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## Isotopic invisibility of protozoan trophic steps in marine food webs

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### Abstract

According to modern oceanographic perspectives that emphasize microbial pathways, phagotrophic protists comprise one to several levels of intermediate consumers between phytoplankton and larger metazooplankton (copepods and krill). However, recent attempts to quantify pelagic trophic structure in the open ocean using nitrogen stable isotope techniques have brought into question whether such measurements adequately account for protistan trophic steps. Here, we use a two-stage chemostat system, with *Dunaliella tertiolecta* and *Oxyrrhis marina* as a predator–prey model, to address this question experimentally. To investigate <sup>15</sup>N trophic discrimination under different conditions of nitrogen availability and recycling, *Oxyrrhis* was fed in the light and in the dark on phytoplankton provided with high and low nutrient ratios of N:P. We used both bulk and amino acids–compound specific isotopic analysis (AA–CSIA) to distinguish trophic fractionation from changes in the  $\delta^{15}\text{N}$  values of phytoplankton (isotopic baseline). Results demonstrate that protistan consumers are not, in fact, significantly enriched in <sup>15</sup>N relative to their prey, a marked departure from the general findings for metazoan consumers. In addition, we show that changes in the isotopic baseline propagate rapidly through the protistan food chain, highlighting the need to account for this variability at ecologically relevant time scales. If protistan trophic steps are largely invisible or significantly underestimated using nitrogen isotope measurements, research that utilize such measurements in ecological, fisheries, and climate change studies may miss a large part of the ocean’s variability in food-web structure and ecosystem function.

Phagotrophic protists (protozoa) consume, on average, two-thirds of primary production in the oceans (Sherr and Sherr 2002; Calbet and Landry 2004). Consequently, temporal and spatial variability in the protistan-consumed fraction of production and the subsequent fate of this material within the microbial food web play a large role in determining whether production can be efficiently transferred to higher trophic levels or to vertical export vs. being largely respired and recycled in the euphotic zone. In our current understanding of marine microbial pathways (Pomeroy 1974; Azam et al. 1983), protistan grazers comprise one to several levels of intermediate consumers between phytoplankton and larger metazooplankton, such as copepods and krill (Calbet and Landry 1999, 2004; Sherr and Sherr 2002). Given that ~ 70% of energy is lost in each trophic step (Straile 1997), a high rate of protozoan grazing on phytoplankton, coupled with multiple levels of protistan trophic steps could lead to an order-of-magnitude less energy flow to higher levels and export for a comparable level of primary production compared with a single trophic step. Given the magnitude of the material and energy channeled through microbial-protistan components of marine food webs, the variable length of protistan grazing pathways is therefore a major uncertainty in understanding of regional differences in elemental cycling and transfer efficiencies, and their likely responses to climate change.

A comprehensive understanding of variability in protistan grazing pathways is lagging because of the difficulty of quantifying trophic steps in complex natural assemblages of interacting microbes. For larger organisms, the systematic increase in  $\delta^{15}\text{N}$  values ( $\delta^{15}\text{N} = \{({}^{15}\text{N}:{}^{14}\text{N}_{\text{sample}}):({}^{15}\text{N}:{}^{14}\text{N}_{\text{atmosphere}} - 1) \times 1000\}$ ) with trophic position (TP; DeNiro and Epstein 1981; Minagawa and Wada 1984; Peterson and Fry 1987) has proven extremely useful for investigating food-web relationships (Boecklen et al. 2011), especially in pelagic ecosystems (Cabana and Rasmussen 1996; Bode et al. 2007) where widespread omnivory greatly enhances the complexity of nutritional sources that would need to be considered in a rigorous dietary analysis (Isaacs 1972). The <sup>15</sup>N content of consumers is determined by the  $\delta^{15}\text{N}$  values of the nitrogenous nutrients taken up by phytoplankton (i.e., the primary producers) and modified by successive metabolic <sup>15</sup>N enrichment of ~ 3–4‰ at each trophic step (Peterson and Fry 1987; Cabana and Rasmussen 1996).

Because heterotrophic protists play such an important role in pelagic food webs, the question of their <sup>15</sup>N trophic enrichment is at the heart of any study that uses nitrogen isotope analysis of suspended particulate organic matter (POM) and bulk plankton to infer trophic structure and connections in the lower food web. Past studies have attributed less-than-expected <sup>15</sup>N enrichment in field observations to a number of factors, including sampling of mixtures of detrital and living material, variable N uptake and release patterns (Rau et al. 1990), overlapping sizes of autotrophs and heterotrophs (Fry and Quiñones 1994), and decreased trophic complexity (fewer steps; Rolff 2000), to name a few. In addition, variability in nitrogen sources and the rapid turnover of phytoplankton make it

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difficult to assess  $\delta^{15}\text{N}$  values at the base of the food web (i.e., the isotopic baseline). In contrast to traditional bulk N isotope analysis of organism tissues or whole organisms, which requires analysis of individual samples of consumer and primary producer to establish relative TP, Amino Acid Compound-Specific Isotope Analysis (AA-CSIA) distinguishes ‘source’ and ‘trophic’ contributions, allowing trophic hierarchical position to be determined from only consumer samples based on the differential  $^{15}\text{N}$  enrichment of individual amino acids (AAs). As generally applied, some AAs (e.g., phenylalanine) retain  $\delta^{15}\text{N}$  values similar to the baseline value for primary producers, while other AAs (e.g., glutamic acid) become highly enriched in  $^{15}\text{N}$  with each trophic transfer (McClellan and Montoya 2002; Chikaraishi et al. 2009).

In natural samples, phagotrophic protists are too small and too intermixed with other organisms to be isolated in sufficient quantities for analysis by either bulk or AA-CSIA methods. However, using AA-CSIA, the contributions of protistan grazers to food-web structure and flows can be explored from the  $^{15}\text{N}$  enrichment in trophic relative to source AAs of larger suspension-feeding metazoans, such as copepods (Hannides et al. 2009) and krill (Schmidt et al. 2006), which feed on, and integrate over, mixed assemblages of primary producers and small consumers. With this approach we can, in principle, overcome the methodological issues arising from rapid phytoplankton turnover, and variable contributions of living and dead material to POM, as well as time lags in the isotopic steady-state between consumer and diet.

