AMINO ACID – COMPOUND SPECIFIC STABLE ISOTOPE ANALYSIS OF

MICRONEKTON AROUND HAWAII REVEALS SUSPENDED PARTICLES ARE AN

IMPORTANT NUTRITIONAL SOURCE IN THE MESO/BATHYPELAGIC

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ABSTRACT

The mesopelagic food web is poorly understood due to its remoteness and difficulty in sampling. Several studies have found that the demand for carbon by the mesopelagic community exceeds the sinking flux of particulate carbon by up to two to three orders of magnitude. This suggests that additional overlooked sources of carbon exist in the mesopelagic. Suspended particles have been suggested as one of these sources but little has been done to estimate their relative contribution to the mesopelagic food web. In this study, we investigate whether suspended particles are an important nutritional source in the mesopelagic using amino acid compound specific nitrogen isotope analysis. Suspended particles have distinct nitrogen isotope values in their source amino acids which can be traced through the food web. The objective of this study was to determine if mesopelagic micronekton around Station ALOHA feed from a suspended particle based food web. Our results suggest that micronekton feed from food webs based on a variety of nutritional sources including surface dwelling phytoplankton and bacteria, sinking particles and suspended particles with micronekton generally becoming more reliant on suspended particles with depth. Several species were identified as feeding from a suspended particle based food web including *Cyema atrum*, *Cyclothone pallida*, *Japatella diaphana*, *Melanocetus johnsonii* and *Serrivomer sector* which were the deepest living micronekton from this study suggesting that in the lower mesopelagic/upper bathypelagic micronekton rely on a nutritional source separate from the fresh surface derived material utilized by epipelagic and upper mesopelagic micronekton. Additionally, we investigated whether species which feed from a suspended particle food web can be identified using bulk tissue nitrogen isotope analysis, a less expensive and less time-consuming method, hypothesizing that these species will have high δ^{15}N values driven by the high δ^{15}N values at the base of the food web. We found that not all species

that feed from a suspended particle based food web have high δ^{15}N values and therefore bulk tissue nitrogen isotope analysis is not a suitable technique for this type of study. Our results indicate that suspended particles are an important nutritional source in the lower mesopelagic and upper bathypelagic and should be taken into account when estimating food and carbon supplies for these communities.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The meso and bathy-pelagic zones of the ocean (200 m – 2500 m) make up the largest habitat on the planet (Robison 2004). However, due to its remoteness and difficulty in sampling, this environment is poorly studied. In particular, little is known about the function and structure of the food web. For example, studies examine the controls on primary production as well as distribution and feeding ecology of top predators in the open ocean, but few studies have been done on the meso/bathy-pelagic zooplankton and micronekton which are the trophic link between these two groups (Brodeur and Yamamura 2005).

It is important to understand the meso/bathypelagic food web because it plays an important role in fisheries and the sequestration of carbon through the biological pump. It is crucial to understand the entire food web for ecosystem-based fisheries management, which links exploited commercial species to their prey and competitors (Choy et al. 2016; Grumbine 1994). In terms of climate change, meso/bathypelagic zooplankton and micronekton play an important role in the biological carbon pump by migrating vertically to the surface at night to feed under the cover of darkness and then returning to depth during the day to hide from predators, carrying organic carbon to depth during this migration (Longhurst et al. 1990; Al-Maturi and Landry 2001; Steinberg et al. 2000; Steinberg et al. 2008b; Hannides et al. 2009a).

Several studies have attempted to estimate the amount of carbon transported to depth via the biological carbon pump as well as the demand for carbon in the meso/bathypelagic and have found that the demand for carbon exceeds the amount of carbon being delivered there by sinking particulate organic carbon (POC) flux by as much as two to three orders of magnitude (Boyd et al. 1999; Steinberg et al. 2008b; Baltar et al. 2009; Burd et al. 2010). There are two leading hypotheses to explain the discrepancy between the carbon supply and the carbon demand. The

first hypothesis is that carbon demand in the meso/bathypelagic is being overestimated. In one

study by Giering et al. (2014) a balanced carbon budget was achieved by excluding vertically

migrating zooplankton from carbon demand estimates based on the premise that migrating

zooplankton are capable of obtaining enough carbon from the surface and do not need further

sources of carbon at depth. Overestimating carbon demand is also thought to be due to

uncertainty in parameters used to estimate metabolic activity, such as assimilation efficiency

(Boyd et al. 1999; Buesseler et al. 2007; Burd et al. 2010). The second is that carbon sources in

the meso/bathypelagic are being underestimated. Many of the studies that estimated carbon

supply used sediment traps to measure the downward flux of carbon from the surface. There are

several limitations, however, when using sediment traps to measure POC flux including free-

swimming organisms entering the sediment traps and particles being swept out by flow within

the sediment traps (Buesseler 2007). Additionally, organisms may sustain themselves through

diel vertical migration or by feeding from a pool of smaller particles (Steinberg et al. 2000; Al-

Maturi and Landry 2001; Steinberg et al. 2008b; Baltar et al. 2009; Giering et al. 2014; Mayor et

al. 2014). For example, Steinberg et al. (2008b) estimated that zooplankton from around Hawaii

were able to transport organic material through their vertical migrations, via feeding at the

surface and defecating at depth, corresponding to up to 15% of the carbon demand of the

mesopelagic around Hawaii. One study in the northeast Pacific estimated that micronekton

could transport up to 23 mg C m^{-2} d^{-1}, which is 26% of the carbon demand to the mesopelagic

around Hawaii (Davison et al. 2013). Since the northeast Pacific is a more productive region

than Hawaii we would expect micronekton around Hawaii to be less abundant and contribute

even less total carbon transport. Including carbon transport by zooplankton around Hawaii and

micronekton from the northeast Pacific in carbon flux still only covers 61% of the carbon demand in the mesopelagic around Hawaii.

Few studies exist that estimate the relative contribution of small particles often referred to as suspended or slowly sinking particles. The pool of these small particles (operationally defined here as 0.7-0.53μm) in the meso/bathypelagic is quantitatively larger than the pool of sinking particles (>0.53μm) (Verdugo 2004; Baltar et al. 2009), and has also been shown to be a major contributor to carbon flux in some places such as the eastern tropical North Pacific (Puigcorbè et al. 2015). Therefore, if eaten small particles could make up for some of the deficit in the carbon supply at that depth. Baltar et al. (2009) found a strong positive relationship between suspended particle concentrations and bacterial activity in the subtropical North Atlantic, suggesting that suspended particles are an important source of organic matter to meso/bathypelagic microbes. However, Baltar et al. (2009) did not investigate how the suspended particles might support higher trophic levels.

A commonly used tool to trace organic matter, such as suspended particles, through food webs is stable nitrogen isotope analysis. The nitrogen isotope composition (δ^{15}N) has often been used as an indicator of relative trophic position because it increases ~2-3‰ with respect to the food source between predator and prey (Heikoop et al. 1998). However, the nitrogen isotope composition of an organism will also vary with the δ^{15}N value of the base of the food web, and in some cases has been shown to be a better indicator of changes at the base of the food web than of trophic position. For instance, Choy et al. (2012) found that lantern fishes and dragon fishes from five different regions of the world had widely varying δ^{15}N values (~6‰) despite stomach content studies suggesting consistent trophic levels across all regions.

Amino acid compound specific isotope analysis (AA-CSIA) is another a tool which can be used to trace organic matter through food webs that can also be used to distinguish between changes in nitrogen isotope composition driven by differences at the base of the food web versus changes in trophic position. Some amino acids, known as "trophic" amino acids (alanine, aspartic acid, glutamic acid, isoleucine, proline, valine), fractionate in a predictable way (~2-7‰) with increasing trophic position, while other amino acids, known as "source" amino acids (glycine, lysine, methionine, phenylalanine, serine, tyrosine) have $\delta^{15}N$ values that change by <1‰ with increasing trophic level (McClelland and Montoya 2002; Chikaraishi et al. 2009). Therefore, source amino acids reflect the nitrogen isotopic compositions of different food sources at the base of the food web such as surface dwelling plankton or deeper water column particles (Hannides et al 2013). Additionally, this technique allows one to estimate the trophic position of an organism from a single sample using the difference between the $\delta^{15}N$ values "trophic" and "source" amino acids (Chikaraishi et al. 2009; Bradley et al. 2015). Choy et al. (2012) was able to use AA-CSIA to confirm that the lantern fishes and dragon fishes from five different regions of the world did in fact have the same trophic positions and that differences in bulk tissue $\delta^{15}N$ values were being driven by differences in the $\delta^{15}N$ values of primary producers in the different regions as recorded in the $\delta^{15}N_{sourceAA}$ values in these fishes.

