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SPECIAL ISSUE: THE HOST-ASSOCIATED MICROBIOME: PATTERN, PROCESS AND FUNCTION

WILEY MOLECULAR ECOLOGY

Nematode-associated microbial taxa do not correlate with host phylogeny, geographic region or feeding morphology in marine sediment habitats

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Abstract

Studies of host-associated microbes are critical for advancing our understanding of ecology and evolution across diverse taxa and ecosystems. Nematode worms are ubiquitous across most habitats on earth, yet little is known about host-associated microbial assemblages within the phylum. Free-living nematodes are globally abundant and diverse in marine sediments, with species exhibiting distinct buccal cavity (mouth) morphologies that are thought to play an important role in feeding ecology and life history strategies. Here, we investigated patterns in marine nematode microbiomes, by characterizing host-associated microbial taxa in 281 worms isolated from a range of habitat types (deep-sea, shallow water, methane seeps, Lophelia coral mounds, kelp holdfasts) across three distinct geographic regions (Arctic, Southern California and Gulf of Mexico). Microbiome profiles were generated from single worms spanning 33 distinct morphological genera, using a two-gene metabarcoding approach to amplify the V4 region of the 16S ribosomal RNA (rRNA) gene targeting bacteria/archaea and the V1–V2 region of the 18S rRNA gene targeting microbial eukaryotes. Contrary to our expectations, nematode microbiome profiles demonstrated no distinct patterns either globally (across depths and ocean basins) or locally (within site); prokaryotic and eukaryotic microbial assemblages did not correlate with nematode feeding morphology, host phylogeny or morphological identity, ocean region or marine habitat type. However, fine-scale analysis of nematode microbiomes revealed a variety of novel ecological interactions, including putative parasites and symbionts, and potential associations with bacterial/archaeal taxa involved in nitrogen and methane cycling. Our results suggest that in marine habitats, free-living nematodes may utilize diverse and generalist foraging strategies that are not correlated with host genotype or feeding morphology. Furthermore, some abiotic factors such as geographic region and habitat type do not appear to play an obvious role in structuring host–microbe associations or feeding preferences.

KEYWORDS
16S rRNA, 18S rRNA, feeding ecology, host-associated microbiome, marine nematodes, metabarcoding

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1 | INTRODUCTION

Studies of host-associated microbiomes have become increasingly ubiquitous in recent years, driven by continued advances in high-throughput sequencing technologies (HTS). The decreasing costs and enormous data outputs associated with HTS approaches (e.g., Illumina HiSeq and MiSeq platforms) have vastly expanded our understanding of microbial taxa associated with various hosts and ecosystems, including the human microbiome [e.g., gut and skin microbiome (Parfrey, Walters, & Knight, 2011; Schommer & Gallo, 2013; Turnbaugh et al., 2007; Walter & Ley, 2011)], vertebrate and invertebrate species such as amphibians (Bataille, Lee-Cruz, Tripathi, Kim, & Waldman, 2016; McKenzie, Bowers, Fierer, Knight, & Lauber, 2012; Walke et al., 2017), marine sponges (Fan, Liu, Simister, Webster, & Thomas, 2013; He, Liu, Karuppiah, Ren, & Li, 2014; Hentschel, Piel, Degnan, & Taylor, 2012), coral reefs (Bourne, Morrow, & Webster, 2016; Thompson, Rivera, Closek, & Medina, 2014; Ainsworth et al., 2015), oysters (Chauhan, Wafula, Lewis, & Pathak, 2014; Lokmer & Mathias Wegner, 2015) and household pets (Dewhirst et al., 2012; Dorn et al., 2017; Swanson et al., 2011; Weese, 2013).

Although the term "microbiome" is often used in reference to any type of sequencing-based assessment of microbial biodiversity [e.g., the Earth Microbiome Project (Gilbert, Jansson, & Knight, 2014) or the troposphere microbiome (DeLeon-Rodriguez et al., 2013)], the term is most commonly used to define any species found in association with a specific host organism, including known bacterial symbionts [(endo- or ectosymbionts such as *Wolbachia* or sulphur-utilizing bacteria (Ainsworth et al., 2015; Nicks & Rahn-Lee, 2017; Sayavedra et al., 2015; Werren, 1997)] specialized microbial populations that are adapted to life in host-associated habitats such as mucus, gut linings or skin (Glasl, Herndl, & Frade, 2016; Larsen, Bullard, Womble, & Arias, 2015; Schommer & Gallo, 2013; Walke et al., 2017; Walter & Ley, 2011; Weese, 2013), and partially digested gut contents and prey items that can be identified via DNA sequencing (De Barba et al., 2014; Deagle, Kirkwood, & Jarman, 2009; Yu et al., 2012; Zeale, Butlin, Barker, Lees, & Jones, 2011). Common microbiome taxa include both parasitic and commensal species (Dheilly et al., 2017; Nunes-Alves, 2015; Parfrey et al., 2011; Schommer & Gallo, 2013), with host-associated species spanning both prokaryotic and eukaryotic domains of life.

For microbiome studies carried out to date, the largest research efforts have focused on microbial associations known to be critical for organismal health or defence [e.g., the human microbiome (Schommer & Gallo, 2013; Walter & Ley, 2011)], as well as in-depth studies of well-characterized symbionts that have co-evolved with hosts [e.g., sponge and coral reef microbiomes (Bourne et al., 2016; Hentschel et al., 2012)]. Similarly, the sulphur-utilizing symbionts associated with chemosynthetic organisms at hydrothermal vents and methane seeps have been extensively characterized using molecular approaches (Dubilier, Bergin, & Lott, 2008; Nakagawa & Takai, 2008), with complete genome sequences available for many symbiont species (Newton et al., 2007; Sayavedra et al., 2015). Far less is known about the microbiome taxa associated with free-living invertebrate hosts, especially those inhabiting remote or comparatively featureless ecosystems such as tropical soils or deep-sea sediments. Emerging evidence from invertebrate taxa has underlined the evolutionary and ecological significance of microbiome assemblages. In the turtle ant species *Cephalotes varians*, bacterial operational taxonomic units (OTUs) recovered from the gut microbiome were shown to cluster within ant-specific lineages, suggesting deep co-evolutionary history between ant hosts and their resident microbiome (Kautz, Rubin, Russell, & Moreau, 2013). According to Poulsen and Sapountzis (2012), this trend of host–microbiome specificity is found across many representatives of the Formicidae, and ant microbiome patterns further appear to be tightly correlated with host tropic level (i.e., herbivores, scavengers and predators). It remains to be seen whether such evolutionary and ecological patterns are consistent for microbiome taxa associated with diverse invertebrate phyla, especially in marine environments where biotic and abiotic constraints are markedly distinct.