Initial efforts to do this with planktonic crustaceans from the North Pacific Subtropical Gyre (NPSG; Hannides et al. 2009) have, however, yielded the unexpected result that protistan trophic steps appear to contribute negligibly to  $^{15}\text{N}$  enrichment of higher level consumers. The phytoplankton community of the NPSG, for example, is dominated by tiny cells ( $< 2\text{--}3\ \mu\text{m}$ , mainly photosynthetic bacteria and small flagellates; Campbell et al. 1997) that fall outside of the normal size range of effective feeding by mesozooplankton. Experimental studies in the region have also demonstrated trophic cascades involving multiple levels of protistan consumers that respond to the top-down predatory effect of larger zooplankton (Calbet and Landry 1999). An active microbial trophic pathway involving intermediate links of protistan grazers is therefore essential for moving biomass and energy to higher levels in such a system (Calbet and Landry 1999, 2004). Nonetheless, in their analyses of large suspension-feeding copepods from the NPSG, Hannides et al. (2009) found AA  $\delta^{15}\text{N}$  values that were not significantly different from those expected for pure herbivores (TP = 2), implying an absence of protistan trophic links.

Motivated by these results, we designed chemostat experiments to test whether protistan grazers themselves or some aspect of protistan grazer systems (e.g., strong trophic coupling [Strom 2002] or high rates of nitrogen cycling [Goldman et al. 1985]) might lead to systematic misrepresentation of ocean trophic structure by nitrogen isotope methods. Such laboratory-based experiments offer an alternative platform for testing food-web-related

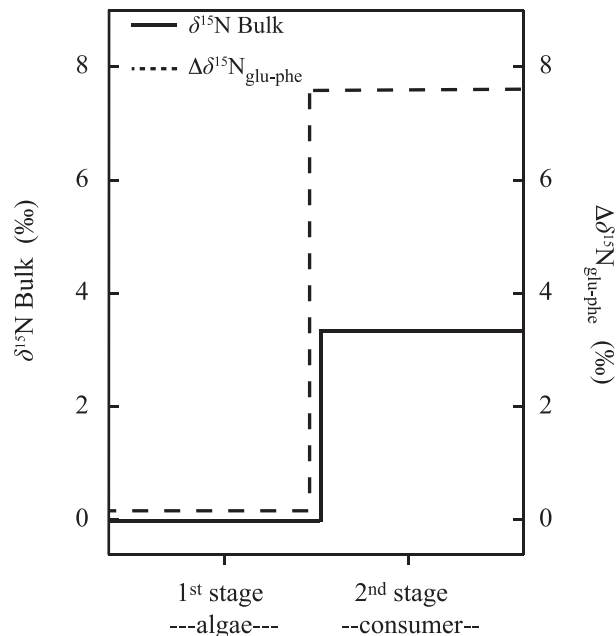


Fig. 1. Schematic representation of the  $^{15}\text{N}$  trophic enrichment in the laboratory protistan food chain expected for bulk and compound-specific nitrogen isotopic compositions of amino acids. According to conventional usage, the  $\delta^{15}\text{N}$  values of bulk consumer tissue are expected to be  $\sim 3.4\text{‰}$  higher than food resources for each trophic step (DeNiro and Epstein 1981; Minagawa and Wada 1984; Peterson and Fry 1987), and the difference in  $\delta^{15}\text{N}$  values of glutamic acid and phenylalanine ( $\Delta\delta^{15}\text{N}_{\text{glu-phe}}$ ) in the consumer should increase by  $\sim 7.6\text{‰}$  for each trophic step (McClellan and Montoya 2002; Chikaraishi et al. 2009).

hypotheses when the complexities of natural, and particularly highly dynamic microbial-protistan systems, preclude robust testing in situ (Kaunzinger and Morin 1998). In the present study, we constructed a simple microbial predator-prey system in a two-stage chemostat, with autotrophic prey growing in the first-stage reactor and feeding continuously to a protistan grazer in the second stage. Figure 1 shows the expected difference in  $\delta^{15}\text{N}$  values between protistan consumers (second stage) and algal food (first stage) if they followed the general rules of  $^{15}\text{N}$  trophic enrichment in bulk tissues found in metazoans (DeNiro and Epstein 1981; Minagawa and Wada 1984; Peterson and Fry 1987) and specific AAs (McClellan and Montoya 2002; Chikaraishi et al. 2009). In addition, we generated different nutrient conditions for phytoplankton growth in the first stage (high and low N:P ratios) and trophic coupling scenarios in the second stage (light and dark) to specifically investigate nitrogen availability and grazing-mediated recycling as potential mechanisms modifying nitrogen isotope discrimination in microzooplankton. We expected that the combination of low nitrogen availability (low N:P) and active algal growth (light) in the second chemostat reactor would lead to significantly reduced trophic isotopic fractionation by the protistan grazer because nitrogen would be efficiently recycled, homogenizing isotopic compositions of producers and consumers. Conversely, excess nitrogen (high N:P) and/or dark

second-stage conditions were expected to short-circuit nitrogen algal reuse of excreted nitrogen, maximizing trophic isotopic fractionation in the protistan grazer.

## Methods

**Experimental design**—Experiments were performed in a two-stage chemostat system under constant light and temperature ( $120 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ ,  $18^\circ\text{C}$ ) in a controlled culture room. Nutrients were pumped first from a 20 liter media reservoir to a 2.2 liter first-stage reactor containing the green alga *Dunaliella tertiolecta* (CCMP 1720) through a platinum-cured silicone tube. Outflow from the first stage was then split into two parallel second-stage reactors (1.8 liters) containing the phagotrophic dinoflagellate *Oxyrrhis marina* (CCMP 1739). One of the second-stage reactors was incubated in the light (same illumination as the first stage) to promote algal growth and nutrient recycling in the presence of protozoan consumers, while the other was incubated in the dark to suppress algal growth and recycling. In all experiments, algal biomass in the second-stage reactors was tightly controlled by grazing, such that the contribution of algae to total biomass remained on average below 0.5% and 4% in light and dark treatments, respectively, for the last 5 d of the experiment. These relatively small contributions of algal biomass allowed us to attribute the vast majority of nitrogen isotope values of particulate organic matter collected in the second stages to the protistan grazer.

