indicated by a circle.

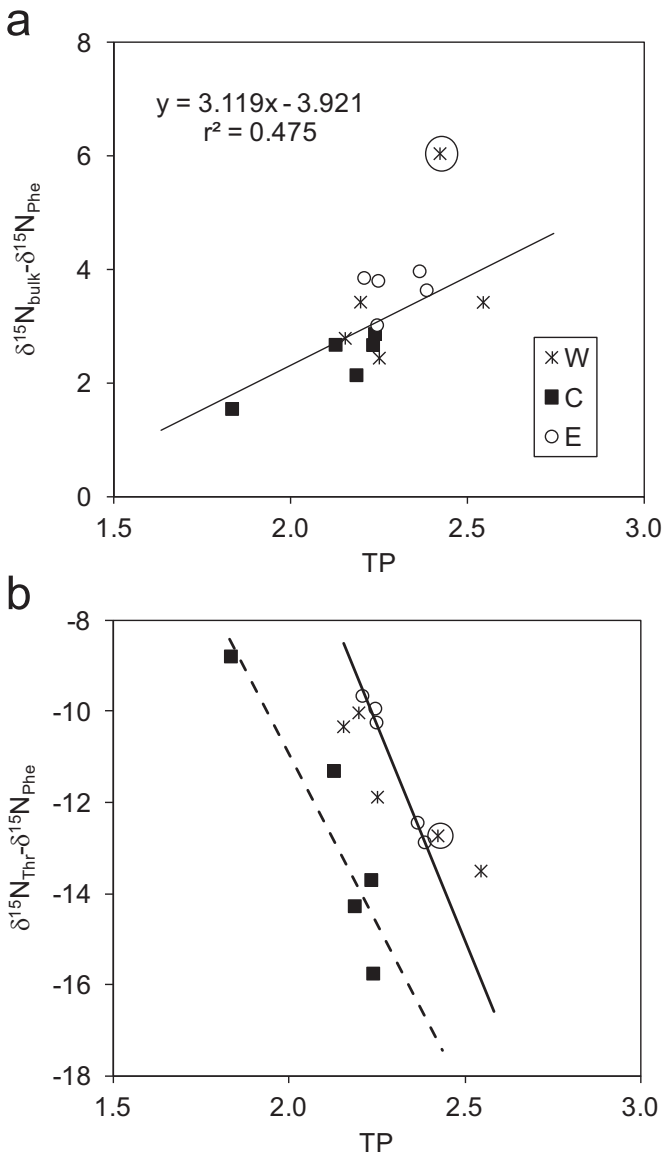

Fig. 5. Relationships between trophic position (TP) and $\delta^{15}N$ values of Thr, normalized to Phe $\delta^{15}N$, as a proxy for baseline $\delta^{15}N$ values ($\delta^{15}N_{Thr} - \delta^{15}N_{Phe}$). Regression lines fitted independently to central (dashed line) or eastern (continuous line) zones are shown (both significant; $P < 0.05$; r = 0.915 and 0.995, respectively). The corresponding regression for the western zone (line not shown) has a slightly higher slope (−8.44), but was not statistically significant. As in prior figures, the outlier $\delta^{15}N_{Phe}$ value not used in the regression computations is indicated by a circle.

The relatively small range of TP found in this study is typical of marine zooplankton estimates using CSI-AA approaches (Hannides et al., 2009; Nakatomi et al., 2013; Hannides et al., 2015). Even maximum values for the largest size fraction were < 3 TP, suggesting a large dependence from organic matter directly derived from primary producers, rather than from carnivory. This result is consistent with current ideas about omnivorous feeding for most zooplankton, particularly involving protozoans as recyclers of primary production (e.g., Calbet, 2001). Low TP values can also result from the presence of phytoplankton in some of the size fractions. For instance, the smallest size fraction (40–200 μm) sampled in this study include almost equivalent abundance of phytoplankton and zooplankton in all zones (Mompeán et al., 2013). Even when large amounts of phytoplankton were not evident by visual inspection of the larger size classes, the presence of some filaments of *Trichodesmium* cannot be discarded. This could cause some underestimation of TP values at least in the central zone, where this cyanobacterium was abundant (Fig. 7d), and where the lowest values of TP were observed (Table 1).