There have been few formal studies of host-associated microbes in metazoan meiofaunal species [i.e., organisms retained on a 38- to 42-μm sieve but passing 1-mm mesh, such as nematodes, tardigrades, kinorhynchs, copepods and a variety of other "minor" phyla that are ubiquitous but poorly described (Bik et al., 2012)]. To date, HTS studies of marine meiofauna (i.e., metabarcoding) have primarily focused on biodiversity patterns across different regions and marine habitats (reviewed in Carugati, Corinaldesi, Dell'Anno, & Danovaro, 2015). Microbial symbionts are known from a few nematode species, although the majority of reported associations represent isolated or anecdotal evidence (Bhadury et al., 2011; Moens et al., 2013). The most well-known examples of nematode symbionts include the genus *Astomonema* and the subfamily Stilbonematinae. *Astomonema* nematodes lack a buccal cavity (mouth) and have an absent or non-functional gut; sulphur-oxidizing bacterial endosymbionts fill almost the entirety of the gut lumen and provide the nematode with nutrition (Musat et al., 2007). Representatives of the subfamily Stilbonematinae (e.g., *Robbea*) are generally associated with specific ectosymbionts (i.e., nematode species have different bacterial phylotypes) which are attached to the outer cuticle and utilized as the primary food source (Bayer et al., 2009). Although *Astomonema* and Stilbonematinae nematodes are not close phylogenetic relatives, their bacterial symbionts display high sequence similarity (Musat et al., 2007).

More extensive investigations of nematode microbiomes have focused on terrestrial habitats or nematode species maintained in laboratory cultures. Baquiran et al. (2013) characterized the microbiome associated with the soil nematode *Acrobeloides maximus* and found that the core microbiome was composed of three main genera, viz. *Ochrobactrum*, *Pedobacter* and *Chitinophaga*, and displayed less overall diversity than that of the nematode model *Caenorhabditis elegans*. Recently, Dirksen et al. (2016) used HTS to characterize the native microbiome of *C. elegans* isolated from soils across Europe, demonstrating that the native microbiome is highly diverse and distinct from other nematode species in the same genus. Furthermore,

the *C. elegans* native microbiome was capable of enhancing nematode fitness under stressful conditions; *Ochrobactrum* taxa were shown to enter and persist in the *C. elegans* gut under reduced food conditions (suggesting a long-term association), and *Pseudomonas* isolates from the nematode microbiome appear to produce antifungal compounds and thus protect worms from pathogens (Dirksen et al., 2016). Within the well-described marine species complex *Litoditis marina*, Derycke et al. (2016) observed a low-diversity core microbiome and detected resource partitioning of food sources for cryptic species that often co-occurred in space and time. Taken together, these studies confirm the presence of distinct host-associated microbial assemblages in metazoan species such as nematodes and suggest species-specific microbiome patterns that may be influenced by both evolutionary and ecological factors.

In this study, we aimed to broadly expand our knowledge of nematode microbiomes by comparing microbial assemblages across host taxonomy/phylogeny, feeding ecology, ocean depth and geographic region. We chose to focus on free-living marine nematode taxa isolated from benthic sediments (intertidal and deep-sea sites), as these taxa can be categorized by mouthparts (e.g., feeding ecology) and many morphological genera have "cosmopolitan" distributions and can thus be easily found at most sampling locations. To investigate nematode microbiomes, single worms were isolated from marine sediments collected from Arctic habitats north of Alaska (Beaufort Sea), Southern California (intertidal and deep-sea sites) and the Gulf of Mexico (methane seep sites, *Lophelia* coral mounds and nonseep background sites). Nematodes were picked and morphologically identified to genus level using strict laboratory conditions designed to minimize microbial contamination, followed by enzymatic digestion of whole nematodes to extract total DNA. Voucher images were used to classify each specimen into one of four discrete feeding groups based on mouth (buccal cavity) morphology defined by Wieser (1953): selective and nonselective deposit feeders with no teeth or buccal cavity armature (groups 1A and 1B) and epistrate feeders, predators and omnivores (groups 2A and 2B) that possess large teeth, mandibles or rows of denticles. Microbiome profiles were generated from DNA extracts of each worm using a two-gene metabarcoding approach to amplify the V4 region of the 16S ribosomal RNA (rRNA) gene targeting bacteria/archaea and the V1–V2 region of the 18S rRNA gene targeting microbial eukaryotes (enabling molecular confirmation of nematode IDs as well as assessment of other eukaryotic taxa associated with each worm).

Using nematode morphology as a variable that could be empirically controlled (e.g., isolating the same morphological genus from disparate sample sites, ecologically classifying nematodes by feeding group), we aimed to test a number of overarching ecological and evolutionary hypotheses. First, we expected nematode microbiomes to show clustering patterns corresponding to Wieser's feeding groups, indicative of different food preferences and gut contents in each group; however, within a sample site we expected to see a subsignature of niche partitioning (selective feeding to avoid competition for food resources) for nematodes within the same feeding group. Second, on a global scale we anticipated that nematode microbiome profiles would show the greatest similarity within a host genus or phylogenetic clade, reflecting species-specific microbiome assemblages and co-evolution with nematode hosts (similar to patterns seen in ants and *C. elegans*; Dirksen et al., 2016; Poulsen & Sapountzis, 2012). Finally, we expected that characterization of nematode microbiomes would reveal novel insights about species interactions in marine sediment habitats, such as nematode–microbe symbioses and ingested prey species.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Sediment samples leveraged in this study were collected from three main geographic regions: the Arctic, Gulf of Mexico and Southern California (Figure 1). Samples were selected from a range of frozen material available at UC Riverside (marine sediments previously collected as part of various research studies and stored at −80°C). The goal of this project was to maximize the geographic range of sample sites and include a variety of benthic habitat types (Table 1), in order to analyse a diverse collection of nematode specimens representing a wide range of feeding morphology and ideally isolate specimens from the same nematode genus at disparate sample sites. Thus, the sample sites included in this study consisted of Arctic shelf/slope ecosystems, methane seeps and nonseep background sediments in the Gulf of Mexico, sediments proximate to cold water *Lophelia* coral mounds in the Gulf of Mexico, anoxic mud near an experimental whale fall site off the coast of Southern California, and intertidal sand and kelp holdfasts in Southern California.