It has recently been discovered that sinking particles and suspended particles have distinct $\delta^{15}N_{sourceAA}$ values throughout the water column at Station ALOHA (Close et al. *in prep*). These distinct $\delta^{15}N_{sourceAA}$ values can be used to trace the relative contributions of carbon from these two sources to the diets of organisms at higher trophic levels. Hannides et al. (2013), measured the $\delta^{15}N$ values of source amino acids in suspended particles and zooplankton finding that $\delta^{15}N_{sourceAA}$ values of suspended particles increased by up to 14‰ with depth, a trend present

but muted in zooplankton that suggested that zooplankton relied on suspended particles by as much as 38% (Hannides et al. 2013). Choy et al. (2015), found that the $\delta^{15}N$ values of three source amino acids (phenylalanine, glycine and serine) also increased with depth in a variety of micronektonic fishes with at least one fish, *Cyema atrum*, which appeared to rely on nitrogen largely from a suspended particle based food web.

While Hannides et al. (2013) and Choy et al. (2015) found evidence for zooplankton and micronekton feeding from a suspended particle based food web neither of these studies had data for sinking particles and the depths and taxa examined were limited. In this study, we further investigate whether suspended particles are an important nutritional source for a diversity of meso/bathypelagic micronekton around Hawaii from depths to 1500 m. This was accomplished by measuring $\delta^{15}N_{sourceAA}$ values in micronekton and comparing these values to known $\delta^{15}N_{sourceAA}$ values of sinking and suspended particles throughout this depth range at Station ALOHA. We hypothesized suspended particles are an important nutritional source in the meso/bathypelagic. We tested this hypothesis by determining if meso/bathypelagic micronekton have similar $\delta^{15}N$ values to the source amino acids in suspended particles. Indeed, this was the case for several species of meso/bathypelagic micronekton indicating that suspended particles are an important nutritional and therefore carbon source in the lower mesopelagic and upper bathypelagic. Additionally we investigated whether micronekton species that fed from a suspended particle food web can be identified using bulk tissue $\delta^{15}N$ analysis because this method is less expensive and less time consuming than AA-CSIA. We hypothesized that micronekton feeding from a suspended particle food web would have high $\delta^{15}N$ values at a given trophic position, driven by higher $\delta^{15}N$ values at the base of the food web. We found that some

species of micronekton, which feed from a suspended particle food web do not have high $\delta^{15}N$ values and therefore AA-CSIA is necessary for this kind of study.

METHODS

Sample collection

Micronekton were collected using a 10 m^2 multiple opening closing net and environmental sensing system (MOCNESS) around Hawaii at Station ALOHA (22.45˚N, 158˚W) in March and August of 2011 and in February and September of 2014 with a few samples from other locations around Oahu in 2011 (Choy et al 2015). Micronekton were collected over five depth ranges between the surface and 1500 m: 0 – 100 m, 100 – 500 m, 500 – 700 m, 700 – 1000 m and 1000 – 1500 m. At sea micronekton were sorted and identified to best taxonomic level, then measured and photographed. Standard length measurements were taken for fish, carapace length and total length were taken for crustaceans and mantel length and total length were taken for cephalopods. Depending on the size of an individual organism, either a piece of white muscle tissue, whole individual, or pool of individuals of the same species were frozen in cryovials in liquid nitrogen. Upon returning to land specimens were transferred to a -80˚C freezer until the samples could be prepared for stable isotope analysis.

Bulk tissue nitrogen isotope analysis

Samples selected for stable isotope analysis represented different combinations of trophic strategies (suspension feeding, zooplanktivores, micronektonivores), depth guilds (epipelagic, mesopelagic, bathypelagic) and migrating behaviors based on available ecological information (e.g. Maynard et al 1978, Clarke 1973). Each sample was freeze dried and ground using a ceramic mortar and pestle. For bulk tissue carbon and nitrogen isotope analysis approximately

0.5 mg of each sample was weighed and placed into a tin boat. Carbon and nitrogen isotopic compositions were determined using an isotope ratio mass spectrometer (Delta^{Plus}XP) coupled to an elemental analyzer (Costech Model 4010). Isotopic ratios are given in δ-notation relative to the international standards VPDB and atmospheric N_2. Accuracy and precision were <0.2‰ based on glycine and homogenized fish tissue analyzed every ten samples.

Amino acid compound specific stable isotope analysis

For AA-CSIA, approximately 15 mg of each sample were hydrolyzed and derivatized following the methods of Popp et al. (2007) and Hannides et al. (2009) to yield trifluoroacetic amino acid esters. For acid hydrolysis, ~0.5 mL of 6N HCl was added to each sample in a 5 mL reaction vial flushed with N_2 gas and sealed with a Teflon-lined cap. Vials were heated at 150˚C for 70 minutes, allowed to cool, and the liquid in each vial was evaporated at 55˚C under a stream of N_2 gas. The residue in each vial was re-dissolved in 1 mL of 0.01N HCl and this solution was filtered and purified through cation-exchange chromatography. Briefly, the 0.01N HCl solution for each sample was pushed through a filter using a syringe into a cation exchange column (Gracepure SPE cation-x 1000mg/6ml (Grace Alltech part number 5141488)), followed by three rinses with 0.01N HCl. Approximately 1 mL of 2N ammonium hydroxide was then added to the cation-exchange column to elute the amino acids, followed by three more rinses of 2N ammonium hydroxide to ensure all amino acids have eluted from the column.

For derivatization, the 2N ammonium hydroxide solution for each sample was evaporated at 80˚C under a stream of nitrogen gas. Approximately 0.5 mL of 0.2N HCl was then added to each vial, flushed with N_2 gas, heated at 110˚C for five minutes, then allowed to dry at 55˚C under a stream of N_2 gas. About 5 mL of acetyl chloride was added to 20 mL (25 mL total) of isopropanol to create a 4:1 isopropanol and acetyl chloride solution, which was added to each

sample vial, which was flushed with N_2 gas and heated at 110°C for 60 minutes. The 4:1 isopropanol and acetyl chloride solution in each vial was then dried at 60°C under a stream of N_2 gas. Approximately 600 μL of methylene chloride and 200 μL of trifluoroacetic acid (TFAA) were then added to each sample vial which were flushed with N_2 gas and heated at 100°C for 15 minutes, then allowed to cool and evaporate at room temperature under a stream of N_2 gas. Approximately 2 mL of P-buffer (KH_2PO_4 + Na_2HPO_4, pH 7) and 1 mL of chloroform were then added to each sample vial and each vial was shaken for 60 seconds allowing the amino acids to transfer to the chloroform and any contaminants to transfer to the P-buffer (Ueda et al. 1989). Each vial was then centrifuged at 600g for five minutes and a glass pipette was used to remove the chloroform layer containing the amino acids from the sample vial into a new clean vial. Chloroform was then evaporated from each new vial and 600 μL of methylene chloride and 200 μL TFAA were added, heated to 100°C for 15 minutes and then stored in the freezer until analysis. Immediately before analysis, each sample was dried at room temperature under a stream of N_2 gas and ~250 to 500 μL of ethyl acetate was added to the sample.