Nutrient media were prepared with autoclaved-filtered seawater (0.1  $\mu\text{m}$  Suporcap capsule) following f/2 Guillard medium recipe for all nutrients except for  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$ , which were added in f/20 proportions to yield N:P mol ratios of 2.4 (expt 1) and 244 (expt 2). A peristaltic pump was used to ensure constant flow at dilution rates of  $0.50 \text{ d}^{-1}$  (first stage) and  $0.30 \text{ d}^{-1}$  (second stages), with day-to-day variability of  $\sim 10\%$  of the dilution rate in all reactors. Experiments were run for 15 (expt 1) and 12 d (expt 2) until populations reached steady state. Samples for particle counts, inorganic nutrients, and bulk isotope analysis were taken daily. Cell counts and biovolumes were measured using an Elzone II particle analyzer with a calibrated  $95 \mu\text{m}$  orifice tube. Cell populations were clearly distinguishable by a unimodal peak in the 6–10  $\mu\text{m}$  size range for *D. tertiolecta* (in the first stage), and in the 10–22  $\mu\text{m}$  size range for *O. marina* (second stage). Aliquots of 25 mL from each treatment were collected on pre-combusted 25 mm glass fiber filter (GFF) filters for subsequent bulk  $\delta^{15}\text{N}$  analyses of POM. The filtrate was frozen at  $-20^\circ\text{C}$  for determination of inorganic nutrient concentrations. For the final AA-CSIA samples, we filtered the entire contents of each treatment bottle onto a pre-combusted 47 mm GFF. All filters were immediately frozen at  $-20^\circ\text{C}$  and later dried at  $60^\circ\text{C}$  for 24 h for isotope analyses.

**Bulk stable nitrogen isotope analysis**—Bulk stable isotope analyses were performed using an on-line carbon–nitrogen analyzer coupled with an isotope ratio mass spectrometer (Finnigan ConFlo II/DeltaPlus). Isotope values are

reported using standard  $\delta^{15}\text{N}$  notation relative to the atmospheric  $\text{N}_2$  standard ( $\delta^{15}\text{N} = \{({}^{15}\text{N}:{}^{14}\text{N}_{\text{sample}}) : ({}^{15}\text{N}:{}^{14}\text{N}_{\text{atmosphere}} - 1) \times 1000\}$ ).

**AA hydrolysis and derivatization**—Samples for AA-CSIA analysis were subject to acid hydrolysis, esterification of the carboxyl terminus, and trifluoroacetylation of the amine group (Macko et al. 1997; Popp et al. 2007; Hannides et al. 2009). Samples were hydrolyzed by adding high-performance liquid chromatography grade  $6 \text{ mol L}^{-1}$  HCl to each sample vial (containing 1–2 mg of zooplankton). Each vial was then flushed with  $\text{N}_2$ , capped with a Teflon-lined cap, and heated at  $150^\circ\text{C}$  for 70 min. Acid hydrolysis destroys tryptophan and cystine, and converts asparagine to aspartic acid and glutamine to glutamic acid. The resulting hydrolysate was evaporated to dryness under  $\text{N}_2$  at  $55^\circ\text{C}$ , re-dissolved in 1 mL  $0.01 \text{ mol L}^{-1}$  HCl, purified by filtration ( $0.45 \mu\text{m}$  hydrophilic filter), and washed with 1 mL of  $0.01 \text{ mol L}^{-1}$  HCl. The hydrolysate was further purified using cation-exchange chromatography with a 5 cm column of resin (Dowex 50WX8-400) prepared in a glass Pasteur pipette (Metges et al. 1996). AAs were eluted with 4 mL of  $2 \text{ mol L}^{-1}$   $\text{NH}_4\text{OH}$  and evaporated to dryness under a stream of  $\text{N}_2$  at  $80^\circ\text{C}$ . Samples were then re-acidified with 0.5 mL of  $0.2 \text{ mol L}^{-1}$  HCl, flushed with  $\text{N}_2$ , heated to  $110^\circ\text{C}$  for 5 min, and evaporated to dryness under  $\text{N}_2$  at  $55^\circ\text{C}$ . Hydrolyzed samples were esterified with 2 mL of 4:1 isopropanol:acetyl chloride, flushed with  $\text{N}_2$  and heated to  $110^\circ\text{C}$  for 60 min. After drying at  $60^\circ\text{C}$  under  $\text{N}_2$ , the samples were acylated by adding 1 mL of 3:1 methylene chloride:trifluoroacetic anhydride (TFAA) and heated to  $100^\circ\text{C}$  for 15 min. The derivatized AAs were further purified by solvent extraction following (Ueda et al. 1989). The acylated AA esters were evaporated at room temperature under  $\text{N}_2$  and re-dissolved in 3 mL of 1:2 chloroform:P-buffer ( $\text{KH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$  in Milli-Q water, pH 7). Vigorous shaking ensured that the derivatized AAs were partitioned into chloroform and that contaminants remained in the P-buffer. The solvents were separated by centrifugation (10 min at 600 g), the chloroform was transferred to a clean vial, and the solvent extraction process repeated. Finally, to ensure derivatization, the acylation step was repeated. Samples were stored at  $-20^\circ\text{C}$  in 3:1 methylene chloride:TFAA for up to 2 weeks until analysis of the full batch was completed.