Arctic sediment samples were collected in the Beaufort Sea as part of the US–Canada Transboundary Fish and Lower Trophic Communities Project during 2012 and 2014 research cruises (Bureau of Ocean Energy Management Study AK-12-04), using either a box core or Van Veen grab aboard a scientific research vessel. Meiofaunal samples were taken from the surfaces of grab and box core samples (0–1 cm sediment depth) using two 5-cm-diameter subcores. Arctic sample depths ranged from 200 to 1,000 m and included a 3-station depth transect (200-, 500- and 1,000-m sites, with three replicate samples taken at 200 m depth). Gulf of Mexico samples were collected aboard the *R/V Ronald H. Brown* in June/July 2007 as part of the USGS Deepwater Program "CHEMO III" [Studies of Gulf of Mexico Lower Continental Slope Communities Related to Chemosynthetic and Hard Substrate Habitats (Ross et al., 2012)]. Box core sediment samples were subsectioned using 10-ml serological pipettes, with 10–30 replicate subsamples collected per box core collection. Serological pipettes containing subsection sediment were immediately sealed at both ends using Parafilm (Bemis Inc., Neenah, WI). Deep-sea samples off Southern California (site WF.623.53; Table 1) were collected aboard the *R/V Western Flyer* in June 2014. Push core samples were collected using the ROV Doc Ricketts deployed at an experimental whale fall site; cores were taken at 0 m distance from the whale carcass and sectioned to retain the 0- to 10-cm depth fraction. Following core sectioning, all sediment

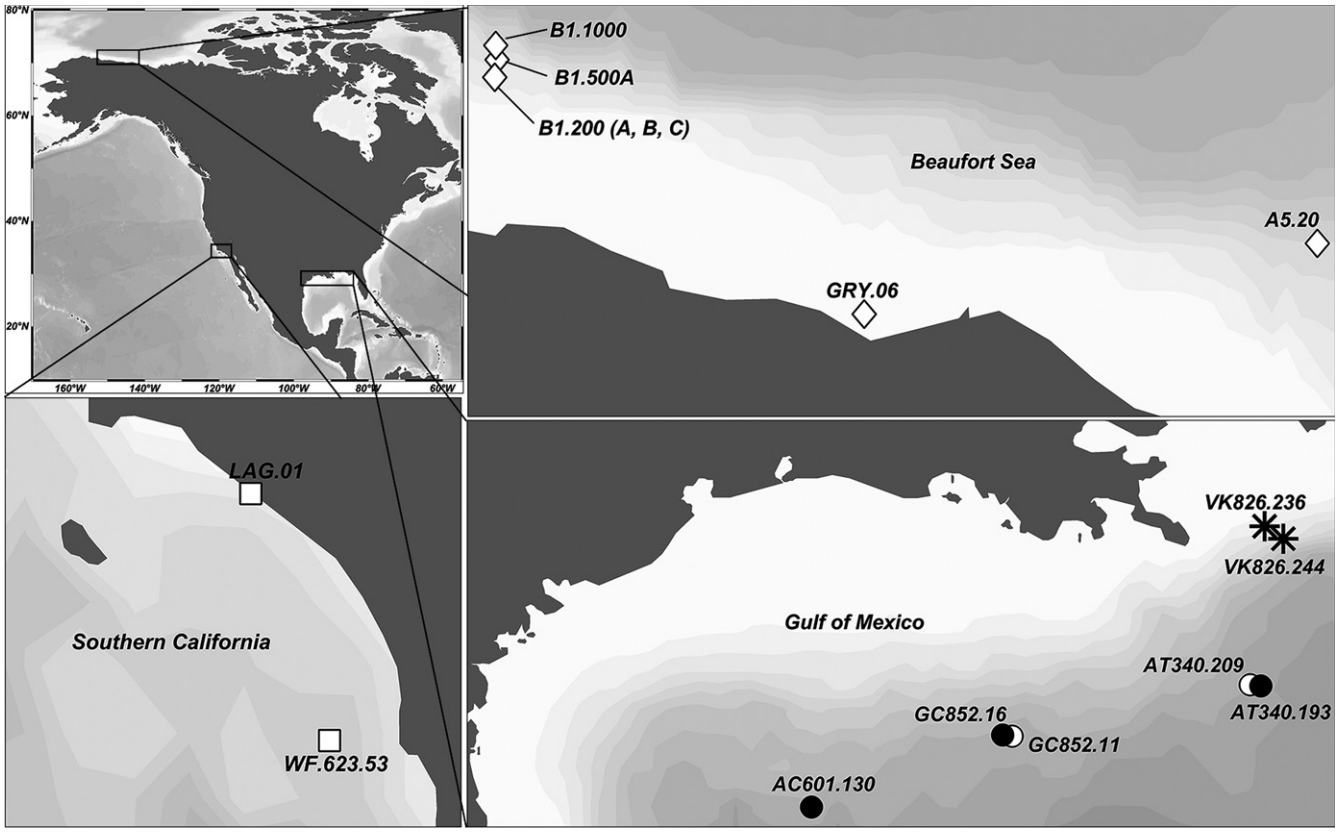

FIGURE 1 Geographic distribution of samples used in the present study. Nematodes were isolated from sediment cores collected from the Beaufort Sea off the North Slope of Alaska (white diamonds; 20–1,000 m depth), methane seeps, nonseep background sites and proximate to *Lophelia* coral mounds in the Gulf of Mexico (black circles, white circles and asterisk shapes, respectively; 495–2,391 m depth), an experimental whale fall site off the coast of Southern California (842 m depth) and an intertidal sandy beach site at Laguna Beach, California. GPS coordinates and site descriptions for all sampling locations are provided in Table 1

samples collected at sea were frozen immediately upon collection at −80°C and transported to university laboratories (University of Alaska at Fairbanks and UC Riverside). Intertidal samples (LAG.01; Table 1) were collected at Laguna Beach, California, in June 2017; washed-up *Macrocystis* kelp holdfasts were collected from the low-water mark and placed in plastic bucket filled with seawater. Collected holdfasts were kept cool and transported back to UC Riverside, and subsequently stored at 4°C for 48 hr before holdfasts were processed to extract live nematodes living in association with *Macrocystis*. GPS coordinates, sediment profiles and site descriptions for all sampling locations are provided in Table 1.