The nitrogen isotope composition of the trifluoroacetic amino acid esters were determined using an isotope ratio mass spectrometer (Thermo Scientific Delta V Plus or Thermo Scientific MAT 253 IRMS) interfaced with a Thermo Finnigan GC-C III. Samples were injected onto a BPx5 *forte* capillary column (60m x 0.32 mm x 1.0 μm film thickness) at an injector temperature of 180°C with a constant helium flow rate of 1.4 mL/min. The column was initially held at 50°C for two minutes and then increased at a rate of 15°C/min to 120°C. Once at 120°C, the temperature was increased at a rate of 4°C/min to 195°C, then to 255°C at a rate of 5°C/min and finally to 300°C at a rate of 15°C/min and was held at that temperature for eight minutes. Each sample was analyzed in triplicate and co-injected with the reference compounds norleucine

(Nor) and aminoadipic acid (AAA). A suite of pure amino acids of known nitrogen isotopic composition (Ala, Thr, Ile, Pro, Glu and Phe) was also injected every three runs as an extra measure of accuracy for the instrument. Reference compounds Nor and AAA as well as the suite of amino acids also served as methods for normalizing the measured isotope values. Standard deviation for all amino acids averaged ± 0.4‰ (range 0-3.1‰).

Data analysis

A weighted average of the $\delta^{15}N$ values of Glycine, Serine, Phenylalanine and Lysine with weighting based on the standard deviation of (at least) triplicate analysis was used to calculate $\delta^{15}N_{sourceAA}$ value for each individual analyzed (e.g., Hayes et al. 1990). A statistical model was created to evaluate the causes of variability in the $\delta^{15}N_{sourceAA}$ values of the micronekton (R version 3.1.2). The model tested the hypothesis that variability in $\delta^{15}N_{sourceAA}$ values of micronekton are due to differences in species, length, depth, region and year. As a preliminary assessment each of the predictors for the model was plotted against $\delta^{15}N_{sourceAA}$ values of the micronekton. Not all variables were found to have linear relationships with the $\delta^{15}N_{sourceAA}$ values of the micronekton suggesting a general additive model (GAM, R-package mgcv) would best explain the variability in the data. A gam was created using $\delta^{15}N_{sourceAA}$ values as the response variables and species, length, depth and year as the predictor variables. A paired t-test was used to determine if season had an effect on $\delta^{15}N_{sourceAA}$ values in species where individuals were captured in both seasons sampled. For *Cyclothone pallida*, linear regressions with depth and length were used to investigate variability in $\delta^{15}N_{sourceAA}$ values.

RESULTS

δ^{15}N values of amino acids were measured for 83 individuals representing 25 different species of micronekton (Table 1). Crustaceans ranged in size from 21 mm total length in *Euphausia* sp. to 102.5 mm total length in *Sergia* sp. Cephalopods ranged in size from 31.1 mm total length in *Histioteuthis* sp. to 290 mm mantle length in *Sthenoteuthis oauliensis*. Fish ranged in size from 22.9 mm standard length in *Hygophum proximum* to 507 mm total length in *Serrivomer sector*. Trophic positions (TP), determined using the method of Bradley et al. (2015), overlapped between the three types of feeding guilds with micronektonivores ranging from a TP of 3.1 to 4.5, zooplanktivores ranging from a TP of 2.6-4.4, and a TP of 3.8 for the single presumed suspension feeder in this study. Bulk tissue δ^{15}N values ranged from 5.4‰ in juvenile *Thunnus albacares* to 13‰ in *Melanocetus johnsonii*.

Table 1. Summary of data collected for each species in this study. The symbol (J) after a species names indicates the individuals were juveniles of the species. For feeding guild column, M=micronektonivore, S=suspension feeder, Z=zooplanktivore. For size type column, SL=standard length, FL=fork length, TL=total length, CL=carapace length, ML=mantle length. Source AA values (mean±standard deviation) were calculated using a weighted mean of Glycine, Serine, Phenylalanine and Lysine δ^{15}N values. Trophic position was calculated using the difference between the trophic amino acids alanine, leucine and glutamic acid, and the source amino acids glycine, lysine and phenylalanine, a β value of 3.6‰, and a TDF_{AA} of 5.7‰ (Bradley et al. 2015).

No.	Species	N	Animal Type	Day Depth Range	Night Depth Range	Depth Ref	Feeding Guild	Size (mm)	Size Type	δ^{15}N Source AA	δ^{15}N	δ^{13}C	Avg. TP
1	*Histioteuthis sp.*	2	Ceph	375-850	100-500	13	M	31.1	TL	2±1.3	10.4±2.1	-20.1±1.1	4.0±1.3
2	*Japetella diaphana*	2	Ceph	725-1065	725-1065	13	Z	48.4-53.2	ML	4.1±0.6	7.6	-20	2.9±0.1
3	*Sthenoteuthis oauliensis*	3	Ceph	650	0	16	M	245-490	ML	-1.2±1.1	8.7±0.7	-18.2±0.1	4.5±0.5
4	*Euphausia sp.*	3	Crust	175	84	9	S	21-40.1	TL	0.5±1.3	6.8±0.8	-19.7±0.4	3.8±0.6
5	*Gennadas sp.*	3	Crust	600-1100	100-1000	12	Z	10.8-11.9	CL	1.3±2	6±0.2	-19.6±0.5	3.8±0.1
6	*Oplophorus gracilirostris*	2	Crust	490-650	60-750	15	Z	11.8-12.2	CL	0.3±0.7	5.4±0.9	-20.8±1	3.3±0.1
7	*Sergia sp.*	3	Crust	800	150	15	Z	22.5-27.2	CL	1.7±2.6	7.2±1.2	-19±1.4	3.8±0.2
8	*Stomatopod*	2	Crust	0-100	0-100	7	Z	12.2-33	TL	0.5±0.9	4.7±0.4	-19.7±0.1	2.8±0.4
9	*Anoplogaster cornuta*	2	Fish	750-1150	275-980	1	M	88.6	SL	-0.5±3.7	7±0	-18.7±0.1	3.5±0.7
10	*Bolinichthys distofax*	4	Fish	490-690	490-690	2	Z	48.6-87.7	SL	0.2±1.1	8.6±0.9	-18.6±0.7	3.8±0.2
11	*Bolinichthys longipes*	2	Fish	525-725	50-150	2	Z	32-46	SL	0.1±0.6	6±0.4	-19±1	2.9
12	*C. hippurus (J)*	1	Fish	0-100	0-100	3,4	Z	180	FL	-0.2	6.2	17.3	3.3
13	*Chauliodus sloani*	5	Fish	480-825	45-225	5	M	52.3-174	SL	0.2±1.4	9.7±2.5	-18±0.8	3.4±0.5
14	*Cyclothone alba*	4	Fish	425-465	425-465	6	Z	24.5-26.5	SL	0.9±1.7	6.4±1.1	-19.9±0.4	2.7±0.1
15	*Cyclothone pallida*	12	Fish	600-1500	600-1500	6,7	Z	24-58.5	SL	4.3±2.7	11±2.6	-19.5±1.9	3.1±0.4
16	*Cyclothone pseudopallida*	1	Fish	525-750	525-750	6	Z	38.1	SL	1.7	8.2	-22.3	2.7

17	*Cyema atrum*	3	Fish	1200-1400	1200-1400	8	Z	103-139	SL	6.9±2.9	7±2	-18.3±1.7	2.9±0.1
18	*Exocoetus sp.*	2	Fish	0-10	0-10	10,11	Z	75-160	SL	-0.4±3.8	8.6±0.2	-17.7±1.3	3.5±0
19	*Hygophum proximum*	4	Fish	500-700	0-300	5	Z	22.9-42.2	SL	1.2±3.6	6.8±0.5	-18.7±1.2	2.9±0.1
20	*Melanocetus johnsonii*	3	Fish	500-1500	500-1500	14	M	21-98	SL	5.5±2	12.7±1.5	-19±0.9	3.9±0
21	*Myctophum lynchnobium*	2	Fish	600-800	0-15	2	Z	24.6-106	SL	-1.4±0.4	8.6±2.3	-18.7±0.8	3.5±0.5
22	*Opisthoproctus soleatus*	3	Fish	450-550	450-550	8	Z	67-85.1	SL	1.9±0.8	9.8±0.6	-20±1.3	4.4±0.6
23	*Scopelogadus sp.*	3	Fish	600-1000	150-250	1	Z	29-37.4	TL	0±1.2	5.6±0.4	-20.4±0.3	2.6±0.1
24	*Serrivomer sector*	3	Fish	550-1500	550-1500	8	M	356-507	TL	4.5±2.9	10.4±2.6	-18.3±1	3.1±0.1
25	*Thunnus albacares (J)*	2	Fish	0-110	0-110	17	Z	139-191	FL	-0.4±0.1	5.4±0.1	-17.4±0.4	3.2±0.4