**Compound-specific stable nitrogen isotope analysis**—AA derivatives were then analyzed by isotope monitoring gas chromatography–mass spectrometry (Popp et al. 2007). We used a Delta V Plus mass spectrometer interfaced with a Trace GC gas chromatograph through a GC-C III combustion furnace ( $980^\circ\text{C}$ ), reduction furnace ( $650^\circ\text{C}$ ), and liquid nitrogen cold trap as described in Hannides et al. (2009) and Dale et al. (2011). Internal reference compounds (aminoadipic acid and norleucine) of known nitrogen isotopic composition were co-injected with samples and used to normalize the measured  $\delta^{15}\text{N}$  values of unknown amino acids. A suite of eight AAs with known isotopic composition was analyzed every three injections for

Table 1. Mean nutrient concentrations and N:P mol ratios in the initial media and chemostat cultures. Vitamins and trace metals were added following *f/2* Guillard medium recipe. The same batch of filtered seawater was used for both experiments. Mean ( $\pm$  SD) calculated for the last 8 d before harvesting the cultures.

Nutrient media	Expt 1 (N:P=2.4)		Expt 2 (N:P=244)	
N:P (mol: mol)	2.4		244	
NO <sub>3</sub> ( $\mu$ mol L <sup>-1</sup> )	88		880	
PO <sub>4</sub> ( $\mu$ mol L <sup>-1</sup> )	36		3.6	
First stage				
NO <sub>2</sub> ( $\mu$ mol L <sup>-1</sup> )	2.53 $\pm$ 0.37		1.24 $\pm$ 0.40	
NO <sub>3</sub> +NO <sub>2</sub> ( $\mu$ mol L <sup>-1</sup> )	29.1 $\pm$ 3.3		816 $\pm$ 39	
NH <sub>4</sub> ( $\mu$ mol L <sup>-1</sup> )	1.04 $\pm$ 1.1		1.68 $\pm$ 2.2	
PO <sub>4</sub> ( $\mu$ mol L <sup>-1</sup> )	6.41 $\pm$ 1.1		0.15 $\pm$ 0.04	
Second stage				
	Light	Dark	Light	Dark
NO <sub>2</sub> ( $\mu$ mol L <sup>-1</sup> )	1.13 $\pm$ 0.12	1.99 $\pm$ 0.36	0.48 $\pm$ 0.07	0.85 $\pm$ 0.31
NO <sub>3</sub> +NO <sub>2</sub> ( $\mu$ mol L <sup>-1</sup> )	5.77 $\pm$ 1.0	24.6 $\pm$ 1.2	773 $\pm$ 41	772 $\pm$ 38
NH <sub>4</sub> ( $\mu$ mol L <sup>-1</sup> )	0.83 $\pm$ 0.79	6.43 $\pm$ 0.68	0.96 $\pm$ 0.68	2.51 $\pm$ 1.01
PO <sub>4</sub> ( $\mu$ mol L <sup>-1</sup> )	8.73 $\pm$ 0.64	14.24 $\pm$ 0.75	0.10 $\pm$ 0.01	0.11 $\pm$ 0.04

additional quality control. Samples were analyzed at least in triplicate (three injections per sample). Typically, all three runs were within 1%, averaging 0.7‰ for all AAs and ranging from 0.01‰ to 3.4‰. All isotope measurements were done at the University of Hawaii, Isotope Biogeochemistry Laboratory.

*Statistical analysis*—Independent *t*-tests were used to compare the mean values of two groups. All statistical analyses were conducted using GraphPad Prism Version 5 (GraphPad Software).

## Results

The mean concentrations of inorganic nutrients established at steady state during the last 8 d of each experiment varied with the different conditions of nutrient availability and light (nitrogen recycling; Table 1). In the low N:P experiment, phytoplankton uptake in the light treatments consumed the majority of the nitrogen pool. Final nitrate

concentrations were  $\sim$  30% and 7% of initial concentration in the first and second light stages, respectively (Table 1). Conversely, nitrogen availability in the high N:P experiment remained far in excess in all treatments (Table 1). The opposite pattern was observed for phosphorus concentrations, with most of the available inorganic phosphorus pool being consumed in the high N:P but not in the low N:P experiments (Table 1). Increased ammonium concentrations in the second-stage dark treatments relative to the first- and second-stage light treatments were indicative of grazing-mediated nutrient regeneration and active phytoplankton uptake, respectively (Table 1).

Differences in nitrogen availability between experiments were evident in bulk  $\delta^{15}\text{N}$  values (Table 2). For instance, the higher  $\delta^{15}\text{N}$  values in the first stage of the low N:P experiment ( $\delta^{15}\text{N} = 3.9 \pm 0.6$ , mean  $\pm$  standard deviation [SD]) compared with those in the high N:P experiment ( $\delta^{15}\text{N} = 2.1 \pm 0.0$ , *t*-test, *t* = 4.5, degrees of freedom [df] = 2, *p* = 0.046) confirm that lower isotopic fractionation by phytoplankton occurs when a greater proportion of the

Table 2. Bulk and AA  $\delta^{15}\text{N}$  values of suspended POM in the first and second stages of the two chemostat experiments (N:P = 2.4 (mol: mol) and N:P = 244). Bulk mean ( $\pm$  SD) values are averaged for the last 2 d of each chemostat experiment. Mean values ( $\pm$  SD) for individual amino acids are for replicate subsamples collected during the last day of each experiment, except for serine and threonine in N:P = 244 Second dark. Estimates ( $\pm$  SD) for these two AA are based on triplicate injections of the same subsample.