However, low TP values could also result from the low isotopic fractionation in some trophic steps. While the average $\delta^{15}N$ increase between subsequent trophic steps for entire food webs is well established (Post, 2002), increases observed for either top consumers (Hussey et al., 2014) or in microbial food webs (Gutiérrez-Rodríguez et al., 2014) are comparatively smaller. Therefore, the application of average TEF values could cause an underestimation of the actual number of trophic steps for individual species in areas with microbial-loop dominated food webs. In our study, the significant regression between $\delta^{15}N_{bulk}$ (after correction for variation in baseline $\delta^{15}N$, via $\delta^{15}N_{Phe}$values), and TP implies a

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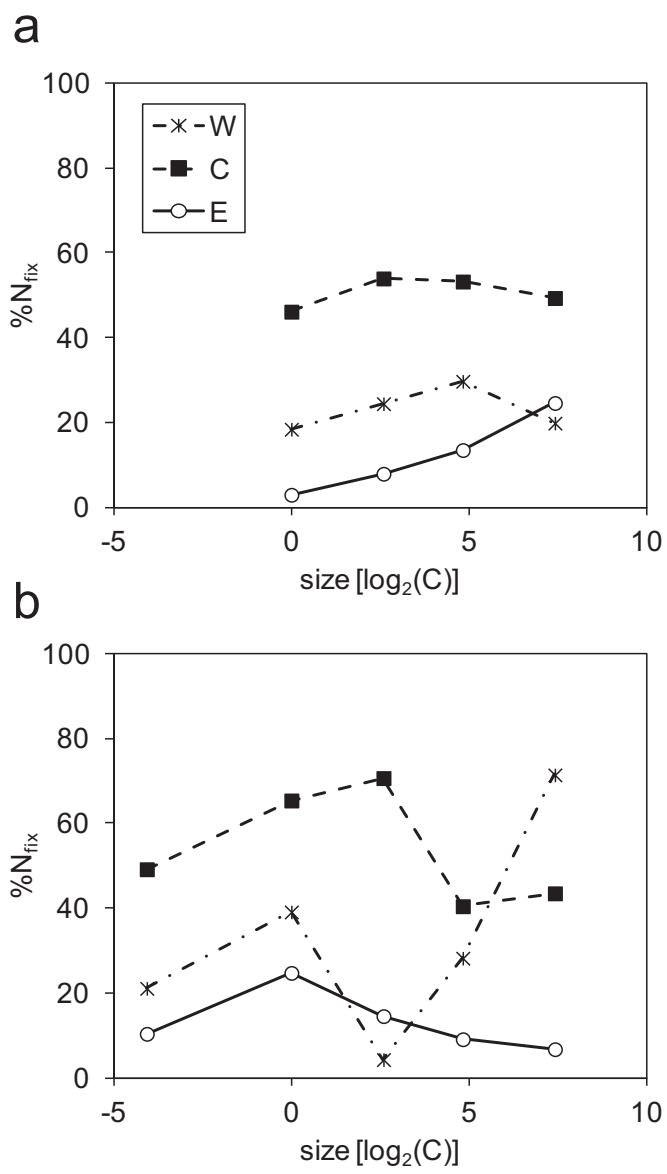

Fig. 6. Compression of estimates of contribution to N fixation (%N_{fix}) to total N in different plankton size samples from western (W), central (C) and eastern (E) zones. Plankton size classes are represented by the mean individual size [$\log_2$(C), μg C indiv^{-1}]. Estimations of %N_{fix} were computed using the mixing model of Montoya et al. (2002) and either (a) measured $\delta^{15}N_{bulk}$ (from Mompeán et al., 2013) or (b) using $\delta^{15}N_{Phe}$ as a proxy for correcting $\delta^{15}N_{bulk}$ for baseline $\delta^{15}N$. Note that the very high value for the largest size class in W zone (indicated by a circle) is likely an artifact, due to the anomalous $\delta^{15}N_{Phe}$ in this sample noted in previous figures. No estimates were made using the measured $\delta^{15}N_{bulk}$ for the smallest size class as reference values for no fixation areas were available only for plankton larger than 200 μm (Mompeán et al., 2013).

constant trophic enrichment of ca. 3‰, and thus within the range reported in the literature (Post, 2002). This supports the validity of the estimations of TP for the range of organisms collected in the different fractions, even when the inclusion of entire food webs, from microbes to top consumers, might require the application of specific TEF values.