2.2 | Sediment processing and nematode isolation

Meiofaunal organisms were extracted from thawed deep-sea sediments using a decantation–flotation method in a 2-L glass cylinder with stopper (Danovaro, 2009). For Arctic and whale fall sample sites, whole sediment cores were processed to extract meiofauna, apart from a ~50 ml volume of raw sediment that was set aside as a genetic archive for each sample. For Gulf of Mexico sites, 10–20 serological pipettes (box core subsamples) were processed from each sample site and the remaining subsamples kept frozen as a genetic archive. Deep-sea samples were decanted over a stainless steel sieve with 63 μm (Arctic samples) or 45 μm (all other samples) mesh size using sterile artificial seawater (Instant Ocean® Spectrum Brands, Inc., USA) prepared with Milli-Q ultrapure water (Millipore Corporation, Billerica, Massachusetts). All material retained on stainless steel sieves was washed into 50-ml Falcon tubes (Fisher Scientific) and stored at −80°C. For the intertidal Laguna Beach site, *Macrocystis* holdfasts were submerged and washed thoroughly with tap water over a 45-μm sieve to detach nematodes (Derycke et al., 2010); live nematodes were processed immediately. All sieves, cylinders, beakers and other equipment used during sediment processing were sterilized in between samples, to minimize the potential for cross-over DNA contamination. Equipment was washed thoroughly with Milli-Q ultrapure water, soaked in a 10% Sodium Metabisulfite (Pro Supply Outlet, CA, USA) solution for 1 hr (Fonseca et al., 2011) and subsequently rerinsed in Milli-Q ultrapure water before the next sample was processed.

Next, single nematodes were isolated and morphologically identified from processed meiofauna fractions, either using fresh material (Laguna Beach) or frozen samples (all other sites) that were thawed as needed. For frozen samples, only one sample was processed (thawed) at a time to minimize sample degradation and cell lysis, as

TABLE 1 Geographic locations and descriptions of samples used in this study

Sample ID	Collecting date	Nematode Num.	Ocean/Region	Depth (m)	Latitude	Longitude	Sediment and habitat description
B1.200A	2012	34	Arctic, Beaufort Sea	200	71.230128	−150.083509	Coarse silt
B1.200B	2012	45	Arctic, Beaufort Sea	200	71.230128	−150.083509	Coarse silt
B1.200C	2012	30	Arctic, Beaufort Sea	200	71.230128	−150.083509	Coarse silt
B1.500A	2012	14	Arctic, Beaufort Sea	500	71.250180	−150.083509	Very fine silt
B1.1000	2012	26	Arctic, Beaufort Sea	1,000	71.283493	−150.083503	Fine silt
GRY.06	2013	8	Arctic, Beaufort Sea	750	70.433460	−138.983388	—
A5.20	2014	46	Arctic, Beaufort Sea	20	70.118850	−145.106900	Medium silt
VK826.236	2007	12	Gulf of Mexico, Viosca Knoll	594	29.149800	−88.019383	Mud; coral mound, *Lophelia* site
VK826.244	2007	22	Gulf of Mexico, Viosca Knoll	495	29.169150	−88.014500	Coral mound, *Lophelia* site
AT340.209	2007	5	Gulf of Mexico, Atwater Canyon	2,222	27.648193	−88.374722	Sand; aggregation of tubeworms
GC852.11	2007	2	Gulf of Mexico, Green Canyon	1,430	27.116600	−91.164000	Bacterial mats
AC601.130	2007	8	Gulf of Mexico, Alaminos Canyon	2,391	26.387180	−93.512932	Cold seep site, presence of worm tubes, sediment with oil slick on surface
AT340.193	2007	8	Gulf of Mexico, Viosca Knoll	2,239	27.645488	−88.362753	Cold seep site, heavy mussel beds
GC852.16	2007	9	Gulf of Mexico, Green Canyon	1,425	27.119967	−91.165083	Cold seep site
LAG.01	2017	10	Laguna Beach, Southern California	0	33.530940	−117.775350	Intertidal sand and kelp holdfasts
WF.623.53	2014	2	Southern California	842	32.776835	−117.488126	Anoxic mud; Whale Fall site

(—) No data.

well as prevent cross-contamination between samples. Taxonomic workflows were designed to minimize the potential for ambient contamination from air, dust, laboratory surfaces, unsterilized equipment and kit reagents. Disposable equipment and gloves were used whenever possible (e.g., glass slides, coverslips, razor blades) and nondisposable equipment (e.g., nematode picks, microscope surfaces) was sterilized with 70% ethanol at the beginning and end of each day as well as in between picked worms and in between samples. Nematodes were individually picked out of samples under a dissecting microscope (Olympus SZX16, Olympus Corporation, Tokyo, Japan), washed in molecular-grade water following the methods of Derycke et al. (2016) (where authors used electron microscopy to verify that this washing procedure was effective in removing most adherent bacteria from marine worms) and subsequently mounted in temporary slides (glass slide, coverslip, and sterile artificial seawater) for morphological identification. A total of 13 of "blank taxonomy samples" were also generated as samples were being processed in the laboratory, whereby the nematode taxonomist (T. Pereira) simulated the full workflow for worm isolation/identification using sterile molecular-grade water instead of an actual sample. While mounted on slides, nematodes were morphologically identified to the lowest possible taxonomic level (family or genus) using a compound microscope (Nikon Eclipse E600; Nikon Corporation, Kawasaki, Japan) and specialized keys for marine nematodes (Platt & Warwick, 1983, 1988; Warwick, Platt, & Somerfield, 1998) including the database NEMYS (Bezerra et al., 2017). A morphological voucher of each specimen was retained in the form of digital photos or through-focus videos prior to DNA extraction (De Ley & Bert, 2002). After identification, nematodes were individually transferred into microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) containing Worm Lysis Buffer (WLB). This entire taxonomic workflow, from initial nematode picking to final digestion and DNA extraction in WLB, took approximately 8 min per nematode specimen to complete. Digital images from the anterior region/buccal cavity were retroactively used to classify nematodes into four feeding groups according to Wieser (1953): selective deposit feeders (1A), nonselective deposit feeders (1B), epigrowth feeders (2A) or predators/omnivores (2B) (Table 2).