	N:P=2.4			N:P=244		
	First stage	Second light	Second dark	First stage	Second light	Second dark
Bulk	3.9 $\pm$ 0.6	5.3 $\pm$ 0.4	4.3 $\pm$ 0.1	2.1 $\pm$ 0.0	3.6 $\pm$ 0.3	2.0 $\pm$ 0.1
Alanine (Ala)	-1.5 $\pm$ 1.3	7.8 $\pm$ 0.5	7.2 $\pm$ 0.2	-4.1 $\pm$ 0.5	4.6 $\pm$ 0.3	1.5 $\pm$ 1.3
Aspartic acid (Asp)	7.8 $\pm$ 0.7	9.1 $\pm$ 0.3	9.0 $\pm$ 0.6	6.4 $\pm$ 0.5	6.0 $\pm$ 0.8	4.9 $\pm$ 0.5
Glutamic acid (Glu)	7.9 $\pm$ 0.4	9.6 $\pm$ 0.5	8.9 $\pm$ 0.1	5.8 $\pm$ 0.1	5.9 $\pm$ 0.1	4.0 $\pm$ 0.8
Leucine (Leu)	3.7 $\pm$ 0.7	6.7 $\pm$ 0.5	5.5 $\pm$ 0.2	1.1 $\pm$ 1.1	3.5 $\pm$ 0.4	0.9 $\pm$ 0.1
Isoleucine (IsoL)	5.1 $\pm$ 0.6	7.7 $\pm$ 1.1	6.9 $\pm$ 0.7	2.3 $\pm$ 0.9	3.2 $\pm$ 0.3	3.9 $\pm$ 3.4
Proline (Pro)	7.0 $\pm$ 1.4	11.8 $\pm$ 0.8	10.0 $\pm$ 0.1	5.8 $\pm$ 0.7	9.4 $\pm$ 0.9	8.2 $\pm$ 0.9
Valine (Val)	7.9 $\pm$ 0.6	9.3 $\pm$ 1.0	8.8 $\pm$ 0.3	5.7 $\pm$ 0.1	6.4 $\pm$ 0.5	4.9 $\pm$ 0.2
Glycine (Gly)	-3.4 $\pm$ 1.9	2.2 $\pm$ 0.4	2.6 $\pm$ 0.8	-4.1 $\pm$ 0.4	1.4 $\pm$ 0.1	-0.9 $\pm$ 1.8
Lysine (Lys)	4.5 $\pm$ 1.9	7.7 $\pm$ 0.2	5.2 $\pm$ 0.4	1.7 $\pm$ 2.5	4.3 $\pm$ 0.6	3.3 $\pm$ 1.8
Phenylalanine (Phe)	3.9 $\pm$ 0.4	5.8 $\pm$ 0.4	3.7 $\pm$ 0.6	0.6 $\pm$ 1.0	2.0 $\pm$ 0.4	0.7 $\pm$ 1.7
Serine (Ser)	3.0 $\pm$ 3.0	1.7 $\pm$ 1.0	1.4 $\pm$ 0.7	-0.5 $\pm$ 0.4	-1.1 $\pm$ 0.3	-3.2 $\pm$ 0.6
Threonine (Threo)	8.4 $\pm$ 0.5	7.7 $\pm$ 0.4	5.7 $\pm$ 0.1	6.1 $\pm$ 1.0	5.8 $\pm$ 0.6	4.0 $\pm$ 0.6

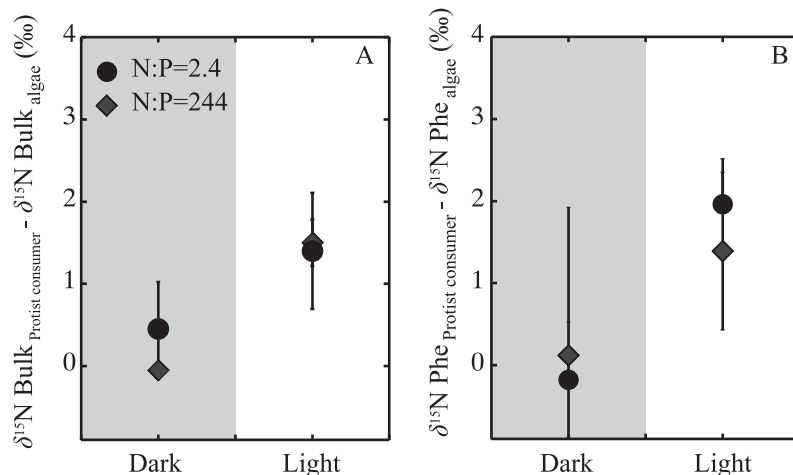


Fig. 2. Bulk  $^{15}\text{N}$  enrichment for a phagotrophic protistan consumer relative to its algal prey. (A) Difference in bulk  $\delta^{15}\text{N}$  values between *Oxyrrhis marina* (second stage) and *Dunaliella tertiolecta* (first stage), mean ( $\pm$  SD) for the last 2 d of the experiments. (B) Difference in  $\delta^{15}\text{N}$  values between the 'source' AA phenylalanine (Phe) in *O. marina* (second stage) and in *D. tertiolecta* (first stage), mean ( $\pm$  SD) of replicate subsamples collected on the last day of the experiments. N:P molar ratios refer to the nutrient composition in the media reservoir.

available nitrogen pool is utilized. These differences in isotopic values remained in the second stages in both light ( $\delta^{15}\text{N}_{\text{low N:P}} = 5.3 \pm 0.4$  vs.  $\delta^{15}\text{N}_{\text{high N:P}} = 3.6 \pm 0.3$ ;  $t$ -test,  $t = 4.71$ ,  $df = 2$ ,  $p = 0.042$ ) and dark treatments ( $\delta^{15}\text{N}_{\text{low N:P}} = 4.3 \pm 0.1$  vs.  $\delta^{15}\text{N}_{\text{high N:P}} = 2.0 \pm 0.1$ ;  $t$ -test,  $t = 32.5$ ,  $df = 2$ ,  $p = 0.0009$ ).

