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ORIGINAL PAPER

Salps in the NW Atlantic Slope Water: metabarcoding and compound-specific stable isotope analysis of diet diversity and trophic interactions

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Abstract
Mesopelagic zones of the NW Atlantic Slope Water support pelagic assemblages with high biomass and high diversity. Species of salps (Phylum Urochordata, Class Thaliacea) are bloom-forming, filter-feeding, gelatinous zooplankton, with significant impacts on pelagic food webs and vertical transport of organic material. Questions remain of their diet diversity, prey selectivity, and timing of feeding with respect to vertical distribution and migration. This study analyzed three salp species, *Soestia (Iasis) zonaria, Salpa aspera*, and *Salpa fusiformis*, collected from the NW Atlantic Slope Water during July and August 2018 and 2019. DNA metabarcoding using V4 and V9 regions of 18S rRNA found dinoflagellates to be predominant prey for all three salp species in both years. Analysis of five prey groups detected by metabarcoding revealed differences in proportions of prey. Compound-specific stable isotope analysis of essential amino acids, which integrates over several months, found diatoms to be the dominant end-member source for all three species. Our findings on salp diet diversity broaden our understanding of trophic pathways in the mesopelagic food web, including sources of productivity and possible impacts of climate change.

Keywords Salps · Diet diversity · Mesopelagic · Food web · DNA metabarcoding · Compound-Specific Stable Isotopes

Introduction

Salps in the Mesopelagic Ecosystem

The mesopelagic depths of the global ocean are characterized by high diversity and high biomass of marine organisms, from microbes to mammals (St John et al. 2016). Also known as the Ocean Twilight Zone (OTZ) and defined as depths from 200 to 1,000 m, these waters are home to numerous species of fish and zooplankton (Koppelmann and Frost 2008; Kaartvedt et al. 2019). To better understand trophic interactions of gelatinous organisms in these communities, this study examined diets of salps in the mesopelagic zone of the Northwest Atlantic Slope Water, a region bordered by the dynamic currents of the Gulf Stream System and the continental shelf (Iselin 1936; McLellan et al. 1953).

Salps (Phylum Urochordata, Class Thaliacea) are important members of the mesopelagic assemblage in terms of both biomass and food web interactions (Vargas and Madin 2004). Salps are filter feeders that pump water through their oral siphon into a fine mucous net, which is then moved toward the esophagus (Sutherland et al. 2010). Salps are known to feed on particles ranging in size over three orders of magnitude (Vargas and Madin 2004), which gives them unusual and potentially important roles in food web dynamics. Salps have important impacts on transport of carbon through the water column and sequestration in the sediment both by diel vertical migration (DVM) and by sinking of their large fecal pellets (Wiebe et al. 1979; Harbou 2010; Henschke et al. 2016; Stone and Steinberg 2016; Lüskow et al. 2020; Decima et al. 2023). Many species of

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salps exhibit strong patterns of DVM, with daytime depths well below the epipelagic waters inhabited at night (Madin et al. 2006). *Soestia* (previously known as *Iasis,* Garic et al. 2024*) zonaria, Salpa aspera*, and *Salpa fusiformis* are all known to exhibit DVM (Stone and Steinberg 2014, 2016), with significantly higher night vs. day biomass in the top 150 m (Madin et al. 1996). *Salpa aspera* vertical migration ranged from 600 – 800 m or deeper during the day, with aggregations near the surface (~ 100 m) at night in the NW Atlantic Slope Water (Wiebe et al. 1979; Madin et al. 2006). Previous studies found abundances of *S. fusiformis* to be negatively correlated with temperature in the mesopelagic (300 – 600 m), suggesting that warming of waters may have negative impacts on populations (Steinberg et al. 2000, 2012; Bianchi et al. 2013). Reverse migration was observed for *S. fusiformis* in the Yellow Sea, associated with patterns of light intensity, chlorophyll concentrations, and other environmental parameters (Liu et al. 2012).

Salps are known to exhibit high spatiotemporal variability, with large swarms or blooms dependent on seasonal and environmental conditions. Salp blooms in the North Atlantic have long been reported for *S. aspera* (Wiebe et al. 1979; Madin et al. 1994, 2006) and *S. fusiformis* (Fraser 1949, 1969; Hunt 1968; Brattstrom 1972). The potential for bloom formation differs among species: *S. zonaria* has been estimated to release up to 420 salps per individual under bloom conditions (Daponte et al. (2013). This is more than twice the rates measured for other salps (Morris et al. 1988), including *S. fusiformis* reported to produce 93–179 zooids (Braconnot et al. 1988) and *Cyclosalpa bakeri* reported to produce 170 zooids per individual (Madin and Purcell 1992).

Salps are efficient filter feeders that contribute substantially to transport of carbon through the mesopelagic, through consumption of prey in surface waters and vertical migration into deep zones (Steinberg et al. 2023). Sequestration of carbon in the sediment occurs via the sinking of the large fecal pellets (Henschke et al. 2016). Several studies have highlighted the large volumes of water filtered by salps. Large (75 mm) individuals of *Cyclosalpa affinis* are capable of rapidly filtering water up to 200 ml / min, compared to 0.76 ml / min for copepods (Henschke et al. 2016). This capacity for rapid, high-volume filtration provides the capacity for bloom formation in dilute prey conditions (Alldredge and Madin 1982). During bloom conditions, salps can substantially impact and alter pelagic ecosystems (Henschke et al. 2016).

Salp species differ in muscle organization, filter efficiency, and swimming speeds, which can also depend upon life stages, whether solitary or aggregate (Sutherland and Weihs 2017). Body size, pulse frequency, and strength of compression are characteristics that determine the amount of water a salp can filter (Madin and Kremer 1995; Sutherland et al. 2010). *Salpa fusiformis* and *S. zonaria* show similarities of thickness and sturdiness of their tests and have been reported to swim faster than other species, due to the overlap of zooids on the chain lying along the chain's axis (Bone and Trueman 1983). Gut contents measured as chlorophyll concentration have been directly related to body size in *S. fusiformis*, which has been reported to consume up to 5% of primary production (Huskin et al. 2003). Salps feed on particles ranging over three orders of magnitude in size (Vargas and Madin 2004; Sutherland et al. 2010; Lawrence et al. 2018), including particles that are four to five orders of magnitude smaller than themselves, thereby bypassing several trophic levels (Fortier et al. 1994).

Salps may be direct consumers of bacteria, ciliates, and autotrophic and heterotrophic dinoflagellates (Vargas and Madin 2004) and may have some of their energy demands met by bacterial biomass (Sutherland et al. 2010) representing the potential for diverse bioenergetic pathways. Prey selectivity by these filter feeders may also be common; salps have been shown to preferentially assimilate smaller heterotrophic prey under low chlorophyll conditions, including flagellates and microzooplankton (Pakhomov et al. 2019). For example, diet studies on *S. fusiformis* have revealed preferential ingestion of eukaryotic cells over prokaryotic cells of similar size (~ 1 µm); calculations of cell carbon content showed 55% came from pico-eukaryotic algae, which comprised 14% of the available carbon in the pelagic community (Dadon-Pilosof et al. 2019). Moreover, studies under high chlorophyll *a* conditions, when diatoms generally dominate the plankton assemblage, suggested non-consumption or dietary exclusion of diatoms by salps (Hughes 1990, 2013).

Studies have demonstrated that salps have varying rates of retention and assimilation of particles of different size and composition (Vargas and Madin 2004; von Harbou et al. 2011; Metfies et al. 2014; Conley et al. 2017; Dadon-Pilosof et al. 2017; Walters et al. 2019). Studies using varied techniques have provided evidence of selective feeding – or selective assimilation – by salps relative to the available prey assemblage (von Harbou et al. 2011; Metfies et al. 2014; Conley et al. 2017; Dadon-Pilosof et al. 2017; Walters et al. 2019; Fender et al. 2023). Tissue fatty acid signatures and metagenomic analysis of stomach contents of *Salpa thompsoni* and *Ihlea racovitzai* suggested a diet based on small flagellates, but not diatoms, which often pass through the salp stomach undigested (von Harbou et al. 2011; Metfies et al. 2014). Salps cannot break hard parts of protected prey items, such as frustules of diatoms, and are continuous feeders, so food items consumed generally have a short stomach residence time – usually a matter of hours (Pakhomov et al. 2006; von Harbou et al. 2011). A high degree of dietary variability and complexity has been observed in salps, but capturing the true spatial and temporal patterns of diet diversity in salp gut contents is challenging due to the lack of diagnostic characters in the prey after digestion. Molecular

and chemical analyses are thus particularly useful in resolving salp trophodynamics and understanding their relevance in the mesopelagic zone.

DNA Metabarcoding of Diets

DNA metabarcoding is the identification of multiple taxonomic groups or species based on high-throughput sequencing (HTS) of short barcode gene regions from environmental samples (Taberlet et al. 2012). Multi-gene metabarcoding has yielded new understanding of marine biodiversity using hypervariable regions of ribosomal RNA (rRNA) genes (Quast et al. 2013; Amaral-Zettler et al. 2014) and several mitochondrial gene regions, including cytochrome oxidase I (COI) (Bucklin et al. 2011, 2021; Questel et al. 2021). DNA metabarcoding of marine organisms has been used to understand sources of productivity of ocean food webs (Zamora-Terol et al. 2020; D'Alessandro and Mariani 2021; Käse et al. 2021; Huggett et al. 2022; Russo et al. 2023). Identification of prey based on metabarcoding of DNA extracted from gut contents has provided more detailed, complete, and accurate characterization of diets of marine organisms (Novotny et al. 2021, 2023; Coker et al. 2023). Gelatinous plankton have been detected by metabarcoding as both predators (Damian-Serrano et al. 2022) and prey (Cavallo et al. 2018). Metabarcoding has been used to examine diets of the salps, *Salpa thompsoni* and *Ihlea racovitzai*; in combination with tissue fatty acid signatures, metabarcoding of stomach contents suggested diets were largely composed of small flagellates, while diatoms were found to pass through salps undigested (von Harbou et al. 2011; Metfies et al. 2014).