2.3 | DNA extraction and PCR from single nematodes and unprocessed sediment

DNA was extracted from single-nematode specimens following the methods of Bik, Thomas, Lunt, and Lambshead (2010), using a simple Worm Lysis Buffer protocol [WLB: 0.2 M NaCl, 0.2 M Tris-HCl (pH 8.0), 1% beta-mercaptoethanol, 800 μg/ml proteinase K]. Nematodes were recovered from temporary slides, transferred to new sterile

TABLE 2 Nematode diversity recovered in the present study (order, family and genus level)

Order	Family	Genus/Species	Feeding group
Chromadorida	Aegialoalaimidae	—	1A
Chromadorida	Ceramonatidae	*Psileonema*	1A
Chromadorida	Chromadoridae	*Dichromadora*	2A
Chromadorida	Chromadoridae	*Euchromadora*	2A
Chromadorida	Chromadoridae	*Neochromadora*	2A
Chromadorida	Chromadoridae	—	2A
Chromadorida	Chromadoridae	*Spilophorella*	2A
Chromadorida	Comesomatidae	*Cervonema*	1B
Chromadorida	Comesomatidae	—	1B
Chromadorida	Comesomatidae	*Sabatieria*	1B
Chromadorida	Comesomatidae	*Sabatieria*	1B
Chromadorida	Comesomatidae	*Setosabatieria*	1B
Chromadorida	Comesomatidae	*Vasostoma*	2A
Chromadorida	Cyatholaimidae	—	2A
Chromadorida	Cyatholaimidae	*Paracanthonchus*	2A
Chromadorida	Desmodoridae	*Desmodora*	2A
Chromadorida	Desmodoridae	—	2A
Chromadorida	Desmodoridae	*Spirinia*	2A
Chromadorida	Desmoscolecidae	*Desmoscolex*	1A
Chromadorida	Desmoscolecidae	*Desmoscolex* sp1	1A
Chromadorida	Desmoscolecidae	*Desmoscolex* sp2	1A
Chromadorida	Desmoscolecidae	*Desmoscolex* sp3	1A
Chromadorida	Ethmolaimidae	*Comesa*	2A
Chromadorida	Leptolaimidae	*Camacolaimus*	2A
Chromadorida	Leptolaimidae	*Leptolaimus*	1A
Chromadorida	Leptolaimidae	—	1A
Chromadorida	Microlaimidae	—	2A
Chromadorida	Selachinematidae	*Halichoanolaimus*	2B
Enoplida	Anticomidae	*Anticoma*	1B
Enoplida	—	—	—
Enoplida	Oncholaimidae	—	2B
Enoplida	Oncholaimidae	*Pontonema*	2B
Enoplida	Oncholaimidae	*Viscosia*	2B
Enoplida	Oxystominidae	*Halalaimus*	1A
Enoplida	Oxystominidae	*Nemanema*	1A
Enoplida	Oxystominidae	—	1A
Enoplida	Oxystominidae	*Oxystominina*	1A
Enoplida	Rhabdodemaniidae	*Rhabdodemania*	1B
Enoplida	Thoracostomopsidae	*Mesacanthion*	2B
Enoplida	Thoracostomopsidae	—	2B
Monhysterida	Diplopeltidae	*Diplopeltis*	1A
Monhysterida	Diplopeltidae	*Diplopeltoides*	1A
Monhysterida	Diplopeltidae	*Diplopeltula*	1A
Monhysterida	Diplopeltidae	—	1A
Monhysterida	Linhomoeidae	*Terschellingia*	1B
Monhysterida	Sphaerolaimidae	—	2B
Monhysterida	Sphaerolaimidae	*Sphaerolaimus*	2B
Monhysterida	Xyalidae	*Amphimonhystrella*	1B
Monhysterida	Xyalidae	*Daptonema*	1B
Monhysterida	Xyalidae	—	1B

Feeding groups are according to Wieser (1953), as defined by buccal cavity morphology and armature. Nematode classification follows Lorenzen (1994) with minor modifications (Warwick et al., 1998).
(—) No data.

glass slide containing 5 μl of WLB, cut into 2–3 pieces with a sterile disposable scalpel and transferred into a 200-μl PCR tube containing an additional 20 μl of WLB. Taxonomy blank samples (i.e., those containing only WLB, where the taxonomist simulated the cutting and transferring of worms into tubes) were also included as a checkpoint for potential sources of contamination (i.e., airborne microorganisms, reagent contaminants) in the laboratory. Worms in WLB were subsequently incubated in a Thermomixer heated shaker block (Eppendorf, Hamburg, Germany) at 65°C and 750 rpm for 2 hr, followed by a 5-min incubation at 100°C to inactivate proteinase K. Lysate was used immediately or stored at −20°C. A separate DNA extraction protocol was carried out on unprocessed Arctic sediment samples a part of a separate study, using the MOBIO PowerSoil DNA Isolation Kit (Catalogue No. 12888-1000; MOBIO Laboratories, Inc., Carlsbad, CA). DNA was extracted from approximately 0.25 g of sediment per sample and lysates were stored at −80°C, according to the manufacturer's protocol.