In contrast to the general rule of trophic isotopic enrichment (Fig. 1), *O. marina* was only modestly enriched in  $^{15}\text{N}$  relative to its food resource (first stage,  $\sim 1.5\%$ ) in the light treatments, and not at all in the dark (Fig. 2A). This isotopic fractionation pattern between protistan consumers (second stage) and algae (first stage) was similar in the two experiments (Fig. 2A) despite the designed differences in nitrogen availability. The observed increase in the  $\delta^{15}\text{N}$  values of *O. marina* in the second-stage light treatments could be due to isotopic fractionation associated with the single trophic step or to a change in the isotopic baseline (Cabana and Rasmussen 1996; Post 2002a; Sherwood et al. 2011). Using the variability in source AAs  $\delta^{15}\text{N}$  values to distinguish between these two mechanisms, we observe that variation in bulk  $\delta^{15}\text{N}$  values of *O. marina* closely follows those for the baseline AA, phenylalanine (Fig. 2B). This indicates that the higher  $\delta^{15}\text{N}$  values of *O. marina* in the light treatments resulted from a change in baseline  $\delta^{15}\text{N}$ , rather than trophic fractionation.

For the consumer *O. marina*, the unique pattern of nitrogen isotope trophic fractionation suggested by the bulk and phenylalanine  $\delta^{15}\text{N}$  values is confirmed by small differences between  $\delta^{15}\text{N}$  values of glutamic acid and phenylalanine ( $\Delta\delta^{15}\text{N}_{\text{glu-phe}}$ ).  $\Delta\delta^{15}\text{N}_{\text{glu-phe}}$  values for *D. tertiolecta* in the first stages are close to that expected for a marine microalga,  $\sim 4\%$  (range: 4.1–5.1%). In contrast, second-stage values for *O. marina* are well below the predicted 7.6‰ enrichment of  $^{15}\text{N}$  for a single trophic step (Fig. 1). In fact, the  $\Delta\delta^{15}\text{N}_{\text{glu-phe}}$  values for *O. marina* ( $\sim 4\%$ ; range: 3.3–5.2‰) are very similar to those of the

algal prey regardless of light or nutrient conditions (Fig. 3). Using published parameter values from Chikaraishi et al. (2009) to calculate mean trophic positions of consumer and prey for the two experiments, we find that they

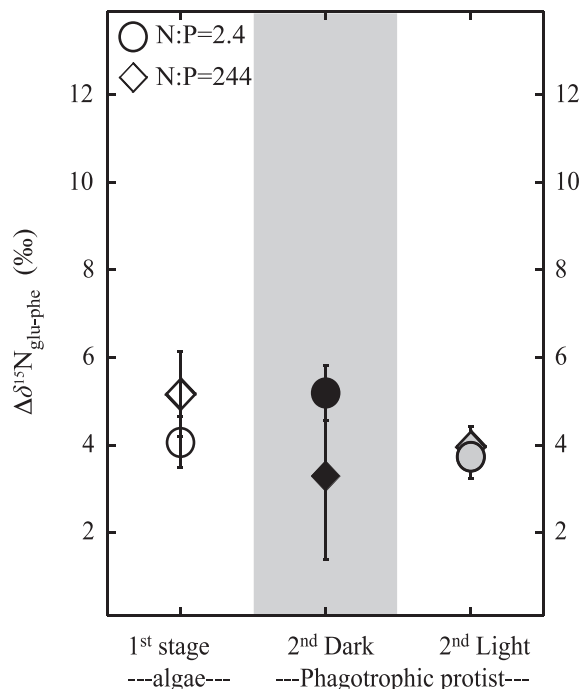


Fig. 3. Comparison of the difference in  $\delta^{15}\text{N}$  values of glutamic acid and phenylalanine ( $\Delta\delta^{15}\text{N}_{\text{glu-phe}}$ ) in the algal prey (*D. tertiolecta*) and the protistan consumer (*O. marina*). Mean values ( $\pm$  SD) for individual amino acids are for replicate subsamples collected during the last day of each experiment (circles and diamonds as in Fig. 2). For metazoan consumers, a  $\Delta\delta^{15}\text{N}_{\text{glu-phe}}$  value of  $\sim 7.6\%$  is expected (McClellan and Montoya 2002; Chikaraishi et al. 2009; see Fig. 1).