The growing availability of reference sequence databases for the various gene regions used as molecular markers is allowing more complete understanding of ocean food webs (Bucklin et al. 2021; O'Brien et al. 2024). Hypervariable regions of the 18S ribosomal RNA (rRNA) gene have been widely used for molecular detection and identification of marine organisms, from microbes to mammals (Amaral-Zetler et al. 2009; Metfies et al. 2014; Hu et al. 2015; Govindarajan et al. 2021, 2023).

Compound-Specific Stable Isotope Analysis

Stable isotopes of carbon (^{13}C) have been used to identify dietary sources and examine food web linkages in mesopelagic ecosystems (Post 2002; Richoux and Froneman 2009; Annasawmy et al. 2020; Richards et al. 2020; Shea et al. 2023; Wojtal et al. 2023). By using specific biochemical compounds such as amino acids (AAs), compound-specific stable isotope analysis (CSIA) allows for greater resolution in diet analysis when compared to bulk isotope analysis (Larsen et al. 2015; Close 2019; Whiteman et al. 2019). AAs that are synthesized de novo at the base of the food web by primary producers and bacteria are called essential AAs, including Isoleucine, Leucine, Valine, Threonine, and Phenylalanine. These AAs are propagated through the food web with little to no fractionation, resulting in the retention of an isotopic signature from basal end members by species at higher trophic levels (Scott et al. 2006; Larsen et al. 2009; Bond and Diamond 2011; Arthur et al. 2014).

CSIA values provide insights into organismal diets based on the turnover rates of the cells in the tissues being analyzed. In the case of muscle tissue, the values typically represent diet averages on the order of a weeks to months (Martínez del Rio and Carleton 2012; Whiteman et al. 2019). While this means recent small scale physical changes may not be represented, CSIA allows examination of diets reflecting the more important and consistent prey items, with less impact of rare or unique prey items.

Previous studies have used CSIA techniques to examine questions of prey choice and selectivity by pelagic predators and to determine sources of productivity of marine food webs based on relative contributions of end members to higher trophic level diets (Budge et al. 2008; McMahon et al. 2016). CSIA has proven useful for tracing sources of productivity for gelatinous organisms, which may rely upon tiny and/or dissolved organic material (Bănaru et al. 2014; Decima et al. 2019; Hetherington et al. 2024). Using these techniques, salp gut contents have been found to contain representatives of diatoms, dinoflagellates, haptophyte flagellates (Prymnesiales and Coccolithophorales), and copepods (Ahmad Ishak et al. 2017).

This study used integrative analysis to provide a more complete view of the roles of salps in the mesopelagic food web. We used a multi-gene DNA metabarcoding approach in conjunction with CSIA to characterize diet diversity and determine trophic relationships of three salp species (*S. aspera*, *S. fusiformis*, and *S. zonaria*) from the Northwest Atlantic Slope Water during the Ocean Twilight Zone (OTZ) program.

METHODS

Hydrography and Sample Collection in NW Atlantic

The mesopelagic ecosystem of the NW Atlantic Slope Water region was surveyed during August 12–19, 2018 and July 27—August 5, 2019 aboard the NOAA research vessel (R/V) *Henry B. Bigelow*. At each station, six conductivity-temperature-depth (CTD) profiles were made with a Seabird 911plus CTD mounted on a small rosette frame with eight 5-L Niskin bottles for collection of water for analysis of chlorophyll, nutrients, and oxygen isotope concentrations. Sample collections were carried out during day and night using a modified Marinovich midwater trawl (MWT; De

Robertis et al. 2017; Jech and Lavery 2018) and 1-m^2 Multiple Opening and Closing Net and Environmental Sensing System (MOCNESS; Wiebe et al. 1985b) (Table 1). For both MWT and MOCNESS tows, individual salp zooids were removed from the samples immediately upon collection to minimize accumulation of gut contents during collection, sometimes referred to as "net feeding". Salps were rinsed in seawater to remove material or organisms attached to the salp test. Despite rapid handling of samples, the possibility remains that some non-prey species will be detected by metabarcoding of DNA extracted from gut contents. After tentative identification and cleaning, samples were immediately flash-frozen in liquid nitrogen to preserve the integrity of tunic tissue and gut content DNA. Samples were stored at -80 °C until the time of analysis. In the laboratory, specimens were partially thawed to allow visual taxonomic identification of the salp species, designation of the life stage (solitary or aggregate), excision of tunic muscle tissue, dissection of the guts, and removal of the gut contents. The tunic muscle tissue was used for analysis of compound-specific stable isotopes and species identification by DNA barcoding of the mitochondrial COI gene. DNA was extracted from entire gut contents for multi-gene metabarcoding of diet diversity.

Salps used for analysis were the most abundant species in the samples collected during 2018 and 2019. *Soestia zonaria* was the most abundant species, with 80% in solitary stage; *S. fusiformis* collected were all in aggregate stage; for *S. aspera* collected, 62% were solitary individuals (Table 2; Suppl. Table 1).

DNA Extraction, Amplicon Generation and Sequencing

Gut contents were placed in elution buffer with Tris–HCl (10 mM), EDTA (100 mM, pH 8), NaCl (200 mM), sodium dodecyl sulfate (SDS, 1%), and Milli-Q water. After homogenization for 15 s, lysates were incubated in a water bath at 55 ºC for 4 h. DNA was extracted from digested samples using phenol:chloroform:isoamyl alcohol (25:24:1); potential PCR inhibitors were removed using a DNEasy PowerClean Pro Kit (Qiagen) according to manufacturer instructions. Fluorometric quantification of DNA was performed with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA).

Purified DNA extracted from gut contents was used to amplify two hypervariable regions of 18S rRNA using primers and protocols designed for eukaryotic organisms: V4 (Metfies et al. 2014) and V9 (Amaral-Zetter et al. 2009). PCR primers and protocols for both 18S r RNA hypervariable regions were not designed for detection of prokaryotic organisms; results presented here include only eukaryotic groups. Forward and reverse PCR primers were altered for multiplexed sequencing by adding 5' adapters (Illumina, Inc., San Diego, CA). PCR amplification of a 450 base-pair (bp) region of V4 used the primers: 528F (Medlin et al. 2006) and 690R (Metfies and Medlin 2008). The PCR reaction used 10 ng of DNA, with KAPA HiFi reagents, 5 µL buffer, 1 µL dNTPs, 0.5 µL HiFi Taq Polymerase, and 1 µL of each primer (10 µM), with the following PCR protocol: one denaturation cycle at 98 °C for 30 s; 10 cycles of 98 °C for 20 s, 57 °C for 30 s, and 72 °C for 15 s; and 15 cycles of 98 °C for 10 s, 66 °C for 30 s, and 72 °C for 15 s; with one extension cycle of 72 °C for 7 min; and hold at 4 °C.

The V9 PCR amplification used primers 1380F and 1510R (Amaral-Zetter et al. 2009). The reaction used 4 µL of DNA template (10 ng), with KAPA HiFi reagents (KAPA Biosystems, Massachusetts, USA): 5 µL buffer containing MgCl, 1 µL dNTPs, 0.5 µL HiFi Taq Polymerase, and 1 µL of each primer (10 µM). PCR protocol included one denaturation cycle at 98 °C for 30 s; 10 cycles of 98 °C for 20 s, 56 °C for 30 s, and 72 °C for 15 s; and 15 cycles of 98 °C for 10 s, 66 °C for 30 s, and 72 °C for 15 s; with one extension cycle of 72 °C for 7 min; and hold at 4 °C. Both V4 and V9 18S rRNA were checked for successful amplification by electrophoresis in a 2% agarose gel with a 50 bp marker.

Library preparation involved adding index and adapter sequences in a second PCR amplification of the purified amplicons using a master mix composed of (per sample): 5.0 µL purified PCR product; 5 µL Nextera XT Index 1 Primer; 5 µL Nextera XT Index 2 Primer; 25 µL 2×KAPA HiFi HotStart ReadyMix; 10 µL PCR-grade water; for a total volume of 50 µL. The PCR protocol was 95 °C for 3 min; 8 cycles of: 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; and 1 cycle of 72 °C for 5 min. The indexed PCR product was purified using AMPure XP beads, with a final elution volume of 25 µL. Libraries were validated for length and adapter dimer removal using the Agilent TapeStation 4200 D1000 High Sensitivity assay (Agilent Technologies, Santa Clara, CA, USA), then quantified and normalized using the dsDNA High Sensitivity Assay for Qubit 3.0 fluorometer (Life Technologies, Carlsbad, CA, USA). Sample libraries were prepared for Illumina sequencing by denaturing and diluting the libraries per manufacturer's protocol (Illumina, San Diego, CA, USA). Samples were pooled separately for each hypervariable region, equally normalized, and run on the Illumina MiSeq sequencer using MiSeq reagent Nano Kit v2 (500 cycles for V4 and 300 cycles for V9; 1 million clusters) spiked with 20% PhiX control library at a final loading concentration of 4 pM. Paired end sequencing was carried out at the University of Connecticut, Center for Genome Innovation (CGI; https://cgi.uconn.edu/).