Overall, our data shows that organism size is the key variable most closely linked to TP, as expected in size-structured marine food webs (Jennings et al., 2008). Even when the increase in TP between size fractions was small (Table 1), there were still significant correlations with $\delta^{15}N$ in source AA (Fig. 3a), and with also CSI-AA TP values (Fig. 3b), with plankton size class across all zones. This result also underscores the precision of CSI-AA TP estimates. Early work by McCarthy et. al. (2007) suggested that CSI-AA TP estimates were likely accurate to <0.5 TP, however the strong correlations we have observed in these plankton size classes suggests that much smaller fractional variations in average CSI-AA TP data are clearly meaningful. However, at the same time there was also variation in specific TP relationships between individual zones. Taken in isolation, among the three zones only the central zone showed an obvious relationship between amino acids $\delta^{15}N$ and size class (Table 1), likely due to the very low values in the smallest size fraction in this region. However, it is most likely that relatively low CSI-AA data density accounts for this, and we hypothesize a larger data set would reveal the same trends in each zone that are clear in the larger total data set. It is also possible that the mixture of organism types (autotrophic and heterotrophic) with different TP size fractions, particularly in the eastern and western zones, may have played a role in blurring relationships between isotope value and TP.

4.2. CSI-AA parameters in oceanic plankton

Our data set of CSI-AA values across multiple plankton size classes represents a unique opportunity to examine CSI-AA parameters in natural, mixed plankton end members crossing an entire ocean basin. These data have important implications for a wide range of oceanographic work. For example, rapidly expanding paleoceanographic CSI-AA applications, as in sediments (e.g., Batista et al., 2014; Larsen et al., 2015) and deep sea corals (e.g., Sherwood et al., 2014; Schiff et al., 2014), rest on assumptions about the CSI-AA signatures in planktonic sources. However, published data for natural plankton populations, and in particular zooplankton, is extremely limited.

The ΣV parameter (McCarthy et al., 2007) has now been widely applied as a proxy for degree of diagenetic resynthesis of amino acids (e.g., Calleja et al., 2013; Hannides et al., 2013; Batista et. al., 2014). This parameter has typically been used as an indicator of relative bacterial AA resynthesis. However, while ΣV has been hypothesized to also increase somewhat with metazoan heterotrophy, only a few prior data points exist for ΣV in zooplankton or mixed plankton tows (McCarthy et al., 2007). Our basin-wide observations represent, to our knowledge, the first comprehensive data on ΣV in natural mixed plankton sources. The relatively narrow ΣV range observed across our entire data set (mean ± sd = 1.4 ± 0.19) therefore is somewhat remarkable. This value for mixed zooplankton, crossing very different ocean regions, establishes a first clear threshold for ΣV values in natural zooplankton, corresponding closely with previously hypothesized values 1.0–1.5). These data support the idea that at least in oceanic material, ΣV values over ~2 represent a clear threshold for major microbial alteration (McCarthy et al., 2007).

The THAA parameter represents a proxy for $\delta^{15}N$ value of total proteinaceous material (e.g., McCarthy et al., 2013). The significant linear regression with slope of ~1between the isotopic signature of total hydrolyzable proteins ($\delta^{15}N_{THAA}$) and that of bulk organic matter $\delta^{15}N$ (Fig. 4a) is therefore consistent with all size fractions having very similar bulk biochemical composition. This further indicates, as would be expected for these samples, that analyzed organic matter is "fresh", derived from local primary production sources, without substantial input of allochthonous detritus. This inference is also supported by the significant correlation between $\delta^{15}N_{Phe}$ and $\delta^{15}N_{bulk}$ (Fig. 4b), which shows that all plankton size fraction $\delta^{15}N$ values co-vary with baseline $\delta^{15}N$ signatures. In addition to confirming similar bulk nitrogenous biochemical composition, this observation also confirms that our CSI-AA TP estimates can be confidently interpreted as representing local plankton.