DNA extracts from single nematodes were used to amplify both the 16S ribosomal RNA gene from bacteria/archaea [515F/806R primers targeting the V4 region (Caporaso et al., 2012)] and 18S ribosomal RNA gene from microbial eukaryotes [F04/R22 primer targeting the V1–V2 region (Creer et al., 2010)]. Dual-index primer constructs were designed by modifying the Earth Microbiome Project Illumina amplicon protocol [http://www.earthmicrobiome.org/ (Caporaso et al., 2012)]. A second barcode region was added to reverse 16S rRNA PCR primers; 18S rRNA primers were designed using the 16S constructs, by modifying the primer linker and replacing the primer sequences themselves. All primer constructs and oligo sequences have been made available on FigShare (https://doi.org/10.6084/m9.figshare.5701090). Both 16S and 18S rRNA genes were amplified using the reagents and PCR conditions in the Earth Microbiome Project (EMP) protocols (http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/). To avoid contamination, PCRs were set up in a dedicated laminar-flow hood that underwent daily sterilization with bleach and UV light. Single-nematode and blank/control sample PCRs had a final volume of 25 μl and contained 1 μl of DNA template, 0.5 μl of each primer (10 μM), 10 μl of Platinum Hot Start PCR Master Mix (2x) (Thermo Fisher) and 13 μl of

molecular-grade water. Both positive and negative controls were included in all PCRs. The ZymoBIOMICS™ Microbial Community Standard (Zymo Research, Irvine, CA) was used as a positive control in both 16S and 18S PCRs; this community standard provided a mix of bacterial and eukaryotic taxa in known concentrations, as a checkpoint for PCR amplification and potential taxonomic or sequencing bias. Negative controls consisted of taxonomy blank samples (worm-free DNA extractions that were manipulated in the same way as single-worm nematode samples) as well as technical PCR blanks where molecular-grade water was used instead of DNA template. The following PCR profile was used for amplification of both 18S and 16S rRNA gene fragments: 94°C for 3 min; 94°C for 45 s, 50°C for 60 s and 72°C for 90 s for 35 cycles; and 72°C for 10 min. PCR amplification success was evaluated with gel electrophoresis (agar 1%) to confirm gel bands of the expected fragment size. Purification of PCR products was subsequently carried out using a magnetic bead purification protocol using Agencourt AMPure XP beads (Beckman Coulter, CA, USA) and following the manufacturer's protocol. In a separate study, unprocessed Arctic sediment samples were subjected to a modified PCR protocol using the EMP primer set to amplify the 16S rRNA gene from environmental microbes [515F/806R primers targeting the V4 region (Caporaso et al., 2012)]; PCR products from sediments were generated using a 2-step PCR protocol (https://github.com/hollybik/protocols/blob/master/16S_rRNA_twostep_PCR.pdf) that also utilized modified dual-index barcode constructs and included separate blank/control samples. Confirmation of PCR amplification success, purification, quantification and sequencing were carried out using the same protocols as the single-nematode samples generated in this study.

After PCR cleanup, sample concentrations were measured using a QUBIT 3.0 FLUOROMETER (Thermo Fisher Scientific) and normalization values were calculated to ensure that approximately equivalent DNA concentrations were pooled across all samples. Libraries were pooled and subjected to a final magnetic bead cleanup step on the final pool, followed by size selection on a BluePippin (Sage Science, Beverly, MA) to remove any remaining primer dimer and isolate target PCR amplicons within the range of 300–700 bp. A Bioanalyzer trace was run on the size-selected pool as a quality control measure, and 18S and 16S rRNA libraries were sequenced in two separate runs on the Illumina MiSeq Platform (2 × 300-bp paired-end runs). Six unprocessed Arctic sediment samples were sequenced on a third Illumina MiSeq run (2 × 300-bp paired-end) generated as part of a separate study. All negative and positive controls were carried through PCR cleanup and pooling and submitted for sequencing alongside single-worm nematode samples.

2.4 | Data processing and bioinformatic analyses

Raw Illumina data were demultiplexed and quality-trimmed using custom scripts for handling dual-index barcode combinations (*0_demultiplexing-dual-index-1*S-FINAL.sh* that calls *Demul_trim_prep_fliped-merge2.1.pl*—available on GitHub (https://github.com/BikLab/ME_microbiome_2017). Data processing parameters were set according to quality-filtering thresholds recommended by Bokulich et al. (2013), and FASTA headers were reformatted for compatibility with the QIIME version1.91 pipeline (Caporaso et al., 2010). Next, OTU picking was independently carried out on 16S and 18S rRNA data sets using an open-reference approach with subsampling (*pick_open_reference_otus.py* in QIIME 1.9.1; Rideout et al., 2014). Open-reference OTU picking uses a combination of database-dependent (reference-based) and de novo OTU picking approaches that maximizes computational efficiency. The 16S rRNA data set was clustered at 97% sequence identity and utilized the QIIME-formatted GREENGENES 13_8 database (McDonald et al., 2012) for reference-based OTU picking steps and taxonomy assignments for representative OTU sequences. Eukaryotic 18S rRNA data were clustered at a more stringent threshold of 99% sequence identity [a standard approach to account for slower gene evolution in eukaryotic rRNA arrays (Creer et al., 2010)] and utilized the QIIME-formatted SILVA 128 database (Quast et al., 2013) for reference-based OTU picking steps and taxonomy assignments for representative OTU sequences. The RDP naïve Bayesian classifier was used for taxonomy assignment for both 16S and 18S OTUs, using a minimum confidence value of 70% (Wang, Garrity, Tiedje, & Cole, 2007). The resulting 16S and 18S OTU tables were filtered at varying levels of stringency, including abundance-based filtering (Bokulich et al., 2013) and OTU subtraction to remove all sequence reads found in taxonomy blanks and technical PCR blanks (representing the potential kit microbiome and laboratory contaminants). For both data sets, representative OTU sequences were aligned using *align_seqs.py* in QIIME version 1.91, using the PyNAST aligner against the GREENGENES or SILVA database and a minimum per cent threshold of 70%. Alignment failures were discarded and gaps were filtered from the resulting alignments, followed by phylogeny construction using either RAxML (Stamatakis, 2014) or the FastTree algorithm (Price, Dehal, & Arkin, 2009) for maximum-likelihood approximation. QIIME outputs were pulled into R for further data exploration and visualization using the PHYLOSEQ (McMurdie & Holmes, 2013) and GGPLOT (Wickham & Wickham, 2007) packages as well as base R functionalities. All commands and scripts for OTU picking, taxonomy assignment, sequence alignment, phylogeny construction and downstream diversity analyses and visualization are available on GitHub (https://github.com/BikLab/ME_microbiome_2017).