Sequence Quality Assessment, Bioinformatics, and Statistical Analysis

Demultiplexed reads for hypervariable regions V4 and V9 18S rRNA were processed using a custom script for the

Table 1 Collection metadata for 2018 and 2019 cruises (HB1805 and HB1907) of the NOAA R/V *Henry B. Bigelow* for samples from which salps were used for metabarcoding and CSIA for this study. Tow # indicates Midwater trawl (MWT) or MOCNESS (MOC, including Net #). Depth indicates stratum sampled by the net (MOC) or maximum depth of tow (MWT). Times are local time at the collection locations (-4 h from Greenwich Mean Time (GMT)

Cruise	Station	Tow # (Net #)	Date	Start Time (Local)	End Time (Local)	Day / Night	Latitude (N)	Longitude (W)	Depth (m)	Volume Filtered (m^3)
HB1805	1	MOC #2 (0)	12-Aug-18	21:34	01:34	Night	40.364	-70.967	0–20	n/a
HB1805	2	MWT #4	16-Aug-18	02:24	03:04	Night	39.327	-70.782	50	n/a
HB1805	2	MWT #5	19-Aug-18	04:24	06:06	Night	39.200	-71.143	600	n/a
HB1907	2	MOC #4 (2)	28-Jul-19	10:14	13:33	Day	39.313	-70.226	800–599	1958.8
HB1907	2	MOC #5 (0)	29-Jul-19	19:05	22:55	Night	39.160	-70.356	0–1000	5496.3
HB1907	2	MWT #1	27-Jul-19	01:23	02:04	Night	39.210	-70.285	66	n/a
HB1907	2	MWT #2	27-Jul-19	06:16	06:56	Day	39.278	-70.318	126	n/a
HB1907	2	MWT #3	28-Jul-19	02:52	03:32	Night	39.341	-70.255	590	n/a
HB1907	2	MWT #4	28-Jul-19	06:22	07:02	Day	39.249	-70.212	487	n/a
HB1907	2	MOC #5 (8)	29-Jul-19	19:05	22:55	Night	39.297	-70.299	26–0	379.8
HB1907	2	MWT #5	30-Jul-19	02:43	03:23	Night	39.119	-70.427	425	n/a
HB1907	3	MWT #7	31-Jul-19	06:31	07:12	Day	39.249	-69.023	451	n/a
HB1907	3	MOC #7 (0)	31-Jul-19	09:47	13:06	Day	39.298	-68.945	0–1000	1391
HB1907	3	MOC #8 (3)	1-Aug-19	06:51	10:38	Day	39.320	-68.959	601–402	2211.1
HB1907	3	MWT #9	3-Aug-19	02:36	03:16	Night	39.274	-69.083	80	n/a
HB1907	3	MOC #11 (0)	3-Aug-19	19:47	23:46	Night	39.302	-68.998	0–1000	5384.1
HB1907	3	MOC #11 (7)	3-Aug-19	19:47	23:46	Night	39.302	-68.998	50–25	325.6
HB1907	3	MWT #10	4-Aug-19	02:52	03:32	Night	39.291	-68.934	475	n/a
HB1907	3	MOC #12 (2)	5-Aug-19	11:19	15:37	Day	39.318	-68.969	799–700	1098.8
HB1907	3	MOC #12 (6)	5-Aug-19	11:19	15:37	Day	39.318	-68.969	499–351	498.6

Table 2 Salp samples collected in 2018 (HB-1805) and 2019 (HB-1907) and analyzed by metabarcoding gut contents for two gene regions (V4 and V9) and by compound species stable isotope analysis (CSIA) of dissected muscle tissue from the same specimen

	HB-1805			HB-1907		
Salp species	V4	V9	CSIA	V4	V9	CSIA
Soestia zonaria	4	5	N/A	17(2)	17 (2)	17
Salpa aspera	3	3	N/A	5(1)	5(1)	8
Salpa fusiformis	3	3	N/A	7(3)	7(3)	6
Totals	10	11	N/A	29	29	31

Mothur pipeline (Ver. 1.44.3; Schloss et al. 2009) modified from Questel et al. (2021) and run on the Xanadu computing cluster of the UConn Computational Biology Core (CBC; https://bioinformatics.uconn.edu/). Contiguous sequences (contigs) were assembled from forward and reverse Illumina MiSeq reads and trimmed; quality Phred scores < 30. Contigs with lengths shorter than 350 bp for V4 and 120 bp for V9 were removed from analysis. For both V4 and V9 unique sequences were aligned against the reference database, SILVA Release 132 (Quast et al. 2013; https://www.arb-silva.de/documentation/release-132/). Any sequences that did not span the entire V4 or V9 region were removed, decreasing the presence of erroneous operational taxonomic units (OTUs) created during clustering. Before clustering of OTUs, the UNOISE method (Edgar 2016) within Mothur (Ver. 1.44.3) was used to remove potential PCR bias that may contribute to errors in biodiversity assessment (Kelly et al. 2019) limiting to 2 bp difference between sequences. These filtering and trimming steps are analogous to the generation of Amplicon Sequence Variants (ASVs) via the DADA2 pipeline (Callahan et al. 2017). Chimeras were detected using VSEARCH command (Rognes et al. 2016); the found chimeras were eliminated for downstream analysis.

Taxonomic identification of OTUs was carried out using a tailored database developed from the SSU 18S SILVA, 132 release (Blanco-Bercial 2020). The modified database includes 18S rRNA sequences for eukaryotic marine organisms acquired from the NCBI GenBank sequence repository available after the release of SILVA 132 (Quast et al. 2013). Taxonomic assignments were determined using a naïve Bayesian classifier algorithm (Wang et al. 2007), based on the highest probability that a given sequence contains kmers specific to a sequence of a known taxonomic identity; default kmer size (ksize) = 8 was used. Taxonomic assignments with identification bootstrap values ≥ 80% after 100 iterations were accepted.

Sequence numbers for both gene regions were listed in taxonomy summary files (Wang et al. 2007) generated by Mothur (Ver. 1.44.3; Edgar 2016). Results for both V4 and V9 were analyzed for five taxonomic groups (Dinoflagellata, Syndiniales, Diatomea, Rhizaria, and Copepoda) previously reported as prey of salps (Madin and Purcell 1992; Harbou et al. 2011; Ahmad Ishak et al. 2017). Sequences classified as salps or fish (Teleostei) in gut contents were not included in analysis of diet diversity, to remove predator and unlikely prey. Gut contents of some salp species yielded only V9 sequences identified as salp; these samples were removed from downstream analysis, reducing sample sizes in some cases. One 2018 sample was excluded from the V4 metabarcoding dataset due to failed amplicon generation.

Diet diversity was analyzed within and among the three salp species by multivariate statistical analysis of V4 and V9 sequence numbers (log10 + 1 values) using MatLab (Ver. 2020B; 9.9.0.1467703). Variation in proportions of the five prey groups between salp species and years was tested using 3-way Analysis of Variance (ANOVA). One distance measure used was the Bray–Curtis dissimilarity coefficient (Bray and Curtis 1957; McCune et al. 2002), with results displayed as cluster diagrams for both years for each species. Differentiation among the three species was analyzed and visualized by Non-Metric Multidimensional Scaling (NMDS) using the FATHOM Toolbox for MatLab (Jones 2017; https://www.usf.edu/marine-science/research/matlab-resources/index.aspx/).

Analysis of Compound Specific Stable Isotopes

Tissue samples were excised from a total of 33 salp specimens of the three species collected in 2019 used for metabarcoding of gut contents; no samples collected in 2018 were analyzed for CSIA (Table 2). Muscle tissue was freeze-dried prior to hydrolysis in 6 M hydrochloric acid, at 110° C, overnight. The hydrolysates were then dried at 60° C, under a stream of nitrogen gas (N_2), then re-suspended in 0.01 M hydrochloric acid before being dried again at 60° C, under a stream of N_2 prior to derivatization with methyl chloroformate. Samples were re-suspended in chloroform and injected onto a gas chromatography (GC) column in split-less mode, using a ramped temperature program from 80° C to 255° C with an injector temperature of 250° C. Amino acids were separated on a VF-23 ms GC column (30 m length, 0.25 mm inner diameter, and 0.25 µm film thickness,) using an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) at Woods Hole Oceanographic Institution. A gas

chromatography-combustion continuous flow interface was used to combust the separated amino acid (AA) peaks at 1030° C before a CO_2 measurement was made on an isotope ratio monitoring mass spectrometer (Thermo Finnigan Mat 253). CO_2 reference gas was pulsed intermittently for standardization. Samples were analyzed in duplicate along with chemical and cod standards with known AA isotopic composition. These standards account for kinetic fractionation and introduction of carbon often paired with derivatization (Silfer et al. 1991). CSIA methods are further described in Walsh et al. (2014). This protocol produced peaks for five essential amino acids: threonine (Thr), isoleucine (Ileu), valine (Val), phenylalanine (Phe), and leucine (Leu), plus five non-essential amino acids: alanine (Ala), glycine (Gly), proline (Pro), aspartic acid (Asp), and glutamic acid (Glu). Mean reproducibility for all individual amino acids from a long-term lab fish muscle standard was maintained at ± 0.5%.

Essential AA $\delta^{13}C$ values were then analyzed using principal component analysis of essential amino acids and visualized using ggplot in R (Wickham 2016). Data were normalized for comparison to essential $\delta^{13}C$ data for bacteria, diatoms, and dinoflagellates. End member data from Stahl et al. (2023) included analysis of nine genera of bacteria (20 samples), three species of diatoms (9 samples), and three species of dinoflagellates (9 samples). Confidence intervals (90%) were calculated for end member data.