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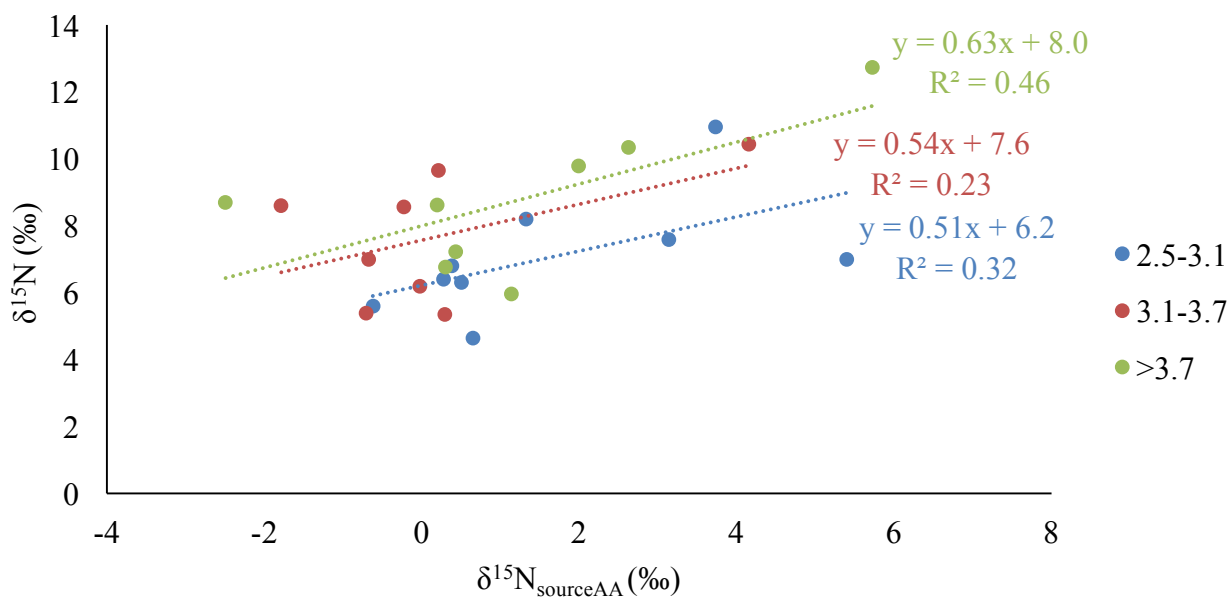
Figure 1. Bulk tissue $\delta^{15}N$ values of micronekton as a function of $\delta^{15}N_{sourceAA}$ values. $\delta^{15}N_{sourceAA}$ values have been averaged for each species. Micronekton have been grouped by trophic position.

Bulk tissue $\delta^{15}N$ values were compared to $\delta^{15}N_{sourceAA}$ values to evaluate their interdependence (Figure 1). For each trophic position group, bulk tissue $\delta^{15}N$ values increased linearly with increasing $\delta^{15}N_{sourceAA}$ values (Figure 1) with similar slopes and a lot of scatter in the relationships. Y-intercepts increased from the group of the lowest trophic positions to the group of the highest trophic positions.
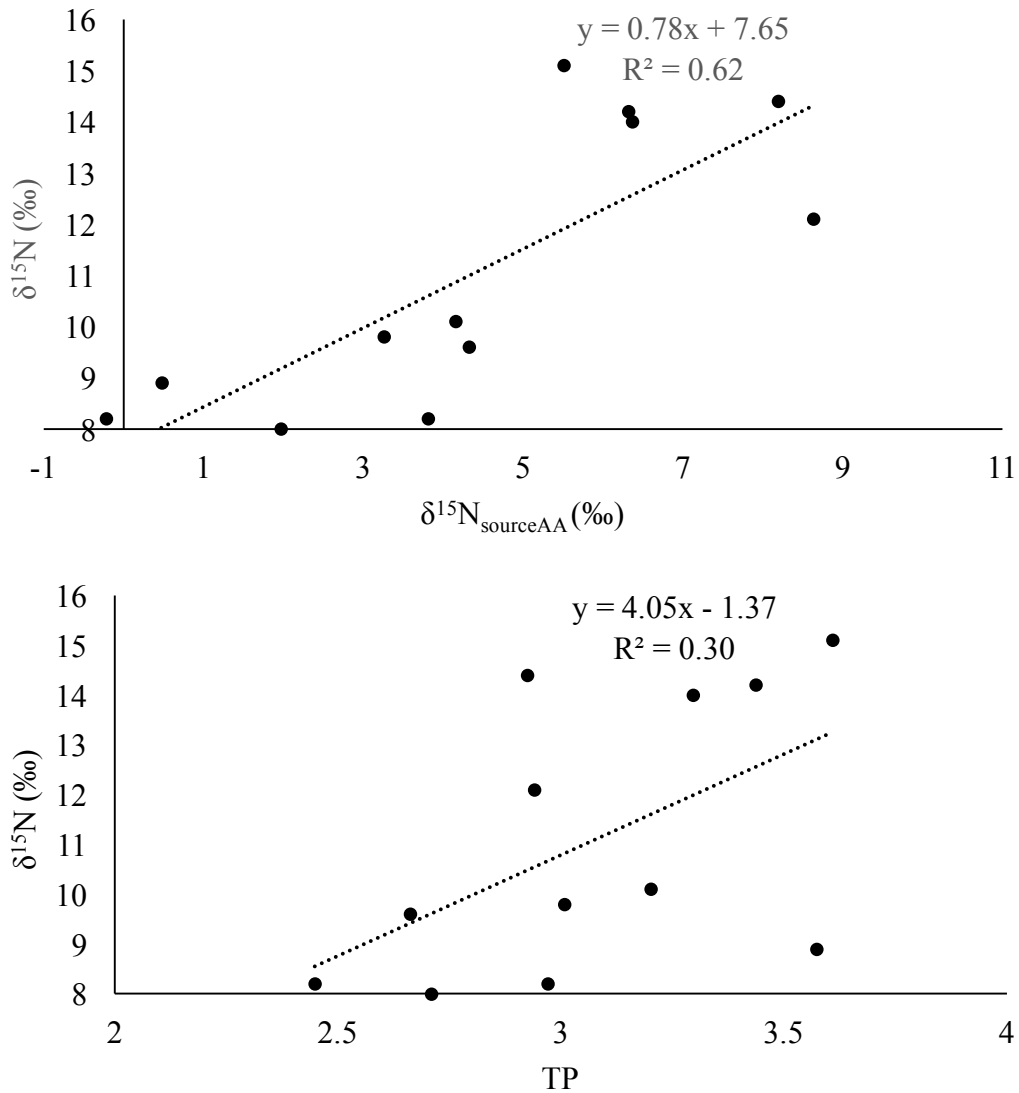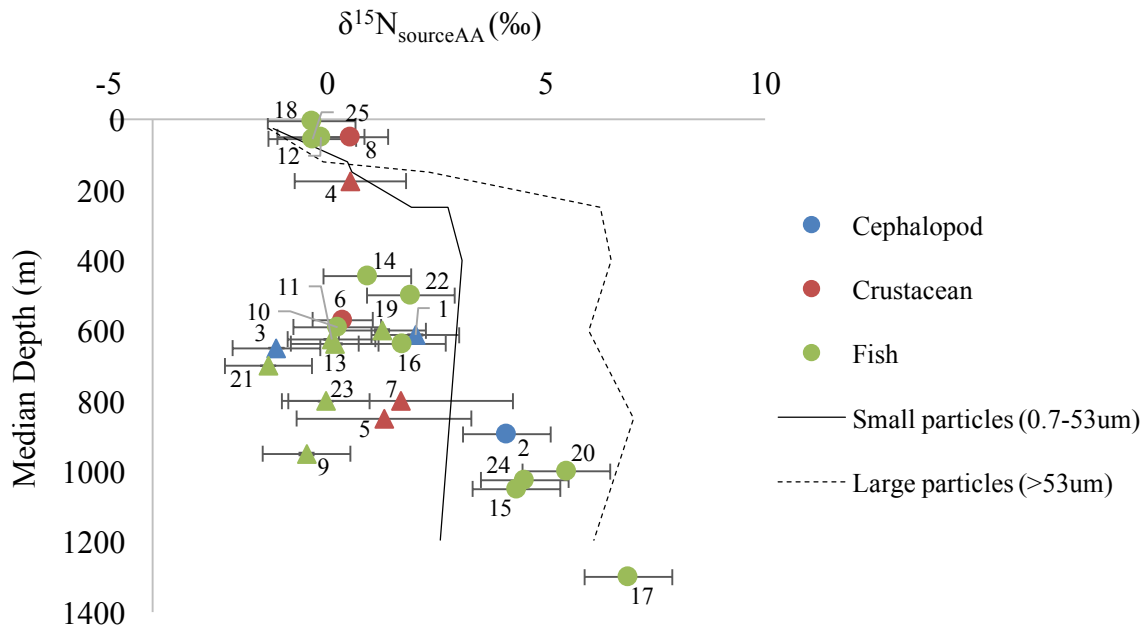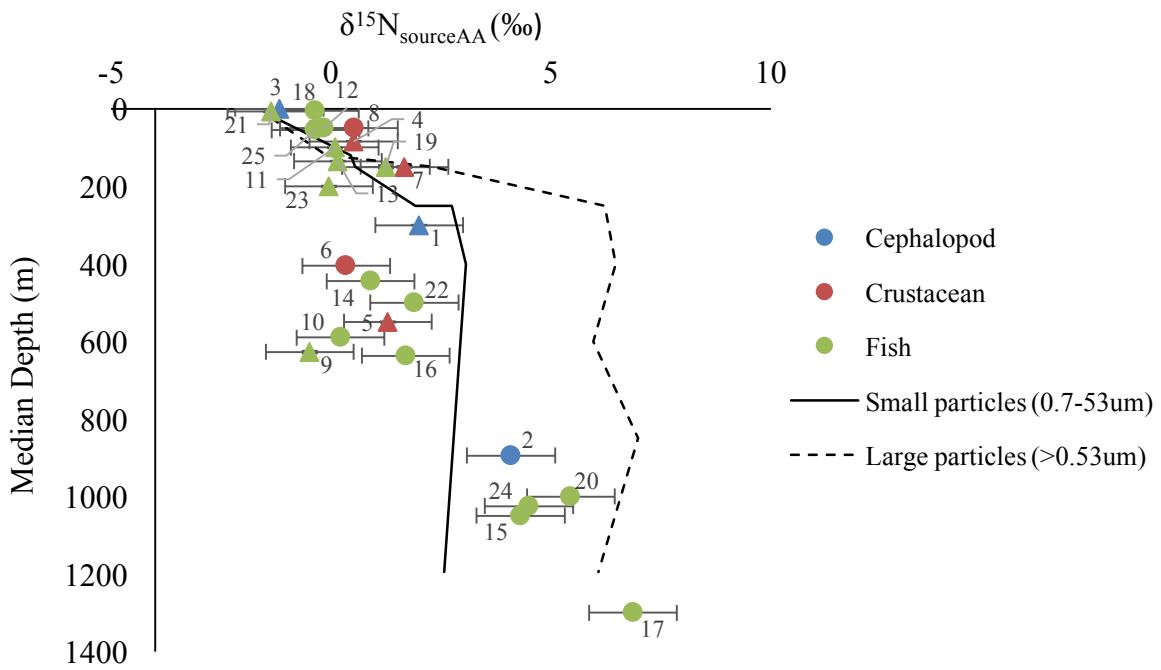
Figure 2. Bulk tissue $\delta^{15}N$ values of *C. pallida* as a function of $\delta^{15}N_{sourceAA}$ values (top) and trophic position (bottom).