The quantitative relationships between THAA, $\delta^{15}N_{Phe,}$ and $\delta^{15}N_{bulk}$ in ocean plankton sources represent critical information

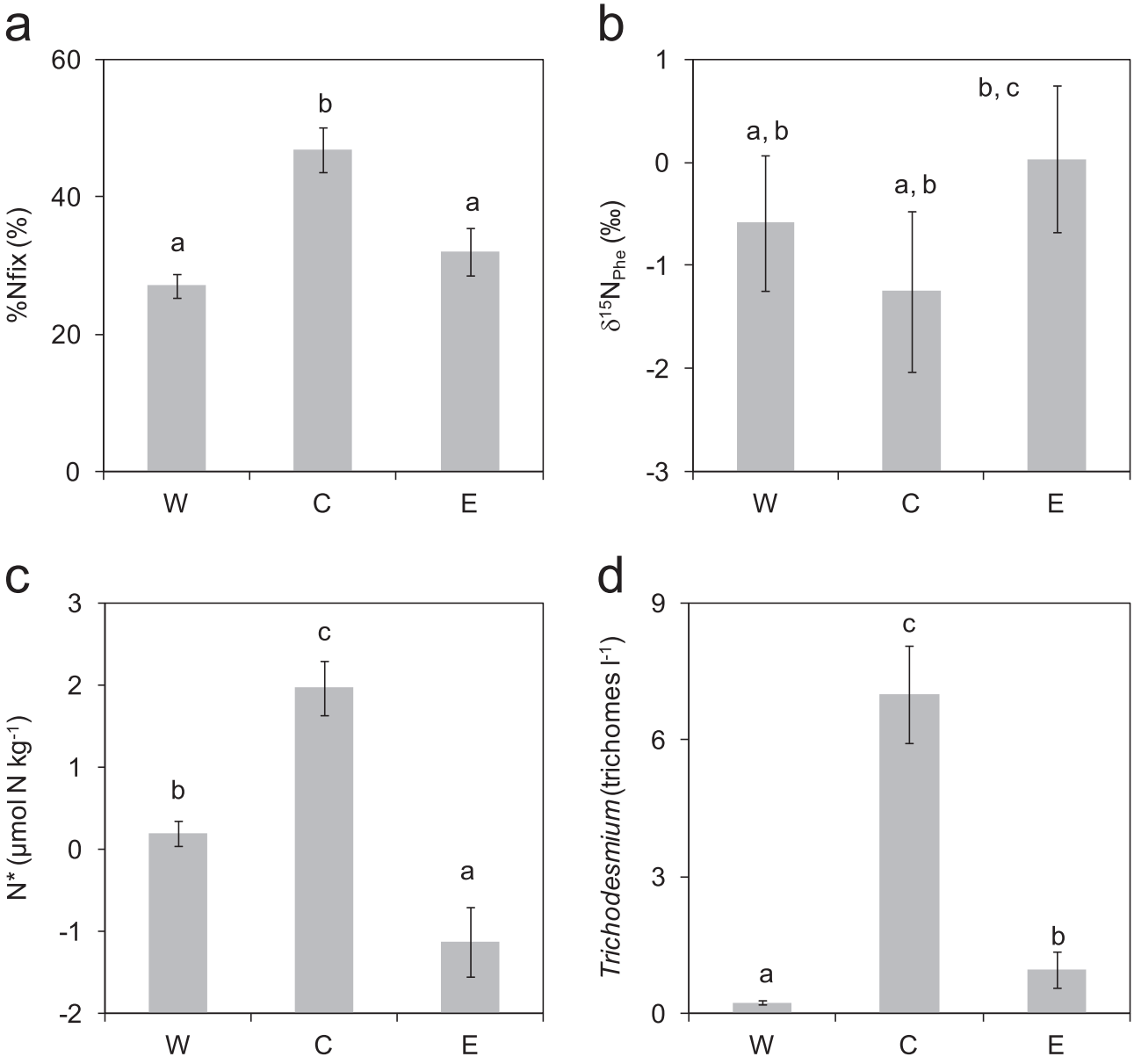

Fig. 7. Nitrogen fixation estimates compared with related parameters from western (W), central (C) and eastern (E) zones. Mean (± se) values of % N_{fix} determined using the corrected value of $\delta^{15}N_{bulk}$ as in Fig. 6b (%, panel (a), $\delta^{15}N_{Phe}$ values (‰, panel b), average N* values (µmol N kg^{-}1, panel c,) and the average abundance of *Trichodesmium* (trichomes l^{-}1, panel d). N*(Gruber and Sarmiento, 1997) was determined from nitrate and phosphate concentrations in the surface layer (0–125 m), extracted from results reported in Mompeán et al. (2013) as were *Trichodesmium* abundance data. Significant differences between zones (ANOVA and Dunnet-C post-hoc tests, $P < 0.05$) are indicated with different letters (a, b, c).

to make both paleoceanographic interrelations from CSI-AA data (e.g., Batista et al., 2014; Sherwood et al., 2014), as well to construct precise isoscapes based on CSI-AA data in modern organisms (Vokhshoori and McCarthy, 2014; Vokhshoori et al., 2014). The significant regressions we observed for both parameters in plankton sampled across the entire Atlantic basin confirm that both, THAA or $\delta^{15}N_{Phe}$ can be reasonably used to reconstruct $\delta^{15}N$ values of plankton sources. The regression intercepts (3.4‰ and −1.7‰, for THAA and $\delta^{15}N_{Phe}$ respectively) represent the key correction factors for deriving baseline $\delta^{15}N$ values from either living organisms or paleoarchives. In particular, the 3.4‰ intercept for THAA vs. bulk $\delta^{15}N$ regression is very similar to the offset found for both phytoplankton and bacteria (McCarthy et al., 2013; Batista et al., 2014). These results imply that, on average, the differential isotope fractionation between total protein and all other cellular nitrogenous molecules (nucleic acids, amino sugars, chlorophyll, etc.) is remarkably similar across multiple oceanic fresh biomass sources. The very strong correlation observed in all samples ($r^2 = 0.93$; Fig. 4a) further indicates a remarkable similarity of this $\delta^{15}N$ offset across