RESULTS

Hydrography of the collection area in NW Atlantic Slope Water

Temperatures and salinities at the sample collection locations in HB-1805 Station #2 and HB-1907 Stations #2 and #3 were similar below 600 m; near the surface, temperatures were cooler and salinities were lower in 2018 than 2019 at Station 2; above 50 m, temperatures were warmer at all three stations, while salinities showed wide variation at Station 2 in 2018 and 2019, with high, but less variable, values in 2019 (Fig. 1). Based on the hydrographic data, Station #2 was in the Slope Water during collections in both years, while higher and more variable temperatures and salinities at Station #3 in 2019 (Fig. 1) indicated it was likely affected by a Gulf Steam meander or warm core ring (Wiebe et al. 1985a).

Metabarcoding analysis of prey diversity

The hypervariable regions of V4 and V9 18S rRNA were sequenced for gut contents of three salp species: *Soestia zonaria*, *Salpa aspera* and *S. fusiformis* collected during HB-1805 and HB-1907 cruises (Tables 1, 2). V4 sequence numbers (Log10 + 1) showed the predominance of five prey groups: Dinoflagellata, Syndiniales, Diatomea, Rhizaria, and Copepoda (Fig. 2, Table 3). The Syndiniales (also known as Alveolates) are an early-branching sub-group of parasitic Dinoflagellata; the two groups were analyzed separately here. Based on V4 identifications of dinoflagellate species, the diets of the three salp species was dominated by *Gonyaulax polygramma* in 2018; in 2019, *Scrippsiella trochoidea* was the most abundant species, followed by *Gymonxanthella radiolarae* and *Pelagodinium bei* for all three salp species (Table 4).

Diatoms were abundant prey for *S. zonaria* based on V4, with higher numbers in 2019 than 2018; diatoms were also important diet components for *S. aspera* in both years and for *S. fusiformis* in 2018 (Table 3). *Thalassiosira* sp. was the predominant diatom species in the diet of *S. zonaria* in 2018, while *Mindiscus trioculatus* predominated in *S. fusiformis* and *S. aspera* in 2018; in 2019, *S. zonaria* samples had similar average proportions of sequences of three diatom species: *Thaliassiosira* sp., *M. trioculatus,* and *Minutocellus polymorphus* (Table 4). The gut contents of *S. fusiformis* included high numbers of V4 sequences for two diatom species: *Meuniera membranacea* (50%) and *Chaetoceros* sp. (Table 4).

Copepoda was one of most abundant groups detected by V4 in 2019 (Table 3). There were notable similarities and differences in the species of copepods found in gut contents of the salp species, with additional differences between years (Table 5). In 2018, the copepods, *Mecynocera clausi* and *Paracalanus parvus*, were found in all three salp species, with the addition of *Metridia pacifica*, *Neocalanus cristatus*, and *Pleuromamma abdominalis* in *S. aspera* and *S. fusiformis*, and *Rhincalanus nasutus* in *S. aspera* (Table 5). In 2019, *Calanus finmarchicus* and *P. parvus* were found in abundance in all salp species; additional copepod species detected in at least one salp species included: *Candacia* sp., *Gaetanus variabilis, Temora discaudata*, and *Undinula vulgaris* (Table 5).

Based on 3-way ANOVA of V4 sequence numbers (Log10 + 1), prey composition ($F = 4.98$, $p < 0.008$) and relative proportions of prey groups ($F = 40.58$, $p < 0.001$) differed significantly between the salp species, but these patterns did not differ significantly between years ($F = 1.34$, $p < 0.249$). The Bray–Curtis cluster plot based on V4 for prey groups showed one distinct cluster including samples of *S. zonaria* from both years, a second cluster intermixing with *S. aspera* and *S. fusiformis*, albeit with a sub-cluster of *S. fusiformis* from 2019 (Fig. 3). NMDS showed separation of prey groups detected by V4 for *S. fusiformis* and *S. zonaria* in 2018, with more intermixing of the two species in 2019; prey groups of *S. aspera* were not clearly resolved in either year (Fig. 4).

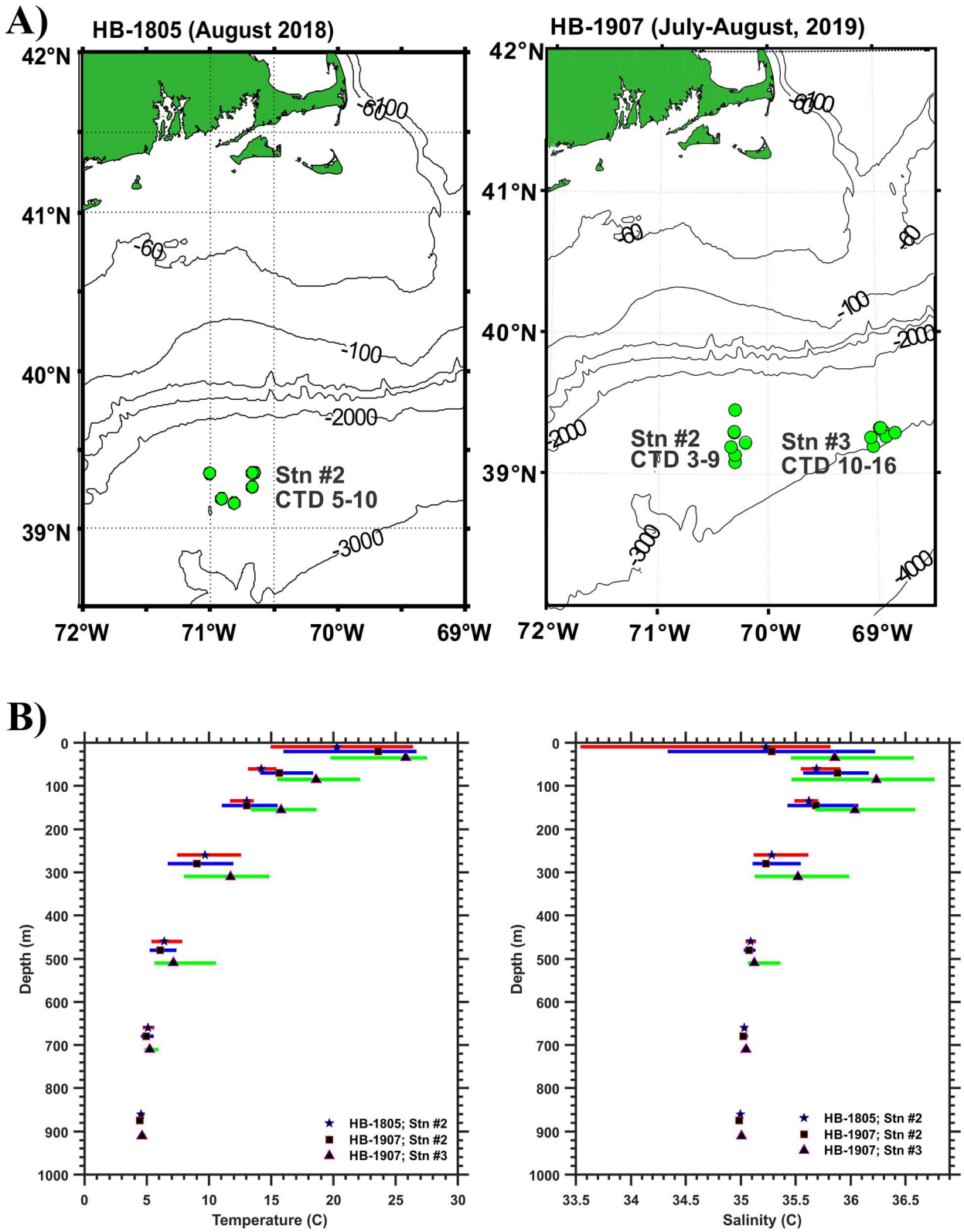

Fig. 1 A) Station locations and bathymetry during R/V *Henry Bigelow* cruises in 2018 and 2019. B) Hydrography based on CTD profiles taken at the stations shown each year. Figures prepared by P.H. Wiebe (Woods Hole Oceanographic Institution)

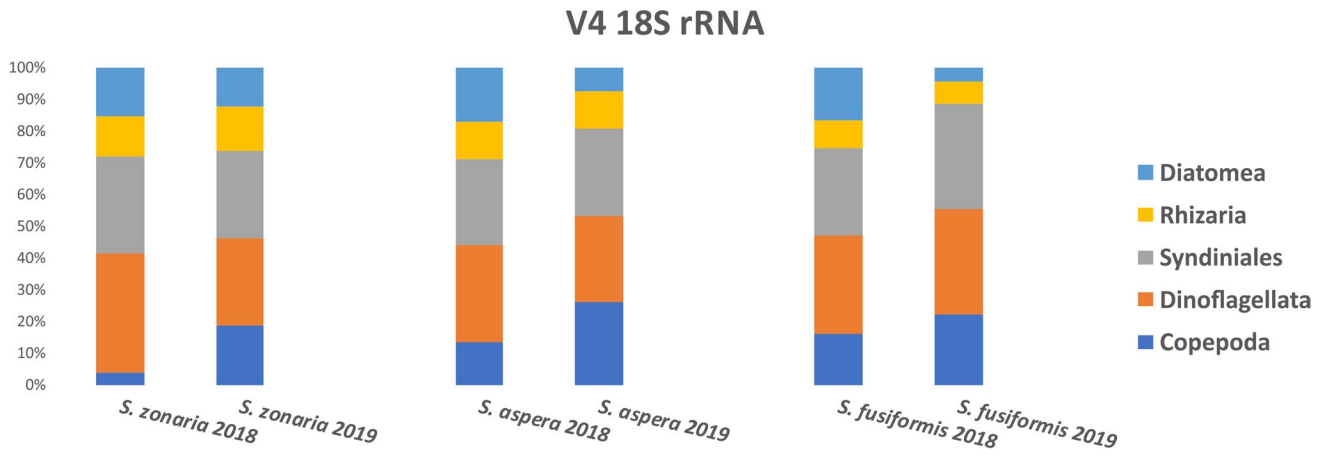

Fig. 2 Stacked bar graph showing average percentages of V4 sequence numbers (Log10 + 1) for five primary prey groups for the three salp species for samples collected during 2018 and 2019

V9 sequence numbers were analyzed for the same five prey groups as for V4: Dinoflagellata, Syndiniales, Diatomea, Rhizaria, and Copepoda. For all three salp species, Dinoflagellata and Syndiniales were the most abundant prey groups in both 2018 and 2019; diatoms were also detected in all three salp species for both years, but with relatively fewer sequences than for V4. Copepod prey were found abundantly in all three species in 2019 (Table 6, Fig. 5). Two additional prey groups were detected in abundance by V9 in 2019, including Gastropoda and Siphonophora in all three salp species; Siphonophora were also found in abundance in *S. fusiformis* in 2018 (Table 6).