Bulk tissue $\delta^{15}N$ values were also compared to $\delta^{15}N_{sourceAA}$ values for *C. pallida* to evaluate their interdependence within a single species (Fig. 2). A strong, positive correlation was found between bulk tissue $\delta^{15}N$ values and $\delta^{15}N_{sourceAA}$ values (ANOVA $p<0.05$, df=12). Additionally, bulk tissue $\delta^{15}N$ values were compared to the trophic position for *C. pallida*. Bulk tissue $\delta^{15}N$ values and trophic position were also found to have a positive correlation but were more weakly correlated than the bulk tissue $\delta^{15}N$ values and the $\delta^{15}N_{sourceAA}$ values.

Figure 3. $\delta^{15}N_{sourceAA}$ values (mean and standard deviation) of several micronekton species plotted against **A)** their median daytime depth of occurrence and **B)** their median nighttime depth of occurrence. For figures A and B, the dashed line indicates the $\delta^{15}N_{sourceAA}$ values of small particles (0.7-0.53μm) and the solid line indicates $\delta^{15}N_{sourceAA}$ values of large particles (>53μm). Circular points represent non-migrating organisms and triangular points represent migrating organisms. Numbers correspond to species in Table 1.

Figure 4. Dendrogram representing cluster analysis on the four source amino acids (Gly, Ser, Phe and Lys) using group average linkage and Euclidean distance. Red lines indicate statistically significant cluster groups (SIMPROF; p<0.05). Samples within a group are more similar to each other. Red diamonds= bathypelagic non-migrators; blue triangles=mesopelagic non-migrators, green triangles=mesopelagic migrators, blue squares=epipelagic non-migrators. Labels

without symbols indicate different particle samples. Small (0.7-53 μm) = suspended particles; large (>53 μm) = sinking particles; numbers indicate depth the particle sample was collected.

$\delta^{15}N_{sourceAA}$ values of micronekton increased with depth, becoming more similar to the $\delta^{15}N_{sourceAA}$ values of suspended particles in the lower mesopelagic/upper bathypelagic (Figure 3). Two general additive models (gam) were used to determine if variability in $\delta^{15}N_{sourceAA}$ values could be explained by depth, species, total length, region of collection, or year of collection. Separate models were made for daytime median depth of occurrence of micronekton and nighttime median depth of occurrence of micronekton. Of these predictors, depth was found to be the only significant predictor (daytime, $p<0.001$ nighttime $p<0.001$) with daytime depth having somewhat less explanatory power (41% of variability in $\delta^{15}N_{sourceAA}$ values) compared to the nighttime depths (50% of the variability in $\delta^{15}N_{sourceAA}$ values explained). Season was also assessed separately for the four species (*B. distofax, C. pallida, C. sloani, M. johnsonii*) where samples were collected in at least two seasons but no significant differences were found (t-test; t=0.64). In the epipelagic, $\delta^{15}N_{sourceAA}$ values of micronekton, such as juvenile *T. albacares* and larval stomatopods, were similar to the $\delta^{15}N_{sourceAA}$ values of small and large particles from the surface to 150 m and this similarity was found to be statistically significant (Figure 4; SIMPROF $p<0.05$, df=55). In the mesopelagic, $\delta^{15}N_{sourceAA}$ values of migrating micronekton, including myctophids and sergestid shrimp, were also found to have a statistically significant similarity to small and large particles in the epipelagic (surface to 150 m; SIMPROF $p<0.05$, df=55), with the exceptions of *Gennadas* sp., *A. cornuta* and *Histioteuthis sp.* which were more similar to mesopelagic non-migrating species. Mesopelagic, non-migrating micronekton had $\delta^{15}N_{sourceAA}$ values that fell between those of large and small particles from the epipelagic and large particles from the mesopelagic and bathypelagic (250-1205 m), except *O. soleatus* and *Histioteuthis* sp. which had statistically significant similarity to large particles from the mesopelagic and bathypelagic. Bathypelagic, non-migrating micronekton had $\delta^{15}N_{sourceAA}$ values that were most

similar to those of small particles from 250 m 1205 m with the similarity being statistically

significant for *M. johnsonii* (SIMPROF p<0.05, df=55).