plankton groups and size classes. Because $\delta^{15}N_{Phe}$ does not change appreciably with trophic transfer, $\delta^{15}N_{Phe}$ has often been taken as a direct proxy for baseline $\delta^{15}N$ (e.g., Vokhshoori and McCarthy, 2014; Sherwood et al., 2014). The slightly lower regression coefficient observed for $\delta^{15}N_{Phe}$ vs. $\delta^{15}N_{bulk}$ ($r^2 = 0.73$) is consistent with zonal variability in TP (and therefore in $\delta^{15}N_{bulk}$) for the different plankton size classes sampled (Fig. 3b). The offset indicated by our ocean-wide regression intercept (−1.7) is therefore expected, based on increase in $\delta^{15}N_{bulk}$ with TP in zooplankton. Nevertheless, the surprisingly strong general relationship across all size classes suggests that this value might be explored to reconstruct average zooplankton $\delta^{15}N$ values from $\delta^{15}N_{Phe}$ values measured in sinking POM or paleoarchives. For instance, the relationship could be used to obtain the expected value of $\delta^{15}N_{bulk}$ for plankton from $\delta^{15}N_{Phe}$ derived

from a sediment sample, which would allow a subsequent estimation of TP.

4.2.1. Threonine

Understanding the $\delta^{15}N$ isotopic values of Thr is currently one of the frontiers of CSI-AA work. Originally classified as a "source" AA based on early results from zooplankton (McClelland and Montoya, 2002), Thr is now recognized as unique, displaying "inverse" $\delta^{15}N$ fractionation behavior with trophic transfer. While it seems clear that Thr has novel tracer potential, its systematics are currently very poorly understood. Values of $\delta^{15}N_{Thr}$ have been suggested to represent an alternate (but inversely fractionating), indicator of trophic position (Bradley et al., 2014; McMahon et al., 2015a), a metabolic indicator for physiological stress or starvation (Hare et al., 1991), and a possibly unique tracer for the metabolism of specific organism groups (e.g. marine mammals; Germain et al., 2013). Our data set measuring $\delta^{15}N_{Thr}$ across plankton size classes therefore represents a unique opportunity to examine the systematics of $\delta^{15}N_{Thr}$, directly in the context of changing TP, across ocean basin-scale populations of similar organisms.