Based on 3-way ANOVA of V9 sequence numbers (Log 10 + 1), prey composition did not differ between the salp species ($F = 1.87$, $p < 0.157$), but proportions of prey were different ($F = 26.37$, $p < 0.001$) and patterns differed between years ($F = 26.46$, $p < 0.001$). The Bray–Curtis diagram based on V9 showed differences between prey groups of *S. zonaria* and *S. fusiformis* in 2018 and 2019, while *S. zonaria* and *S. aspera* showed intermixing and lack of separation (Fig. 6). Clear discrimination of diet composition of *S. fusiformis* and *S. zonaria* was observed based on NMDS analysis of prey groups detected by V9 sequence numbers for 2018, with no evidence of separation in 2019 (Fig. 7).

Compound Specific Stable Isotope Analysis

Salp samples used for CSIA were collected during the 2019 cruise and were the same specimens used for metabarcoding of gut contents for all three salp species, *S. zonaria*, *S. aspera*, and *S. fusiformis* (Table 2). Principal Component Analysis (PCA) showed no clear differentiation among the three salps and very little separation among samples of each species collected at different stations (Fig. 8). Based on PCA, PC1 explained 64% of the variance and PC2 explained 22% of the variance. Samples for all three salp species grouped close to or within the diatom 90% confidence interval. Some salp samples were outside the dinoflagellate ellipse, but there was no overlap between any salp sample and the end-member clusters for dinoflagellates or bacteria.

DISCUSSION

NW Atlantic Slope Water

The NW Atlantic Slope Water is a well-defined band between coastal waters and Gulf Stream, comprising waters from the surface layers of the Gulf Stream, surface coastal waters, Labrador waters and deep Atlantic waters which have upwelled (Iselin 1936; McLellan et al. 1953). This study is based on collections of samples and data at two stations in the NW Atlantic (Fig. 1). Based on hydrographic data collected, one of the locations (Station #2) was in characteristic Slope Water in both 2018 and 2019; a second location (Station #3) was sampled only in 2019 and showed higher and more variable temperatures and salinities, indicating it was likely affected by impingement of an offshore feature such as a Gulf Steam meander or warm core ring (Wiebe et al. 1985a).

The design of the field program, including use of samples collected using different net systems, MWT

Table 3 Average percentage of V4 sequence numbers (Log10 + 1) for prey groups for the three salp species for samples collected during A) HB-2018 and B) HB-2019. Five prey groups selected for analysis are shown in yellow highlight

A)

2018	Salp Species		
Prey groups	*Soestia zonaria*	*Salpa aspera*	*Salpa fusiformis*
Eucarida	0.00	3.73	0.62
Amphipoda	0.00	0.00	0.00
Copepoda	2.79	8.51	9.48
Salpida	29.11	28.19	25.73
Teleostei	2.55	0.74	5.09
Gastropoda	0.70	0.00	0.62
Siphonophorae	0.00	2.73	9.74
Ciliophora	0.00	0.00	0.00
Dinoflagellata	25.13	19.68	18.00
Syndiniales	20.75	17.47	15.98
Rhizaria	8.52	7.74	5.13
Diatomea	10.45	11.21	9.61

B)

2019	Salp Species		
Prey groups	*Soestia zonaria*	*Salpa aspera*	*Salpa fusiformis*
Eucarida	1.59	1.24	1.47
Amphipoda	0.00	0.00	0.00
Copepoda	11.22	14.37	11.59
Salpida	21.84	22.03	30.06
Teleostei	2.15	1.86	0.59
Gastropoda	4.57	8.69	4.13
Siphonophorae	8.71	8.70	11.23
Ciliophora	0.00	0.00	0.00
Dinoflagellata	16.89	15.64	17.47
Syndiniales	16.58	15.68	17.37
Rhizaria	8.69	7.11	3.62
Diatomea	7.76	4.68	2.46

and MOCNESS, did not allow evaluation of impacts of depth or time of collection on the diet diversity of the salp species. No consistent differences in prey composition were observed between salps collected by MWT versus MOCNESS or between those collected at different depths during vertically stratified tows of the MOCNESS. The conclusions from this study are intended to allow identification of primary sources of productivity for the salp species based on comparative analysis of gut contents of salps collected in the same net samples, with full recognition of the importance of additional variables, including differences between salp species in vertical distribution, DVM behavior, swimming speed, and possible prey selectivity.

DNA Metabarcoding of Salp Diets

The two gene regions used, V4 and V9 hypervariable regions of 18S rRNA, allow similar analytical approaches for metabarcoding of biodiversity. The highly conserved V9 region allows detection of prey groups across a broad spectrum of eukaryotes (Amaral-Zettler et al. 2009). The

Table 4 Average proportion of V4 18S rRNA sequence numbers (Log10 + 1 values) for dinoflagellate and diatom species detected in gut contents of salps collected in 2018 and 2019. Sequence numbers (Log10 + 1) values > 10 are highlighted in yellow

	2018			2019		
	S. zonaria	***S. aspera***	***S. fusiformis***	***S. zonaria***	***S. aspera***	***S. fusiformis***
	N=4	N=3	N=3	N=17	N=5	N=7
Dinoflagellate species						
Alexandrium affine	0.6	0.0	5.2	1.6	3.5	2.0
Alexandrium tamiyavanichi	9.8	1.8	9.5	2.7	4.5	1.2
Amphidoma languida	1.3	3.9	1.4	4.1	1.0	0.4
Goniodoma polyedricum	5.5	0.0	4.3	7.5	6.0	4.8
Gonyaulax polygramma	34.2	32.6	27.8	13.7	13.3	8.3
Gonyaulax spinifera	0.0	0.0	0.0	3.2	1.0	1.0
Gymnodinium microreticulatum	0.0	0.0	0.0	2.8	1.1	1.1
Gymnoxanthella radiolariae	4.9	1.8	4.1	9.8	13.8	15.2
Heterocapsa niei	0.0	0.0	4.5	6.9	6.6	9.8
Pelagodinium beii	12.7	12.3	16.7	7.0	15.3	14.5
Protoperidinium bipes	12.8	17.0	5.6	8.8	3.3	0.9
Protoperidinium divergens	3.3	0.0	0.0	3.8	2.3	2.3
Protoperidinium elegans	0.0	7.3	0.0	0.2	1.8	1.8
Protoperidinium pellucidum	0.6	4.7	1.6	1.8	2.0	2.0
Scrippsiella trochoidea	12.7	18.7	19.4	20.8	18.6	23.0
Thoracosphaera heimii	1.5	0.0	0.0	5.2	5.9	11.8
Diatom species						
Chaetoceros sp.	0.0	2.5	2.5	8.1	18.0	37.5
Cyclotella sp.	0.0	2.5	3.4	4.2	3.5	0.0
Fragilariopsis sp.	0.0	0.0	0.0	2.2	0.0	0.0
Meuniera membranacea	0.0	12.1	2.5	6.3	0.0	50.0
Minidiscus trioculatus	11.4	41.4	42.0	24.1	25.4	12.5
Minutocellus polymorphus	14.8	13.8	7.5	28.1	3.7	0.0
Psammodictyon constrictum	0.0	2.2	6.9	2.4	0.0	0.0
Pseudonitzschia sp.	0.0	0.0	0.0	2.8	4.9	0.0
Thalassiosira sp.	73.7	25.5	35.2	21.9	44.4	0.0

Table 5 Average proportions of V4 18S rRNA sequence numbers (Log10 + 1 values) for copepod species detected in gut contents of salp species for 2018 and 2019. Sequence numbers (Log10 + 1) values > 10 are highlighted in yellow

	2018			**2019**		
	S. zonaria	***S. aspera***	***S. fusiformis***	***S. zonaria***	***S. aspera***	***S. fusiformis***
	n=2	n=3	n=3	n=15	n=5	n=6
Copepoda						
Calanus finmarchicus	0.0	2.6	0.0	34.7	30.4	19.2
Candacia sp.	0.0	0.0	0.0	0.0	3.1	20.1
Centropages typicus	0.0	0.0	0.0	6.9	1.1	0.0
Gaetanus variabilis	0.0	2.6	0.0	3.3	3.2	11.0
Heterorhabdus tanneri	0.0	0.0	0.0	0.0	2.2	0.0
Mecynocera clausi	50.0	6.6	16.7	0.5	0.0	0.0
Metridia curticauda	0.0	0.0	0.0	0.0	2.6	0.0
Metridia pacifica	0.0	12.0	16.7	10.4	3.2	0.0
Neocalanus cristatus	0.0	27.6	11.1	14.6	1.9	1.3
Paracalanus parvus	50.0	11.9	16.7	15.3	22.5	10.4
Pleuromamma abdominalis	0.0	14.1	38.9	2.9	0.0	0.0
Rhincalanus nasutus	0.0	22.4	0.0	0.3	9.6	0.0
Subeucalanus pileatus	0.0	0.0	0.0	5.2	2.5	0.0
Temora discaudata	0.0	0.0	0.0	6.0	4.5	16.7
Undinula vulgaris	0.0	0.0	0.0	0.0	13.1	21.3