3 | RESULTS

3.1 | Morphological identification of nematode samples

A total of 281 single nematodes were physically isolated from 16 sediment samples in this study. Morphological voucher images were collected for a subset of specimens to assign feeding group morphology and determine taxonomic identifications; video capture images also served as a checkpoint against conflicting information from molecular data (e.g., misidentifications). We were able to isolate nematode species with buccal cavity morphology corresponding to

all four feeding groups (Wieser, 1953), recording 87 nematode specimens assigned to feeding group 1A (selective deposit feeders; Figure 2a), 75 specimens for group 1B (nonselective deposit feeders; Figure 2b), 58 specimens for group 2A (epistratum feeders; Figure 2c) and 27 specimens within group 2B (predators/omnivores; Figure 2d). A subset of nematode specimens were assigned to a fifth category ("unknown" feeding group) due to the unavailability of video capture images or a damaged/obscured head region in the slide mounted worm. "Unknown" feeding groups represented a small minority of specimens; we estimate that >95% of nematodes selected for downstream QIIME analysis were in good morphological condition when initially viewed under the microscope (body intact, no tears or spillage of gut contents). The marine nematodes isolated in this study spanned the three major nematode orders commonly found in marine environments (Enoplida, Monhysterida and Chromadorida), with specimens representing 33 morphologically distinct genera spread across 18 families (Table 2). However, as anticipated the true diversity of our nematode sample set appears to be significantly higher (Figure S1); molecular phylogenies revealed genetic diversity and clade substructuring among nematodes with similar buccal cavity morphology (e.g., nematodes morphologically classified within the same genus or family), and the presence of distinct phylogenetic subclades was often observed for nematode genera isolated from the same ocean region and/or sediment sample.

3.2 | Removal of OTUs corresponding to blank samples

Two amplicon libraries were independently generated from DNA extracts of single nematodes. The same DNA template was used to amplify 16S rRNA targeting bacteria/archaea as well as 18S rRNA targeting microbial eukaryotes, resulting in a total of 9,664,164 and 10,614,592 raw sequence reads, respectively, that matched to known dual-index barcode combinations (Table S1). Raw reads were subjected to further quality-filtering and OTU picking in QIIME, resulting in a total of 9,644,151 reads in the 16S rRNA OTU table clustered at 97% sequence identity and 10,314,234 sequence reads in the 18S rRNA OTU table clustered at 99% sequence identity (Table S1). Unfiltered OTUs were subsequently subjected to a rigorous filtering strategy whereby all OTUs associated with any blank or control sample (OTUs present in taxonomy blanks, technical PCR blanks or Zymo mock community-positive PCR controls) were subtracted from the OTU tables in QIIME; this filtering method removed a subset of OTUs from each single-nematode sample (those OTU with nonzero read counts in blank/control samples) while retaining all other OTUs uniquely present in the nematode microbiome profiles. This strategy was designed to remove the signature of residual DNA and living microbes derived from human skin, airborne sources and the "kit microbiome" of DNA extraction kits and common laboratory reagents (Salter et al., 2014). For the 18S rRNA data set, one additional filtering step was carried out to remove the genetic signature of nematode taxa, which may have otherwise confounded analyses of microbiome patterns in closely related host species (e.g., where the phylogenetic distance of OTUs derived from nematode cells would be low). The 18S OTU table was secondarily filtered on the basis of taxonomy assignments derived from the SILVA database, whereby OTUs assigned to "Metazoa" or "Nematoda" (phrases present in taxonomy hierarchies) were subtracted from the OTU table. This approach was specifically designed to be overly stringent; most marine nematode species are not well represented in the SILVA database, and nematode OTUs from environmental samples can often only be assigned to high taxonomic levels (e.g., "Metazoa" or "Nematoda" with no lower-level classification, even when using the most common 18S rRNA barcoding regions). Downstream analysis of eukaryotic OTUs were thus focused on nonmetazoan microbiome taxa (protist lineages, fungi, diatoms, algae, etc.), as informatic challenges related to taxonomy strings and phylogenetic distance hindered our ability to include metazoan taxa (e.g., nematode/metazoan prey species) in microbiome community analyses. After removal of control/blank OTUs, the final filtered OTU tables contained 1,385,902 sequence reads from 16S rRNA) and 196,366 sequence reads from 18S rRNA (nonmetazoan OTUs only).

Average sequencing depth across samples was 31,790 reads (median 13,025 reads) for 16S rRNA and 34,916 reads (median 16,343 reads) for raw reads that matched known barcode combinations upon initial demultiplexing of the Illumina data sets (Table S1). This

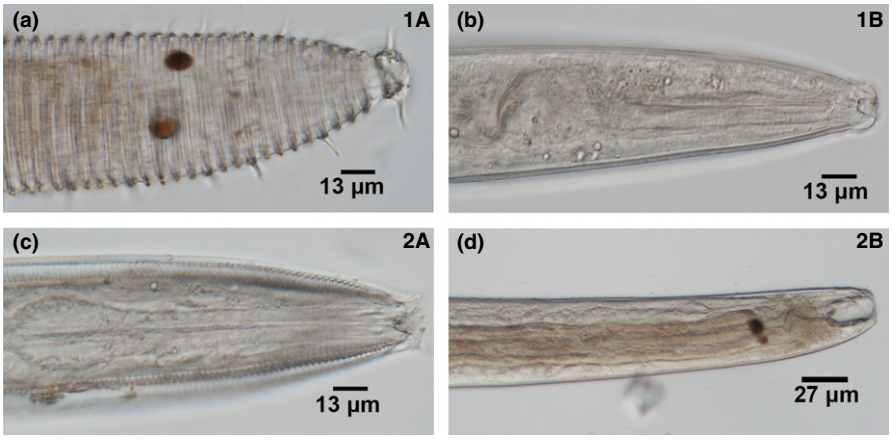

FIGURE 2 Nematode feeding groups according to Wieser (1953), as defined by buccal cavity morphology and armature. Representative nematode images were generated from specimens isolated and photographed in the present study. (a) Group 1A, selective deposit feeders (*Desmoscolex* sp1). (b) Group 1B, nonselective deposit feeders (*Sabatieria* sp.). (c) Group 2A, epigrowth feeders (*Neochromadora* sp.). (d) Group 2B, predators/omnivores (*Pontonema* sp.) [Colour figure can be viewed at wileyonlinelibrary.com]