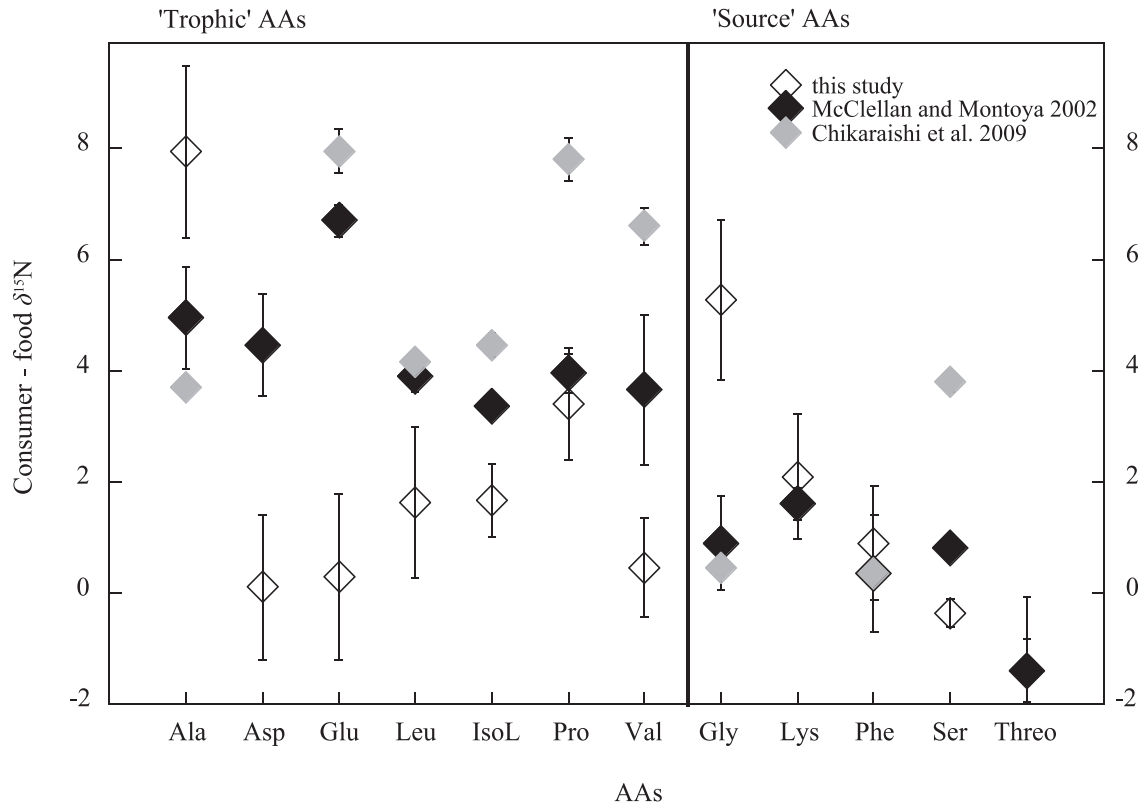


Fig. 4. Comparison of amino acid  $^{15}\text{N}$  enrichment in phagotrophic protistan (this study) and metazoan consumers (McClellan and Montoya 2002; Chikaraishi et al. 2009) relative to their algal prey. Mean ( $\pm$  SD) of the four protist–algae experiments from the present study (Table 1); mean of duplicate metazooplankton–algae experiments from McClellan and Montoya (2002) and Chikaraishi et al. (2009).

are essentially identical within measurement error: 1.18 ( $\pm$  0.10) for *D. tertiolecta* (first stage, primary producer), 1.06 ( $\pm$  0.02) for *O. marina* in the light (Fig. 2A, consumer), and 1.12 ( $\pm$  0.19) for *O. marina* in the dark (Fig. 2B, consumer). These results indicate that the trophic interaction between a protistan consumer and algal prey in our controlled chemostat system was isotopically invisible according to conventional assessments by bulk and AA-specific nitrogen isotopic methods.

A broader analysis of the isotopic composition of AAs in *O. marina* reveals a trophic fractionation pattern that is fundamentally different from that found for metazoans, particularly for the ‘trophic’ AAs (Table 2). In Fig. 4, we show our data in comparison with results from two well-known AA–CSIA studies for diverse metazoan consumers (McClellan and Montoya 2002; Chikaraishi et al. 2009). In the present study, trophic AAs for a protistan grazer were substantially less enriched in  $^{15}\text{N}$  relative to source AAs than those for metazoans. The one exception was alanine, which was markedly enriched in  $^{15}\text{N}$  by *O. marina* compared with metazoans.

## Discussion

The coupling of rapid production, consumption, and recycling processes under nitrogen limitation conditions has been hypothesized to explain the homogenization of

nitrogen isotopic compositions of primary producers and consumers within highly dynamic marine microbial food webs (Bode et al. 2007; Hannides et al. 2009). Although our results do not allow direct rejection of this hypothesis, the systematic lack of  $^{15}\text{N}$  trophic enrichment for very different conditions of nitrogen availability and cycling (Table 1; Figs. 2, 3) suggests another mechanism. The low trophic isotopic fractionation pattern observed across most AAs (Fig. 4) indicates that there was minimal physiological transformation and isotopic discrimination of the AAs absorbed from digested algal food before incorporation into tissue growth by our model protistan consumer. A similar pattern has been observed in bacteria, where a lack of  $^{15}\text{N}$  enrichment in AAs during trophic transfer (termed ‘salvage incorporation,’) has been found. The implication is that the carbon and nitrogen skeletons of digested compounds remain largely intact during uptake and incorporation into protistan biomass (Calleja et al. 2013).

Salvage incorporation of AA was thought to be restricted only to bacteria, but here we show an analogous process that occurs in protistan grazers. The interesting AA exception is alanine, which was substantially enriched in  $^{15}\text{N}$  (5.5–8‰) by *Oxyrrhis* relative to source amino acids in its food in all treatments (Fig. 4; Table 2). We hypothesize that this particular amino acid may be a potentially robust and unique tracer of protistan trophic steps, and we have recently begun a project to study this relationship further

(M. Décima and M. R. Landry pers. comm.). A lack of isotopic fractionation for most amino acids is consistent with high assimilation efficiency and direct incorporation into growth. Heterotrophic protists exhibit characteristically high growth and division rates that, unlike metazoan grazers, allow them to efficiently crop and control actively growing phytoplankton stocks. The observed dramatic differences in the trophic fractionation patterns for nitrogen isotopes between protistan and metazoan grazers could, therefore, reflect fundamental differences in their physiologies and biogeochemical transformations in pelagic ecosystems.

The increase in bulk and baseline AA phenylalanine  $\delta^{15}\text{N}$  values between first and second stage in the light (Fig. 2A, B) is almost certainly due to additional phytoplankton growth on different nitrogen sources in the second stage before being consumed by *O. marina*, propagating the increase in the baseline  $^{15}\text{N}$  from producer to consumer. Such a shift can arise from progressive  $^{15}\text{N}$  enrichment of residual dissolved nitrogen (Hoch et al. 1996) due to preferential removal of  $^{14}\text{N}$  by actively growing phytoplankton (Waser et al. 1998) in the first stage. This  $^{15}\text{N}$ -enriched pool of nitrogen entered the second stage where, given adequate light conditions, it could be taken up by photosynthetically active algae and transferred into grazers. Another mechanism that could explain the second-stage change in  $^{15}\text{N}$  is de novo synthesis of 'source' AAs by *O. marina*. However, we are unaware of any study documenting the ability of *O. marina* to synthesize these AAs. While *O. marina* cultures have been maintained in the absence of autotrophic prey, the complex media used to ensure survival by osmotrophy included all of these AAs (Droop 1959). The fact that we observed no significant difference between  $\delta^{15}\text{N}$  values of algae (first stage) and grazers (second stage;  $t$ -test,  $\text{N:P}_{\text{LOW}} t = 1.11$ ,  $\text{df} = 2$ ,  $p = 0.38$ ;  $\text{N:P}_{\text{HIGH}} t = 1.0$ ,  $\text{df} = 2$ ,  $p = 0.42$ ) when second-stage dark conditions prevented photosynthesis and nutrient uptake from affecting the isotopic baseline, is also consistent with our interpretation that the second light-stage increase in  $\delta^{15}\text{N}$  values is due to change in the  $^{15}\text{N}$  content of the phytoplankton prey.