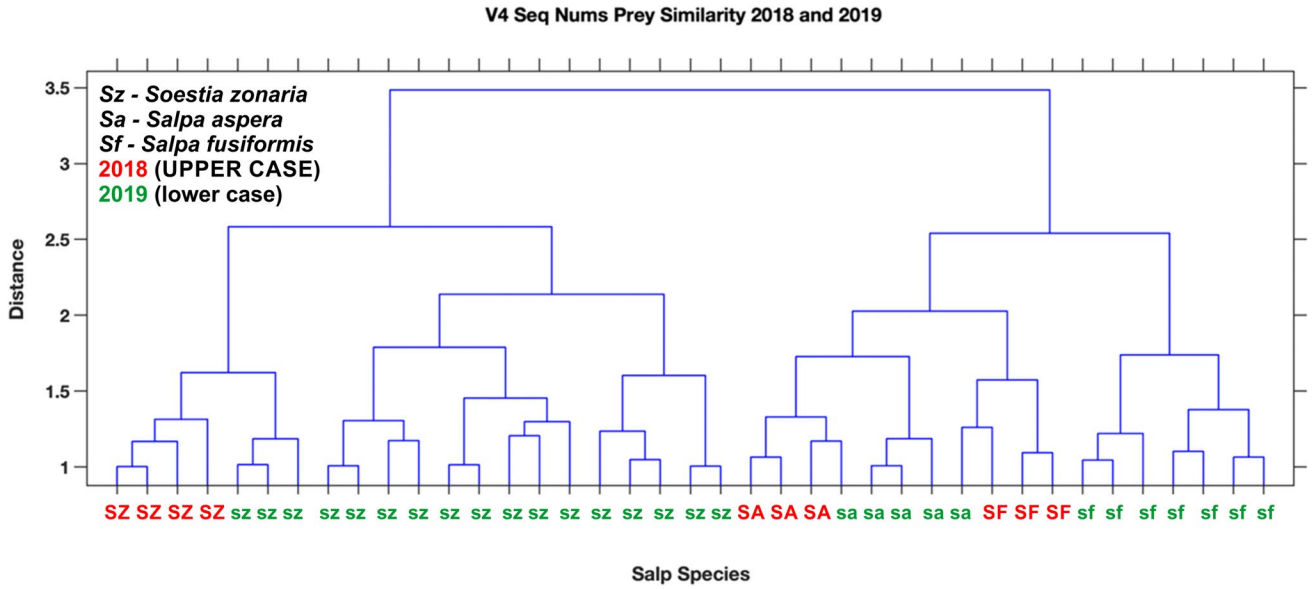

Fig. 3 Bray Curtis similarity cluster plots of V4 sequence numbers (Log10 + 1) for prey groups of three salp species *Soestia zonaria*, *Salpa aspera,* and *Salpa fusiformis* from 2018 (red font, upper case) and 2019 (green font, lower case)

more variable V4 region allows identification of species within some – but not all – taxonomic groups. In this study, V4 and V9 detected the same set of five prey groups with highest sequence numbers: Dinoflagellata and the sub-group Syndinales, Diatomea, Rhizaria, and Copepoda. The higher taxonomic resolution possible with V4 allowed accurate identification of dinoflagellate species. Comparison of V4 and V9 sequence numbers provides useful insights into prey diversity and can be used for semi-quantitative analysis of relative prey importance (Figs. 2, 5). These findings should be interpreted cautiously, due to exceptionally high and variable copy numbers of 18S rRNA genes for these groups (deVargas et al. 2015).

Patterns of variation in prey groups among salp samples, species, and years were similar based on the two gene regions. Proportional sequence numbers for dinoflagellates and diatoms showed similar patterns of variation between salp species and years for both gene regions (Tables 3, 6). Notably, metabarcoding revealed differences in prey group proportions in gut contents of *S. zonaria* and *S. fusiformis* collected at Station #2 in both 2018 and 2019, although it is not clear whether the differences are due to prey availability or prey choice and how these may be affected by morphological characteristics, including test thickness and muscle band arrangement. Comparison of phytoplankton counts from water samples with relative prey abundances in salp gut contents is a goal for future analysis with larger sample sizes and ancillary data sets.

The finding of Copepoda in salp gut contents raises several questions, including whether salps consume copepods as prey. Alternative possibilities might include the consumption of freely-spawned eggs, suspended cells, tissue fragments from molting, and marine snow. Numerous factors may underly the marked variation in prevalence of copepod prey between years, including the abundance of species that freely spawn eggs (e.g., *Calanus finmarchicus, Paracalanus parvus*; Table 5) and variable egg production rates (Kang and Kim 2023).

Compound Specific Stable Isotope Analysis of Salps

Analysis of salp tunic tissue by CSIA provides a useful view of the species' trophic relationships within the mesopelagic food web averaged over timescales of weeks to months, in contrast to the temporal snapshot from metabarcoding of gut contents. The lack of separation between salp species indicates that salp species were occupying similar niches to one another over the few months before the sampling period. Based on the comparison with end members (Fig. 8), the diets of all three salps consisted primarily of diatoms or species that were feeding primarily on diatoms. In contrast to metabarcoding results, CSIA showed little to no separation between samples collected from different stations in 2019.

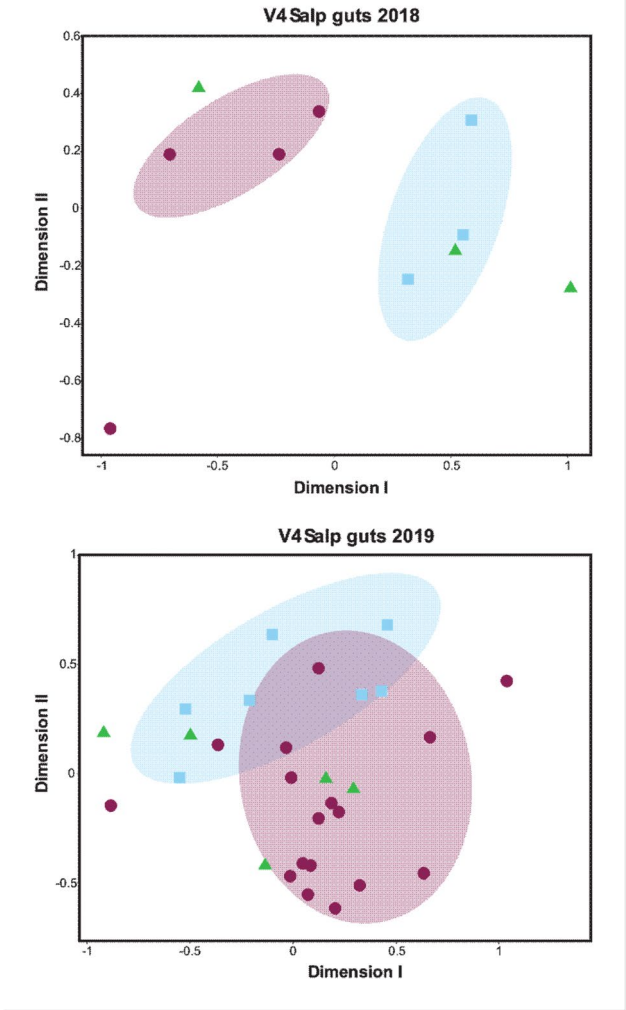

Fig. 4 Non-Metric Multi-Dimensional Scaling (NMDS) for V4 sequence numbers (Log10 + 1) for five prey groups for salp species *Soestia zonaria* (blue squares), *Salpa aspera* (green triangles) and *Salpa fusiformis* (red circles) collected in 2018 and 2019

The most likely reason for the differences between CSIA and metabarcoding results is the timescale difference between the methods. The predominance of diatoms in CSIA analyses may be an indicator of local phytoplankton community structure, reflecting the presence of a diatom bloom in the months preceding sample collection in the NW Atlantic Ocean. Seasonal patterns of phytoplankton diversity and biomass are well-studied in the region (e.g., Bolanos et al. 2020), and baseline information supports the likelihood of diatoms as a primary food resource, although no field samples are available for the months prior to sample collection for this study. Despite reports that salps cannot digest diatoms efficiently due to the outer test, the CSIA findings confirm that salps can process and incorporate diatom material into their tissues. The predominance of diatoms as end-member sources may result from assimilation from suspended organic matter, marine snow, and fecal pellets.

Previous studies have determined marine prokaryotic organisms (e.g., bacteria, *Prochlorococcus*) to be sources of food for salps and other pelagic tunicates (Sutherland and Thompson 2022). These organisms are more quickly digestible due to higher surface-area-to-volume ratios; despite being smaller than the salp mesh size (1.4 µm), and can satisfy salp energetic needs (Sutherland et al. 2010). This study did not evaluate the possible role of prokaryotes in salp diets or the mesopelagic food web, since metabarcoding protocols were not designed to detect prokaryotic prey groups and CSIA results were not compared with microbial end-members.

In the case of salps, no significant differences have been observed between stable isotope values of the whole body compared to only the tunic (stomach removed) or only the stomach (Pakhomov et al. 2019). Salps naturally do not store high amounts of lipids (< 1%; Hagen 1988), and this is reflected in the stable isotope values of defatted and non-defatted salps. Hence, they do not generally require any prior treatment (e.g., lipid removal), or usage of particular body parts/organs for the stable isotope analyses.