The depletion in the $\delta^{15}N_{Thr}$ with increasing TP of plankton (Fig. 5) supports results from other consumers (e.g. Styring et al., 2010; Sherwood et al., 2011; Germain et al., 2013), consistent with a fundamental difference between the biochemical transamination pathways for Thr vs. all other AA (McMahon et al., 2015a). Our data also indicates surprising large negative TEF values for Thr (~ -14 to -16‰, based on Phe normalized regressions), which are far greater than those originally reported for zooplankton. McClelland and Montoya (2002) originally reported TEF_{Thr} values of ~ -1‰ in limited plankton feeding experiments. Such modest TEF values were the reason for the initial classification as a "source" AA, but were also consistent with early observations of $\delta^{15}N_{Thr}$ values in sinking particles from the equatorial Pacific (McCarthy et al., 2007). However, the regressions calculated for ($\delta^{15}N_{Thr} - \delta^{15}N_{Phe}$) vs. TP in our data (Fig. 5) indicate negative TEF values for zooplankton heterotrophy at least an order of magnitude greater. The offset between the two regression lines in Fig. 5 is mainly a reflection of the offset in baseline values between the central zone (with maximum N fixation) vs. the other zones. The predicted $\delta^{15}N_{Thr} - \delta^{15}N_{Phe}$ values at TP=1 (3.99 and 13.33‰, respectively for the central and eastern zones) should indicate the average offset of $\delta^{15}N$ values of Thr vs. Phe in autotrophic primary production (i.e., the relative β values of these AA) in each zone. The values in the central zone are very similar to values observed in algal growth experiments across multiple species (~3.5‰; McCarthy et al., 2013), however the intercept in the eastern zone is inconsistent with any previous culture data, and so seems less reasonable.

While the regression results for the eastern zone suggest caution in comparing exact values, the significance of the two regressions at least implies that characteristically different TEF_{Thr} values may exist between ocean regions. If correct, this would imply that specific TEF_{Thr} values may be linked to planktonic ecosystem structure. As noted above, recent feeding experiments in fish (McMahon et al., 2015a) have suggested that variations in amino acid TEF values are strongly mechanistically linked to diet composition (in particular to relative protein content), as opposed to being linked to trophic level per se. In that data, Thr had one of the most extreme ranges in TEF values of any amino acid, (~ -2 to -10‰, depending on diet composition). The higher end of this TEF_{thr} range found in teleost fish is in fact generally similar to the TEF_{thr} implied by in our data regressions, making the very large differences in TEF_{thr} vs. the original McClelland and Montoya (2002) work puzzling. However, the apparent differences between zones do imply that $\delta^{15}N_{Thr}$ values in plankton (or sinking particles, and in turn the sedimentary record) may be linked to local food webs, suggesting future work is required to explore interpretation of $\delta^{15}Nt_{Thr}$ values in specific oceanographic regimes.

Overall, this unique data set offers several novel conclusions, but also points to a number of major uncertainties for future research. It seems clear from both regressions and directly measured values that TEF_{Thr} values in zooplankton consumers are in fact not universally low, as was indicated by early research (McClelland and Montoya, 2002; McCarthy et al., 2007). The approximate TEF_{Thr} values derived from our regressions with TP instead are more similar to the highest TEF_{Thr} values recently reported in controlled feeding experiments with fish (McMahon et al., 2015a). Together with apparent offsets in TEF between ocean zones, this may indicate far more variability in TEF_{Thr} than for other AA, suggesting food-web specific interpretation of Thr values may ultimately be required. Such differences may also underlie the large ranges in $\delta^{15}N_{Thr}$ values observed in different organisms with similar trophic levels (e.g., deep sea corals; Sherwood et al., 2014 vs. harbor seals; Germain et al., 2013), as well as differences in region-specific $\delta^{15}N_{Thr}$ values in plankton tows and sinking particles (McCarthy et al., 2007; Batista et al., 2014).