The observed baseline variation in  $\delta^{15}\text{N}$  values between first and second light stages (Fig. 2B) is an important aspect of our overall results. Phytoplankton biomass in the second-stage light treatments remained extremely low (0.5% of total biovolume) and tightly controlled by grazing in both experiments. Yet, despite being virtually absent in terms of absolute biomass, their active growth and nitrogen consumption was evident in the  $\delta^{15}\text{N}$  values of their predators. Because primary producers tend to have diverse nitrogen sources with distinct isotopic compositions even within the same ecosystem, an adequate characterization of the isotopic baseline is critical for inferring trophic processes from the isotopic compositions of consumers (Cabana and Rasmussen 1996; Post 2002a). Recent oceanographic studies have shown how seasonal and inter-annual variability of the nitrogen isotopic baseline is reflected in the composition of higher trophic levels (Chouvelon et al. 2012; Décima et al. 2013). The increase in  $\delta^{15}\text{N}$  values of phenylalanine in the illuminated second

stage (Fig. 2B) further emphasizes this point, stressing how rapidly changes in the baseline isotopic composition can propagate through lower trophic levels and confound environmental interpretation of the  $\delta^{15}\text{N}$  values of bulk tissues. Given the remarkable coupling between microzooplankton and phytoplankton trophic interactions (Strom 2002) and the high turnover rates of nutrients (Karl 2002) characteristic of oceanic ecosystems, the rapid propagation of baseline isotopic variations reported here is likely to be the norm in microbial–protistan food webs. This highlights the need to account for such effects in ocean field measurements (Cabana and Rasmussen 1996; Post 2002a).

Here, we provide the first direct experimental evidence that phagotrophic protists do not necessarily follow the systematic  $^{15}\text{N}$  trophic enrichment that is well-established for metazoan consumers (DeNiro and Epstein 1981; Peterson and Fry 1987; Boecklen et al. 2011). These laboratory results, though limited, are fully consistent with multiple bulk tissue or whole organism nitrogen isotope measurements that have failed to detect significant  $^{15}\text{N}$  enrichment among size-fractionated POM field samples encompassing various trophic levels (Rau et al. 1990; Fry and Quiñones 1994; Rolff 2000), and with AA-specific  $\delta^{15}\text{N}$  measurements of field-collected zooplankton from the NPSG, which lacked an obvious  $^{15}\text{N}$  enrichment effect for the protistan consumers that dominate energy pathways in that system (Hannides et al. 2009). Similarly, unrealistically low TPs of large suspension-feeding zooplankton from the California Current Ecosystem estimated from AA-CSIA analysis and the standard (Chikaraishi et al. 2009) trophic enrichment factor of 7.6‰ (Décima et al. 2013) further supports the ecological relevance of the isotopic invisibility of phagotrophic protists unveiled here.

We are aware, however, that others have shown an elevated AA-CSIA trophic signal for Antarctic krill, which were observed to be feeding on heterotrophic protists (Schmidt et al. 2006). Interpretations of these Southern Ocean measurements have some complications, such as the reported large changes in isotope baseline values during the period of sampling, the unknown dietary contributions from metazoan prey that would enhance the carnivory signal in krill, and a temporal mismatch between body composition and ingested prey in this long-lived, cold-water species. It is also possible, however, that real differences could exist between tropical and polar ecosystems because of temperature effects on organic matter turnover rates or predator–prey coupling (Rose and Caron 2007).

Over the past 3 decades, oceanographic research highlighting the importance of microbial organisms and processes (Pomeroy 1974; Azam et al. 1983) has profoundly altered our understanding of community ecology, food-web dynamics, and biogeochemical transformations in the oceans. Prior to this microbial revolution, perceptions were blinded by the inability of existing methods to visualize the vast number of bacterial cells that reside in seawater (Azam et al. 1983). Despite progress in many areas, the results presented here suggest that contemporary methods similarly continue to obscure the full realization of microbial effects on food-web structure and energy transfer.



Because most ocean productivity flows through microbial–protist pathways (Pomeroy 1974; Sherr and Sherr 2002; Calbet and Landry 2004), the present results have important implications for understanding long-term as well as regional variations in the structure and function of pelagic ecosystems. Climate change predictions of expanding areas of oligotrophy in the oceans (Behrenfeld et al. 2006), for instance, envision enhanced roles and complexities of trophic flows through the microbial food web. Such changes will likely affect the ocean’s capacity to take up atmospheric carbon dioxide by vertical export of biogenic carbon (Legendre and Rassoulzadegan 1996), the efficiency of nutrient cycling and contaminant bioaccumulation (Post 2002b), the production–respiration balance of the oceans, and the production and cycling of climate active gases (Simó 2001) other than CO<sub>2</sub>. Trophic level determination has been especially central to documenting global effects of historical human effects on exploited stocks, such as ‘fishing down the food web’ (Pauly et al. 1998), and recommended by recent frameworks as a useful index to monitor and evaluate the health of the oceans (Garcia et al. 2003). Such metrics, whether based on stable isotope assessments or stomach contents of higher level organisms (where delicate protists are often overlooked) cannot adequately represent the energy fluxes from lower trophic levels that support fisheries if they do not account for ocean variability in flows through microbial food-web complexes. Further, recent studies have demonstrated that even modest reevaluation of the trophic status of an individual species (e.g., increasing the trophic level estimate of anchoveta from 2.2 to 2.7) can profoundly affect the interpretation of exploitation trends in global marine fisheries (Branch et al. 2010). Given such sensitivities, the uncertainties associated with missing one or more trophic levels using stable isotopic and other techniques significantly challenge our understanding of food-web structural effects in the ocean and need to be carefully considered in investigations of historical and future trends in ocean biogeochemistry and fisheries.

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