Future Challenges and Opportunities

Mesopelagic zones will continue to present challenges for researchers and managers seeking to understand the key patterns and processes, including biodiversity from microbes to mammals and the complex array of biotic interactions, of these under-studied communities (St John et al. 2016; Sutton et al. 2017; Kaartvedt et al. 2019). Time-series observations of taxonomic composition and relative abundances of organisms at all trophic levels, from primary producers to top predators, are necessary to understand dynamics of deep-sea ecosystems. Increased attention is needed for gelatinous forms, including salps, which have significant impacts on pelagic food webs and transport of organic material (Hereu et al. 2010; Sutton 2013; Henschke et al. 2016; Stukel et al. 2021; Luo et al. 2022; Decima et al. 2023; Orlov and Pakhomov 2024). More complete understanding of pelagic community structure, trophic dynamics, and predator–prey relationships will require observations over various time scales, from snapshots of who-eats-whom to integrative analyses of sources of productivity (Ohkouchi et al. 2017). Important questions remain regarding whether salp species exhibit prey choice and selectivity over

Table 6 Average percentage of V9 sequence numbers (Log10 + 1) for prey groups for the three salp species for samples collected during A) HB-2018 and B) HB-2019. Five prey groups selected for analysis are shown in yellow highlights

A)

2018	Salp Species		
Prey groups	*Soestia zonaria*	*Salpa aspera*	*Salpa fusiformis*
Eucarida	1.61	0.75	3.47
Amphipoda	3.71	0.48	3.00
Copepoda	2.36	8.81	9.49
Salpida	26.98	31.09	25.26
Teleostei	4.08	2.41	7.09
Gastropoda	1.80	0.78	2.15
Siphonophorae	0.37	4.01	7.79
Ciliophora	5.11	0.68	1.47
Dinoflagellata	22.48	17.52	15.61
Syndiniales	16.81	15.32	12.18
Rhizaria	6.61	8.53	4.71
Diatomea	8.07	9.61	7.78

B)

2019	Salp Species		
Prey groups	*Soestia zonaria*	*Salpa aspera*	*Salpa fusiformis*
Eucarida	2.81	2.67	2.71
Amphipoda	1.36	1.99	1.57
Copepoda	10.67	12.37	11.93
Salpida	20.30	19.29	25.17
Teleostei	4.00	2.91	2.58
Gastropoda	6.96	9.27	5.62
Siphonophorae	7.37	7.57	9.16
Ciliophora	3.79	3.56	0.89
Dinoflagellata	14.38	13.32	14.55
Syndiniales	13.12	12.17	13.14
Rhizaria	7.71	8.02	5.42
Diatomea	7.52	6.89	7.26

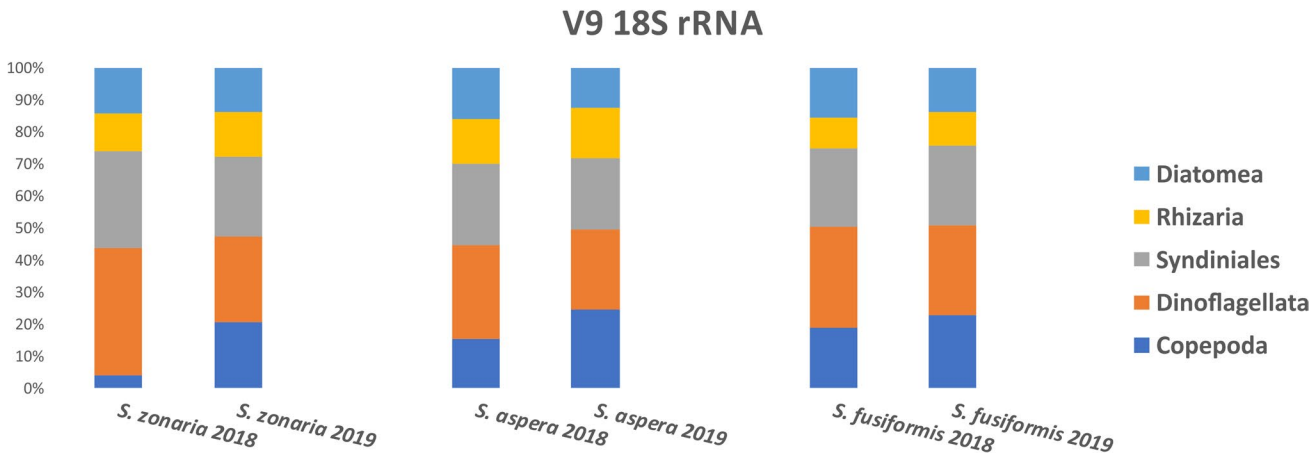

Fig. 5 Stacked bar graph showing average percentages of V9 sequence numbers (Log10 + 1) for five primary prey groups for three salp species collected in 2018 and 2019

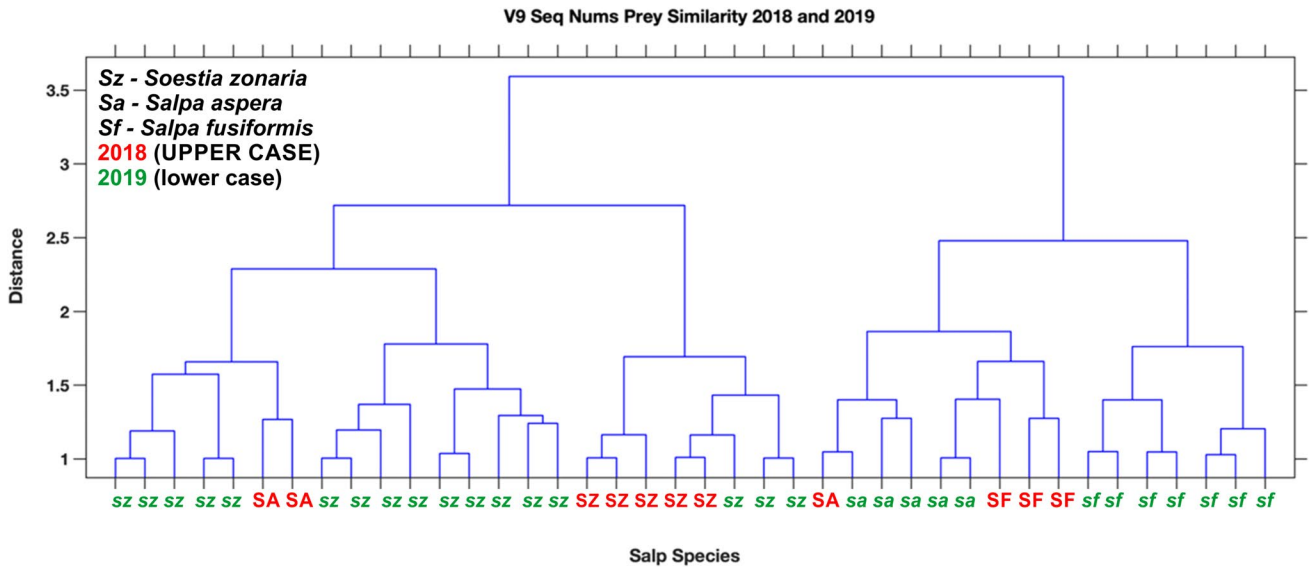

Fig. 6 Bray Curtis cluster plot based on V9 sequence numbers (Log10 + 1) for five prey groups of three salp species *Soestia zonaria* (Sz), *Salpa aspera* (Sa) and *Salpa fusiformis* (Sf). for 2018 (red font; upper case) and 2019 (green font; lower case)

both the short term detected by metabarcoding and the longer term inferred from CSIA. The samples analyzed for this study did not allow evaluation of the impacts of sampling time (day versus night) and depth of collection on salp diet diversity, since in many cases the three salp species analyzed were collected in different net tows (Suppl. Table 1). Future oceanographic cruises should provide opportunities to design more intensive sampling, including replicate tows taken during both day and night, with the goal of capturing larger sample sizes of the salp species of interest, with associated hydrographic data and analysis of pelagic diversity, microbes to mammals.

Assessment and monitoring of mesopelagic ecosystems and food webs should take full advantage of emerging technologies, including molecular, biogeochemical, optical, and acoustic methodologies (Alberdi et al. 2017; Thorrold et al. 2021; Ohkouchi 2023; Grassian et al. 2024). These investigations will provide essential information to design successful responses to numerous challenges (Levin et al. 2020; Kourantidou and Jin 2022; Morzaria-Luna et al. 2022), understand impacts of environmental variation and climate change (Robison 2009; Gamfeldt et al. 2015; Hallegraeff et al. 2021), and ensure the protection and