4.3. Propagation of diazotrophic N along the planktonic food web

Our new computations of $\%N_{fix}$ using CSI-AA resulted in a major increase of estimated importance of diazotrophy for the oligotrophic N Atlantic in the west and east zones, while the values estimated the CSI-AA vs. bulk $\delta^{15}N$ approaches in the central zones are both fairly similar (Fig. 6a and b). Previous estimations using $\delta^{15}N_{bulk}$ indicated than up to 65% of nitrogen in zooplankton could be derived from biological fixation of atmospheric nitrogen in the central zone, but in west and east average zonal values based on $\delta^{15}N_{bulk}$ were slightly lower (Montoya et al., 2002; Landrum et al., 2011; Mompeán et al., 2013, Fernández et al., 2014). These earlier estimates were based on the comparison of $\delta^{15}N_{bulk}$ of areas with and without significant rates of nitrogen fixation, and thus critically dependent on assumptions about $\delta^{15}N$ values for subtropical areas, rather than actual measured data. CSI-AA based $\%N_{fix}$ estimates in this study reproduced the main observation and the values of highest impact of N fixation in the central zone, however the differences in eastern and western zones suggest that $\%N_{fix}$ estimates based on broad assumptions of $\delta^{15}N_{bulk}$ values must be replaced by those based on $\delta^{15}N_{Phe}$, used as an internal, molecular level, record of baseline $\delta^{15}N$ values. According to the new estimates $\%N_{fix}$ represents up to 30% of plankton N in most of the subtropical Atlantic.

As noted above (see Section 2) the exact values for our CSI-AA based $\%N_{fix}$ approach also depend on the offset between $\delta^{15}N_{bulk}$ and $\delta^{15}N_{Phe}$ in autotrophic sources (β_{Phe}). While a single exact value of β_{Phe} cannot be definitively known for such broadly distributed natural samples, the use of $\delta^{15}N_{Phe}$ as an internal, direct proxy for baseline $\delta^{15}N$ value for every sample should yield more accurate $\%N_{fix}$ patterns. Further, variation in β_{Phe} between currently published averages would mainly slightly shift the total $\%N_{fix}$ estimates either higher or lower, but it should not affect the pattern of intra-sample offsets. Therefore, we hypothesize that CSI-AA based estimates are fundamentally more precise in their ability to indicate intra-sample variations in $\%N_{fix,}$ between different plankton size classes, or samples from different regions. The sample-specificity of correcting for baseline $\delta^{15}N$ in the CSI-AA approach is therefore in direct contrast to $\delta^{15}N_{bulk}$ based estimates, which must apply sweeping assumptions about end member $\delta^{15}N$ values to entire sample sets.

These new CSI-AA estimations of $\%N_{fix}$ in fact suggest a fairly constant transmission of the diazotrophic signal up the food web in all zones, as the $\%N_{fix}$ values showed little variation with plankton size within zones. In contrast, the estimates using the

traditional $\delta^{15}N_{bulk}$ approach showed an almost uniform contribution of diazotrophic nitrogen across plankton size fractions only in the central zone while there was an apparent increase of $\%N_{fix}$ with size in the other zones. While the increase of the diazotrophic signal in large plankton was attributed in previous studies to the effect of migrations and differences in turnover time (Landrum et al., 2011; Mompeán et al., 2013, Fernández et al., 2014) our results agree with a uniform transmission of nitrogen sources up the food web.

Finally, we note that averaging the impact of $\%N_{fix}$ across size classes in the different zones, our overall results correspond closely with several independent indicators of nitrogen fixation (Fig. 7). The filament-forming *Trichodesmium* was abundant mostly in the central zone, however also was present in some stations of the eastern zone (Mompeán et al., 2013). However, there were also other organisms responsible for nitrogen fixation, as the study of Benavides et al. (2103) showed significant nitrogen fixation rates in the $<10\,\mu m$ plankton size fraction across the same transect. Moreover, a recent study of samples from the same cruise showed the presence of different types of microbial diazotrophs in addition to *Trichodesmium* across the transect (Benavides et al., 2016). We therefore hypothesize that such small nitrogen fixers are most likely responsible for the relatively low $\delta^{15}N_{Phe}$ measured in the western zone, which clearly indicates the an isotopically low baseline nitrogen source. Another indicator of the importance of nitrogen fixation, N^*, clearly pointed to the central zone as a region with high potential for N fixation, in close agreement with our minimum $\delta^{15}N_{Phe}$ and maximum $\%N_{fix}$ values. It is also possible that low $\delta^{15}N$ values in the source nitrogen can also result from a strong fractionation of inorganic nitrogen during phytoplankton uptake (e.g., Waser et al., 1998), or from combined nitrogen in atmospheric deposition (Knapp et al., 2010). However, isotopic fractionation does not occur when dissolved nitrogen concentrations are very low (i.e., conditions of essentially complete inorganic N utilization), as in our study area (Mompeán et al., 2013), while atmospheric deposition of inorganic nitrogen is expected to be lower in the open ocean than in areas near the continents.