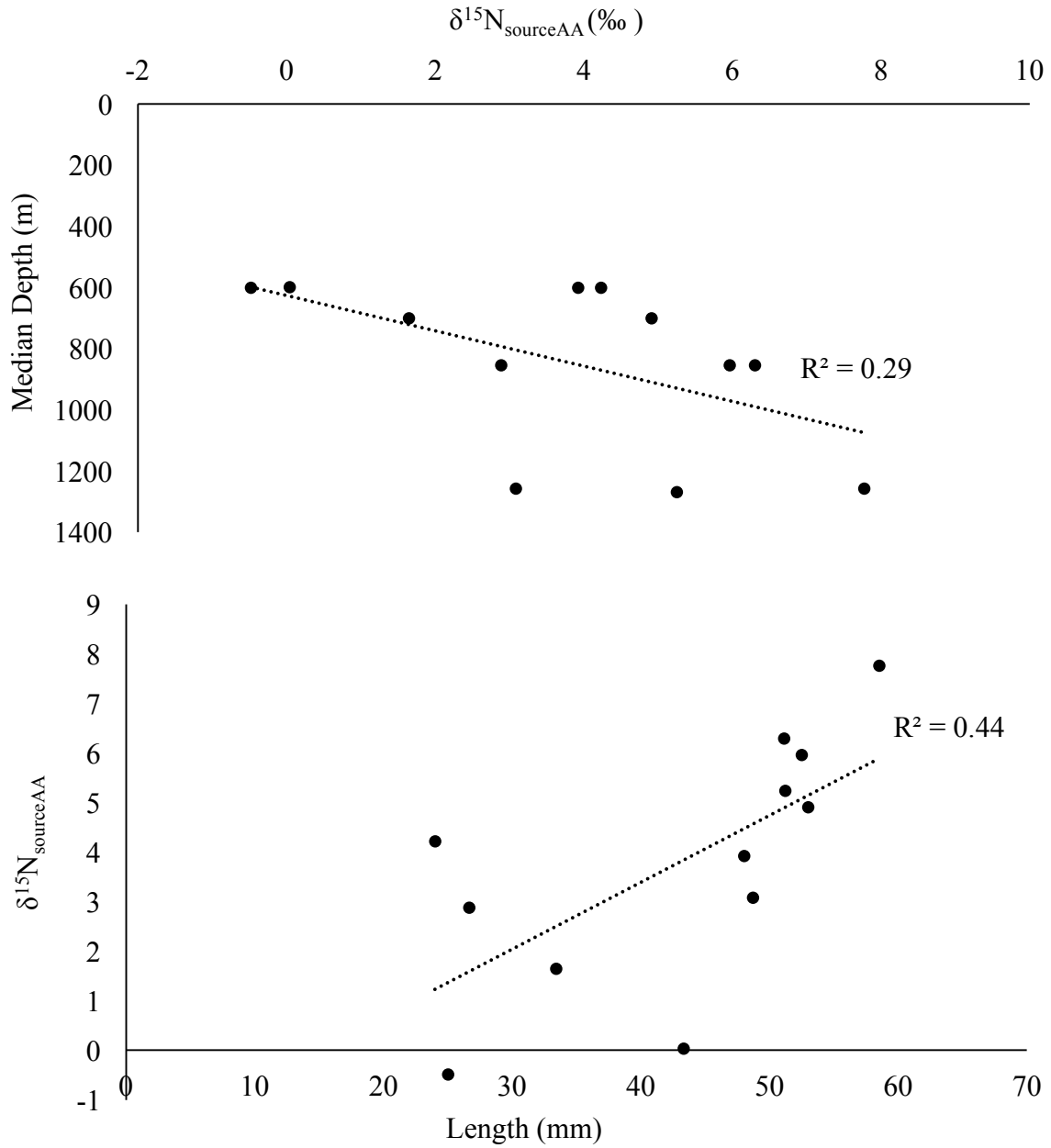**Figure 5.** $\delta^{15}N_{sourceAA}$ of twelve individuals of *C. pallida* plotted against depth (ANOVA p<0.1, df=12) (top) and length (ANOVA p<0.05, df=12) (bottom).

A large number of *Cyclothone pallida* were analyzed permitting a more detailed examination of intraspecific variability in isotopic values of amino acids. $\delta^{15}N_{sourceAA}$ values of twelve individuals of *C. pallida* were found to be highly variable (Figure 5). $\delta^{15}N_{sourceAA}$ values increased with depth as well as with length for this species (Figure 5b. The effects of season, year or capture location could not be fully explored because ten of the twelve individuals were captured at Station ALOHA in the summer of 2014. To test that variability in the $\delta^{15}N_{sourceAA}$ values of *C. pallida* was not being caused by the presence of cryptic species four individuals, two with unusually high $\delta^{15}N_{sourceAA}$ values for their median depth of capture and two with unusually low $\delta^{15}N_{sourceAA}$ values for their median depth of capture were genetically barcoded however all individuals were confirmed to be *C. pallida* (Appendix). The variability found in *C. pallida* $\delta^{15}N_{sourceAA}$ values was not unusual with four species of micronekton having standard deviations higher than that of *C. pallida* including *C. atrum*, *H. proximum*, *Exocoetus* sp. and *S. sector* (Table 1; Figure 3).

DISCUSSION

The goal of this study was to determine if suspended particles were an important carbon source for mesopelagic and bathypelagic micronekton around Hawaii. Suspended particles have been overlooked as a source of carbon at these depths. Using results of AA-CSIA, we were able to find evidence of micronekton from around Hawaii feeding from a suspended particle based food web by tracing amino acids from particles into micronekton. This was made possible by the fact that suspended particles in the mesopelagic and bathypelagic have distinct $\delta^{15}N$ values in their source amino acids which are higher than large sinking particles or particles at the surface of the ocean, a result of fractionation from heterotrophic degradation (Altabet and McCarthy

1986; McCarthy et al. 2007; Hannides et al. 2013), which allowed us to identify micronekton that feed from a suspended particle food web by looking for the distinct suspended particle signal in their source amino acids.

$\delta^{15}N_{sourceAA}$ values of micronekton increased from the epipelagic to the upper bathypelagic suggesting that with increasing depth micronekton begin to rely more on a suspended particle based food web distinct from the surface water food web. This pattern of increasing $\delta^{15}N_{sourceAA}$ values with depth generally followed patterns found in particles and zooplankton from the same depth range (Hannides et al. 2013; Choy et al. 2015). $\delta^{15}N_{sourceAA}$ values of epipelagic micronekton (*Exocoetus* sp., *T. albacares* (J), *C. hippurus* (J), stomatopods) were similar to the $\delta^{15}N_{sourceAA}$ values of both small and large particles at the surface, indicating that these species feed on a food web based on fresh surface produced material comprised of aggregate particles, fecal pellets, phytoplankton, heterotrophic bacteria and small protists. Hannides et al. (*in prep*) has shown that epipelagic zooplankton (0-200 m), have similar $\delta^{15}N_{sourceAA}$ values to the epipelagic micronekton and therefore are likely a trophic step between the particles and the micronekton.