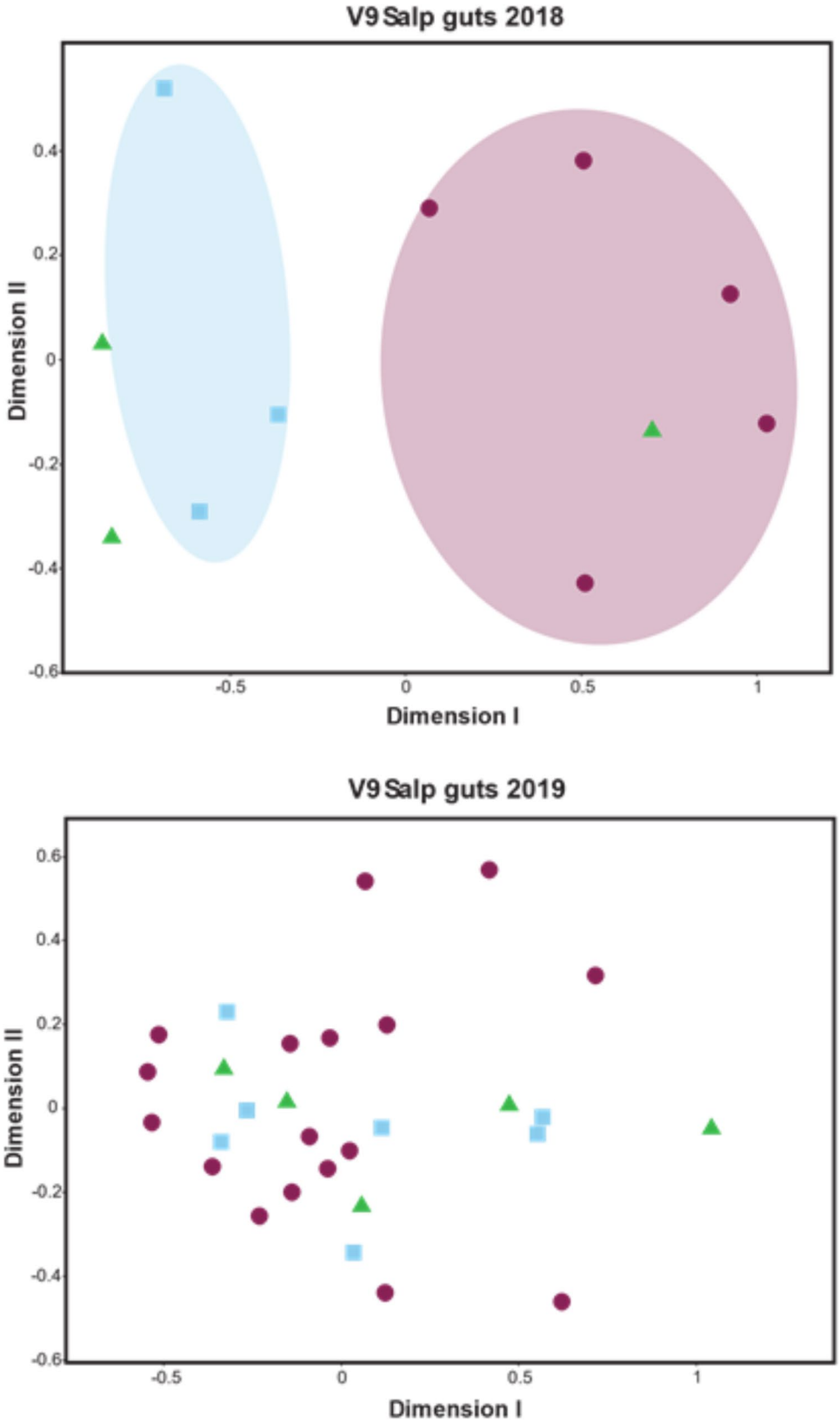

Fig. 7 Non-Metric Multi-Dimensional Scaling (NMDS) for V9 sequence numbers (Log10 + 1) for five prey group for salp species *Soestia zonaria* (blue squares), *Salpa aspera* (green triangles) and *Salpa fusiformis* (red circles) collected in 2018 and 2019

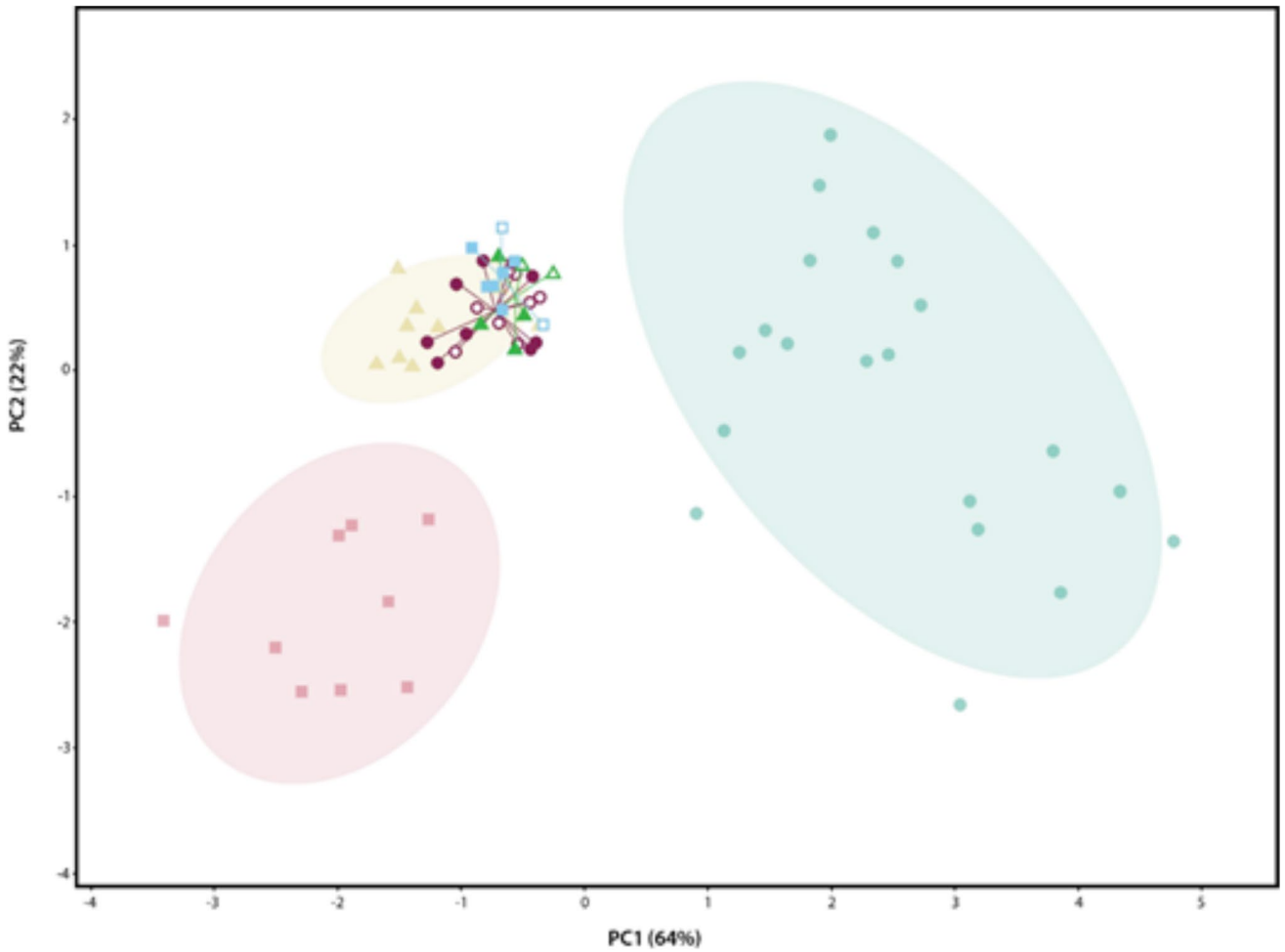

Fig. 8 Principal Component Analysis (PCA) plots of normalized δ (^{13}C) values from Compound-Specific Stable Isotope Analysis (CSIA) of salp species. Salp species are shown in star plots, where the center represents the average of the PCA data for each species: *Soestia zonaria* (red circles), *Salpa fusiformis* (blue squares), and *Salpa aspera* (green triangles). Unfilled symbols represent samples collected at HB1907 Station 2; filled circles represent samples collected at HB1907 Station 3. Normalized δ (^{13}C) values of end members with 90% confidence intervals (data from Stahl et al. 2023) are shown in colored symbols bacteria (teal circles), diatoms (yellow triangles), and dinoflagellates (pink squares)

preservation of mesopelagic communities and ecosystem services of the deep-sea.

Conclusions

DNA metabarcoding of gut contents and compound specific stable isotope analysis (CSIA) was carried out for three salp species, *Soestia zonaria*, *Salpa aspera*, and *S. fusiformis*, collected from the NW Atlantic Slope Water during July and August 2018 and 2019. Metabarcoding results for V4 and V9 18S rRNA were analyzed for five primary prey groups: Dinoflagellata, Syndiniales, Diatomea, Rhizaria, and Copepoda. The two gene markers, which differ in levels of variation and taxonomic resolution, both revealed significant differences between salp species in proportions of prey groups; differences between years were observed for V9, but not V4. CSIA provides a longer-term view of food web interactions, typically weeks to several months. Based on analysis of the same samples, CSIA indicated diatoms as the primary source of carbon for all three salp species, which did not separate by species or station in the end member space. The complementary analyses were designed to provide new insights into the role of salps in the mesopelagic food web. Metabarcoding provides a snapshot of the prey groups present in salp guts at time of collection, including detection of prey across a variety of pelagic planktonic taxa, for which microscopic identification is nearly impossible. CSIA results average the consumed plankton over longer periods of time. The differing results most likely represent a shift in local community structure of

the plankton community of the NW Atlantic Slope Water. Future studies are needed to characterize diversity and structure of mesopelagic communities, including collection of hydrographic data and samples in time-series programs covering deep-sea ecosystems of the global ocean.

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Data Availability DNA metabarcoding sequence data files for V4 and V9 18S rRNA were deposited in FASTQ format in the Short Read Archive of NCBI GenBank (https://www.ncbi.nlm.nih.gov/sra/) with BioProject #PRJNA941071 and Accession Numbers SRR23747471—SRR23747509 for V4 sequences and SRR23770897—SRR23770936 for V9 sequences. The data will be available upon acceptance of the manuscript for publication. Compound-Specific Stable Isotope (CSIA) data and analyses will be available via GitHub folders, with open access upon acceptance of the manuscript for publication.

Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

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