Overall, our data further supports the increasing evidence of a larger prevalence of nitrogen fixation in most of the subtropical N Atlantic than has been previously appreciated (Benavides et al., 2013; Fernández et al., 2014), in contrast to prior studies focused mainly on the western region with maximum abundances of *Trichodesmium* (Montoya et al., 2002, 2007; McClelland et al., 2003; Luo et al., 2012). These new results also point to the key role of zooplankton in the transmission of the diazotrophic nitrogen up the food web.

5. Overview and conclusions

The measurements in this basin-scale sample set represent, to our knowledge, the most extensive data for CSI-AA patterns in oceanic plankton. The strong correspondence in $\delta^{15}N$ values between bulk organic matter and protein-AA indicated that the relative $\delta^{15}N$ value offsets between different nitrogenous biochemical classes in plankton is remarkably homogeneous across ocean regions, and also across plankton size classes spanning several orders of magnitude. The specific offsets identified between bulk and both total protein $\delta^{15}N$ and $\delta^{15}N_{Phe}$ values also provide new and well-founded calibration relationships, necessary for reconstruction of baseline $\delta^{15}N$ values from CSI-AA data measured in paleoarchives (Sherwood et al., 2011, 2014; Batista et al., 2014). Similarly, the narrow range observed in the ΣV parameter across all samples is consistent with a low degree of microbial reworking of the organic matter collected in the size fractions, as predicted by previous studies (McCarthy et al., 2007). The obtained values can be further used as a threshold for unaltered plankton for comparison with ΣV values in processed organic matter in sinking particles and sediments. Finally, the rapid decrease of $\delta^{15}N$ values in Thr with plankton size class indicates TEF_{Thr} values far lower than previously reported for zooplankton, similar in fact to those reported in top consumers. Together with apparent offsets in TEF_{Thr} between our ocean zones, these data also implies that food web specific interpretation of Thr values should be investigated. We suggest that our new plankton data should be crucial in mechanistically understanding $\delta^{15}N_{Thr}$ systematics, and that $\delta^{15}N_{Thr}$ may be a useful new parameter to compare trophic structure of communities driven by different nitrogen sources.

While previous studies used single species or size classes (McClelland and Montoya, 2002; McClelland et al., 2003; McCarthy et al., 2007; Kruse et al., 2015), our analysis showed for the first time that CSI-AA of ocean-basin scale natural zooplankton populations can represent a realistic indicator of the sources of nitrogen at the base of the food web, and also of the relative trophic position of plankton size classes. Our CSI-AA based estimates also revealed far more homogeneous spatial patterns of nitrogen fixation relative to bulk $\delta^{15}N$ estimates. We hypothesize that because CSI-AA based estimates of N fixation are internally normalized to $\delta^{15}N$ baseline values for every sample, they are inherently more precise, removing uncertainties associated with the broad assumptions generally required with bulk $\delta^{15}N$ values, CSI-AA in plankton size fractions indicated that atmospheric nitrogen inputs affected biological production in larger areas of the subtropical N Atlantic than previous estimates have suggested. Even in regions influenced by upwelling of nutrient-rich deep waters (as in the eastern basin), or in the absence of common blooms of nitrogen-fixing organisms (as in the western zone), the isotopic signal effectively traced the relative magnitude of nitrogen sources most likely from atmospheric origin. With a trophic structure largely related to organism size rather than nitrogen source, this implies that zooplankton integrate the inputs of nitrogen fixation across time and space, ultimately transferring diazotrophic N to top consumers.

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