In the mesopelagic, migrating species which have depths of occurrence that cluster around 600 m during the day have similar $\delta^{15}N_{sourceAA}$ values to epipelagic species consistent with mesopelagic migrators that feed from a food web based on surface derived material at night in surface waters as has been suggested in past diet studies (Clarke 1978; Hopkins et al. 1996). For example, Hopkins et al. (1996) reported that Myctophids fed primarily on Euphausids and copepods, especially the copepod *Pleuromama xiphias*. In this study, $\delta^{15}N_{sourceAA}$ values of myctophids *B. longipes, H. proximum* and *M. lynchnobium* had $\delta^{15}N_{sourceAA}$ values similar to both *Euphausia* sp. and *P. xiphias* (Hannides et al. *in prep*). The predatory fangtooth, *A. cornuta,* is

unique amongst the sampled migrating mesopelagic micronekton because it migrates from the lower mesopelagic to the upper mesopelagic rather than migrating all the way to surface but still has $\delta^{15}N_{sourceAA}$ values similar to surface derived material which would seem to indicate that this species feeds on micronekton which migrate to the surface. However, *A. cornuta* does not exhibit reverse migrations. The shallowest end of the depth range for *A. cornuta* overlaps with the deepest part of the depth range for several species of mesopelagic micronekton therefore it is likely that *A. cornuta* feeds during times when these depth ranges overlap.

Mesopelagic and upper bathypelagic non-migrators (600-1200 m; Fig. 2b) had $\delta^{15}N_{sourceAA}$ values that were similar to those found in either sinking or suspended particles demonstrating that there are micronekton that feed on a food web that is based on both suspended and sinking particles. One exception to this was *B. distofax*, which had $\delta^{15}N_{sourceAA}$ values which were similar to surface derived material. Clarke et al. (1973) reported that no *B. distofax* (formerly known as *B. superlateralis*) larger than 53 mm were collected above the day depth range of the species indicating that large individuals of this species did not migrate. The *B. distofax* collected in this study ranged in size from 48.6-87.7 mm and for this reason we classified this species as a non-migrator. However, one of the individuals in this study was captured between 100 m and the surface indicating that *B. distofax* in this size range must migrate which is consistent with their $\delta^{15}N_{sourceAA}$ values similar to that of surface derived material. Amongst the remaining mesopelagic non-migrator, species with median depths between 500 and 700 m had $\delta^{15}N_{sourceAA}$ values similar to sinking particles suggesting that these species feed from a sinking particle based food web. Species with median depth of capture between 700 m and 1300 m had $\delta^{15}N_{sourceAA}$ values that were more similar to suspended particles indicating that these species feed from a suspended particle based food web. These results indicate that suspended particles are an

important carbon source in the mesopelagic and bathypelagic because if these particles only made up a small part of the diet of a few small organisms we would not expect the suspended particle signal to be seen in the higher trophic level micronekton. Furthermore, this suggests that we have overlooked a potentially important food source and that suspended particles supply enough energy to be shuttled up to higher trophic levels. Is this energy, though, shuttled to trophic levels higher than those of micronekton? Choy et al. (2015) measured $\delta^{15}N_{sourceAA}$ values in commercially important fishes around Hawaii and found that $\delta^{15}N_{sourceAA}$ values ranged from 0.04 – 3.04 (‰), which are more similar $\delta^{15}N_{sourceAA}$ values to the surface material or larger sinking particles. This is likely due to the fact that very few species dive deep enough to access the micronekton that feed from the suspended particle food web. Two commercially important fish species, Smith's escolar (*Lepidocybium flavobrunneum*) and the coveted Blue Fin Tuna (*Thunnus thynnus*), have been documented to have foraging depths of 1000 m or more, deep enough to access the micronekton which feed from the suspended particle based food web (Kerstetter et al. 2008; Wilson and Block 2009), however Smith's escolar was found to have a $\delta^{15}N_{sourceAA}$ value of 1.4‰, similar to sinking particles in the mesopelagic (Choy et al. 2015). The $\delta^{15}N_{sourceAA}$ values of Blue Fin Tuna around Hawaii have not been measured. The suspended particle food web is clearly an important nutritional source for lower mesopelagic and upper bathypelagic micronekton which is separate from the fresh surface derived material utilized by epipelagic and upper mesopelagic micronekton, however it likely does not influence populations of commercially important, deep foraging fishes.

It is important to point out that $\delta^{15}N_{sourceAA}$ values were highly variable within several species of micronekton. One species which was found to have particularly high variability in its $\delta^{15}N_{sourceAA}$ values was *C. pallida*. The presence of cryptic species did not seem to generate the

observed variability in $\delta^{15}N_{sourceAA}$ values of these individuals as genetic barcoding confirmed that four" outlier" individuals which had either unusually high or unusually low $\delta^{15}N_{sourceAA}$ values were in fact *C. pallida* (Appendix). For *C. pallida*, almost half of this variability could be explained by the length of the individuals. Additionally, almost a quarter of the variability in the $\delta^{15}N_{sourceAA}$ values could be explained by the median depth of capture of the individuals. However, Maynard et al. (1978) observed that larger *C. pallida* tend to occur deeper implying that length and depth are two closely related variables. One thing to consider for these individuals is their median depth of capture comes from a large depth range. For example, an individual with a median depth of capture of 1250 m could have been caught anywhere between 1500 m and 1000 m. In this study, median depth of occurrence was found to explain 50% of variability in $\delta^{15}N_{sourceAA}$ values for all micronekton species. It is possible that by using more discreet depth ranges for sampling *C. pallida* we would find that depth of capture would explain even more variability in the $\delta^{15}N_{sourceAA}$ values for this species. Another possibility for the variability in the $\delta^{15}N_{sourceAA}$ values of *C. pallida* is that these fishes do not exist at discreet depths but rather undergo periodic vertical migrations within their depth range (600-1500 m). For example, Sutton et al. (2008) found the North Atlantic species *Cyclothone microdon*, which were a dominant species between 1500 and 2300 m, were also found frequently above 750 m. However, if all individuals of *C. pallida* fed throughout their depth range, i.e., where the sinking particle food web is dominant (500-700 m) as well as where the suspended particle food web is dominant (700-1300 m) we would expect the $\delta^{15}N_{sourceAA}$ values from these two food webs to average out leading to more similar $\delta^{15}N_{sourceAA}$ values amongst individuals.

As part of this study, we wanted to determine if, with some insight from AA-CISA, bulk isotope data could be used to identify organisms that fed from a suspended particle based food

web. Bulk isotope analysis is a less time consuming and considerably less expensive technique than AA-CSIA. Choy et al. (2015) identified several species of micronekton from around Hawaii which had very high bulk tissue $\delta^{15}N$ values (e.g. *C. pallida* (10.4‰) and *S. sector* (9.2‰)) similar to those of large pelagic fishes such as tuna and billfish (9.2‰ - 13.4‰) suggesting a different isotopic baseline, i.e. suspended particles, for the smaller lower trophic level fishes. We found several other species that also had very high bulk tissue $\delta^{15}N$ values including the mesopelagic, non-migrating *O. soleatus* (9.8‰), the bathypelagic, non-migrating *M. johnsonii* (13‰), and the mesopelagic, migrating species *Histioteuthis sp.* (10‰) and *C. sloani* (9.3‰). Since the bulk tissue $\delta^{15}N$ value of an organism reflects the trophic position of the organism as well as $\delta^{15}N$ values at the base of the food web, we would expect that the high bulk tissue $\delta^{15}N$ values in these micronekton are the result of a similar trophic position to the large pelagic fishes, and/or that they are feeding from a food web with an elevated isotopic baseline – e.g. suspended particles (Hannides et al. 2013). Choy et al. (2015) reported that the trophic positons of the large pelagic fishes ranged from 4.3-5.0. Using the same method to calculate trophic position, we found that several of the micronekton species with high $\delta^{15}N$ values had trophic positions within the range or similar to the trophic positions of the large pelagic fishes. For example the spookfish *O. soleatus* had a trophic position of 4.4 (Bradley et al. 2015). *O. soleatus* is a small mouthed, sluggish fish which probably has a high trophic position because it likely feeds on predatory siphonophores (Mauchline and Gordon 1983). However, other micronekton species with high $\delta^{15}N$ values, such as *S. sector* (TP=3.1), had lower trophic positions but high $\delta^{15}N_{sourceAA}$ values likely being driven by feeding from a suspended particle food web. Thus high bulk $\delta^{15}N$ values could be interpreted in multiple ways.

Indeed, we found that $\delta^{15}N_{sourceAA}$ values and trophic position explained variability in bulk tissue $\delta^{15}N$ values but poor correlation between $\delta^{15}N_{sourceAA}$ values and bulk tissue $\delta^{15}N$ values (TP 2.7-3.1, ANOVA, p>0.1; TP 3.1-3.7, ANOVA, p>0.1; TP >3.7, ANOVA, p>0.05) indicate that there may be other factors influencing bulk tissue $\delta^{15}N$ values in micronekton. Therefore, we were not able to use bulk tissue $\delta^{15}N$ values to unequivocally identify species that were feeding from a suspended particle based food web. However, $\delta^{15}N_{sourceAA}$ values were able to explain 62% of the variability in the bulk tissue $\delta^{15}N$ values of *C. pallida* implying that within a species the isotopic baseline has a stronger influence on bulk tissue $\delta^{15}N$ values than trophic position.

The main objective of this study was to determine if any micronekton feed from a suspended particle based food web. We suggest that *C. pallida*, *J. diaphana*, *C. atrum*, *S. sector* and *M. johnsonii* feed largely from a suspended particle based food web because these species have $\delta^{15}N_{sourceAA}$ values that match those of the suspended particles at the depths that these micronekton live. Choy et al. (2015) found similar results for *C. pallida* and *C. atrum.* Results of our AA-CSIA suggest that suspended particles are an overlooked food source to the meso and bathypelagic food web that could make up a portion of the "carbon deficit" found for mesopelagic and bathypelagic fauna. Further work should focus on a broader evaluation of the role of suspended particles in the deep mesopelagic food web, including microbes and zooplankton, in order to quantify their relative contribution to carbon budgets.

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APPENDIX

Rationale. High variability in $\delta^{15}N_{sourceA}$ was observed in *C. pallida*. Given prior reports of larger fish living deeper in the water column (Maynard 1982) and prior observations of cryptic species present within the genus *Cyclothone* (Miya and Nishida 1997), the presence of cryptic species in our material was one possible explanation for wide variation in observed isotopic values in *C. pallida*. In order to evaluate whether cryptic species may be present, we obtained DNA sequences of a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I (mtCOI) from four individuals of *C. pallida*, with specimens chosen based on unusually high or low $\delta^{15}N_{sourceAA}$ values and across depth from 600 – 1250m median depth of capture. Information on the individuals selected for barcoding is provided in the table below.

Table 2. Summary of data collected for the individuals of *C. pallida* selected for barcoding. All individuals were collected at Station ALOHA in the summer (August/September) of 2014.

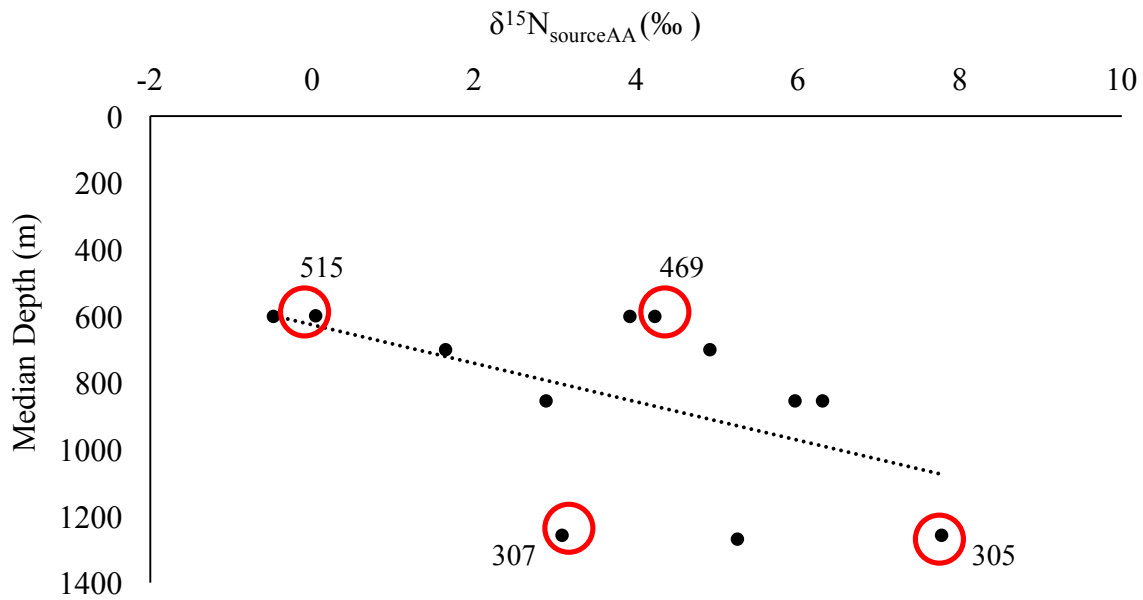| Sample ID	Median Depth of Capture (m)	Length (mm)	$\delta^{15}N_{sourceAA}$ (‰)
515	600	43.3	0.04
469	850	51.1	6.30
305	1250	58.5	7.76
307	1250	48.7	3.08

Figure 6. $\delta^{15}N_{sourceA}$ (‰) values of *C. pallida* plotted against median depth of capture. Individuals selected for barcoding have been circled and labeled by their sample ID number.

Methods. DNA was extracted using the DNeasy Bood & Tissue kit (Qiagen) following the manufacturer's recommended protocols, using freeze-dried and ground tissue. A 624-bp fragment of mtCOI was amplified in polymerase chain reaction (PCR) using primers described in Ward et al. (2005). Reactions were run in 20 µl volumes with 10 µl MangoMix (Bioline), 0.15 µl Bovine Serum Albumin (BSA, NEB Biolabs), and 2 µl of template DNA. PCR cycle conditions were as follows: 95 ℃ for 2 min, followed by 40 cycles of 95 ℃ for 30 sec, 45 ℃ for 30 sec, 72 ℃ for 1 min, with a final extension at 72 ℃ for 4 min. PCR reactions were purified using shrimp alkaline phosphatase and exonuclease I, and Sanger sequences were obtained from both strands on an ABI 3730XL. Sequences were edited, aligned, and trimmed using Genious v7.1.8 (Biomatters), with a final alignment using MUSCLE (Edgar et al. 2004) and including sequences from NCBI of *C. microdon*, *C. atraria*, and *C. pacifica*, *C. acclinidens*, *C. parapallida*, *C. pseudopallida*, *C. braueri* (EU148134- EU148139 (Zhang et al. unpublished),

FJ164515- FJ164523 (Steinke et al. 2009), GQ860355- GQ860358 (Devaney unpublished), GU071722 (Bucklin et al. 2010), GU071728 (Bucklin et al. 2010), GU071729 (Bucklin et al. 2010), GU071740 (Bucklin et al. 2010), GU071741 (Bucklin et al. 2010), GU440298 (Hastings and Burton 2008), HQ010054 (Hastings and Burton 2008), JN640877- JN640879 (Ward et al. 2005), KF929802 (Bentley and Wiley unpublished). Kimura 2-parameter (K2P) genetic distances were calculated in MEGA v6.06 (Tamura et al. 2013).

Results and Discussion. Two haplotypes that differed by a single substitution were observed in our *Cyclothone* specimens, with one of these haplotypes a 100% sequence match to a *C. pallida* sequence reported from an animal collected in the California Current [GU440298]. K2P genetic distances between *C. pallida* and other species in the genus *Cyclothone* ranged from $d = 0.168 - 0.299$, and between *C. pallida* populations in the Atlantic and Pacific Oceans of $d = 0.004-0.008$ (11-14 substitutions), suggesting sufficient polymorphism in this mtCOI fragment to detect species-level differentiation within the genus. We find no evidence of cryptic species in our material.

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