coverage was reduced to an average of 10,660 reads (16S rRNA) and 1,510 reads (18S rRNA) remaining in the final, filtered OTU tables where all blank, control and nonmetazoan OTUs were removed (Table S1). Although this stringent filtering process dramatically lowered the number of sequence reads generated from single worms, laboratory contamination and "kit microbiome" taxa pose significant challenges for microbiome studies such as this one (Salter et al., 2014), where DNA extractions are typically low yield and host-associated microbial communities are anticipated to show limited diversity. Given that the previous nematode microbiome studies reported low numbers of host-associated microbial OTUs and were able to accurately recover microbiome profiles using older sequencing technologies [e.g., 454 and Sanger sequencing (Baquiran et al., 2013; Derycke et al., 2016)], we were confident that this strict filtering strategy would be able to adequately recover the nematode microbiome profile while eliminating the potentially confounding effects of contaminant OTUs. We also note that blank/control samples typically reported much lower read counts compared to the majority of single-nematode samples (Table S1), which supported the presence of host-associated microbial assemblages in single-nematode DNA extracts (e.g., where higher cellular/DNA content resulted in higher PCR amplification and read counts for experimental samples).

3.3 | Manual confirmation of nematode identities

Illumina data from the 18S rRNA data set was used to confirm the taxonomic identity of individual nematodes isolated in this study. The F04/R22 primer set used in this study was chosen because of its ability to amplify a broad range of taxa (nematodes and >20 metazoan phyla, as well as fungi, algae and unicellular eukaryotes); this universal primer set is also commonly used in nematode DNA barcoding approaches to generate Sanger sequences from individual worms (Bik et al., 2010; Blaxter et al., 1998). Unfiltered OTU tables resulting from 99% clustering of 18S rRNA data were split by sample to determine the eukaryotic OTUs assigned to each single-worm sample. The taxonomy assignments for 18S OTUs recovered from each individual nematode specimen were manually examined to check that a nematode OTU was recovered within the top ~10 most abundant OTUs in that sample, and confirm that the SILVA-derived taxonomy assignment [assigned in QIIME using the RDP classifier (Wang et al., 2007)] was in agreement with the morphological taxonomy assignment. The expected nematode genus was recovered as the most abundant 18S OTU for the majority of specimens; discrepancies were found in only 26 nematode samples used in the final analysis (Table S3). These discrepancies reflected sparse representation of certain nematode families/genera in public sequence databases (e.g., five *Diplopeltula* specimens; this nematode genus is represented by only a single 18S reference sequence in GENBANK) or potentially indicate ecological interactions such as nematode–fungal associations or prey species. All discrepancies between QIIME taxonomy assignments and morphological nematode identifications were manually checked using BLAST searches on the NCBI website. In some cases, the molecular OTU was used to "correct" or refine the nematode identification derived from morphology, particularly for damaged or degraded specimens where key taxonomic features were missing. Only a small number of samples (22 single worms) contained no OTUs corresponding to nematode taxa or had OTU assignments corresponding to a nonmarine nematode species such as *C. elegans* (one sample only); in all cases, these samples were removed from all further downstream diversity analyses and statistical tests.

For manually confirmed nematode specimens (259 single-worm samples), one OTU representative sequence corresponding with the morphological species ID—oftentimes the most abundant 18S OTU—was assigned as the DNA barcode for that nematode specimen. All OTUs assigned as specimen barcodes (one DNA sequence per worm) were mined from QIIME output files of aligned OTU sequences and used to construct a phylogenetic tree in RAxML (Stamatakis, 2014) using the GTRCAT model of nucleotide substitution with 1,000 bootstrap replicates (Figure S1). One contaminated sample assigned to *C. elegans* (MEMB.nem.11) was used as a marine nematode outgroup to root the tree. Phylogenetic parameters used for tree construction are listed in the *raxml-FINAL.sh* script on GitHub.

3.4 | Beta-diversity analyses

To investigate patterns in nematode microbiome taxa, we carried out beta-diversity and multivariate analyses in the QIIME and PRIMER software packages, respectively. Unfiltered 16S and 18S rRNA data sets were first subjected to weighted/unweighted UniFrac principal coordinates analysis (PCoA) before removal of blank/control OTUs; both data sets were rarified at 1,000 and 10,000 sequences per sample. In these initial PCoAs, the Zymo mock community (positive PCR control of known microbial taxa) exhibited distinct clustering compared to all other samples, including blank samples (Figure S2). In contrast, taxonomy blanks and negative PCR controls that fell above rarefaction thresholds did not cluster distinctly from single-nematode samples. Thus, all further downstream diversity and community analyses were carried out on stringently filtered OTU tables where control OTUs were subtracted (16S rRNA data set) or both control OTUs and nonmetazoan taxa were removed (18S rRNA data set). Final filtered OTU tables were subjected to weighted and unweighted UniFrac principal coordinates analysis (a phylogenetic metric that takes relative abundance into account or uses only presence/absence of taxa, respectively), and a series of PCoAs were carried out using increasingly stringent rarefaction values of 100, 500, 1,000 and 10,000 sequences per sample. UniFrac PCoAs showed that host-associated microbial communities did not cluster by any discernible metadata category: samples did not group by ocean region (Figures 3a and 4a), sample site, nematode feeding group (Figures 3a and 4a) or nematode taxonomic identification at any level (order, family or genus; Figures 3b and 4b). This lack of any pattern was consistent across 16S rRNA (Figure 3) and 18S rRNA (Figure 4). To assess whether our global data set was obscuring site-specific patterns, we generated additional UniFrac PCoAs using all nematode specimens isolated from a single site; this process was repeated for

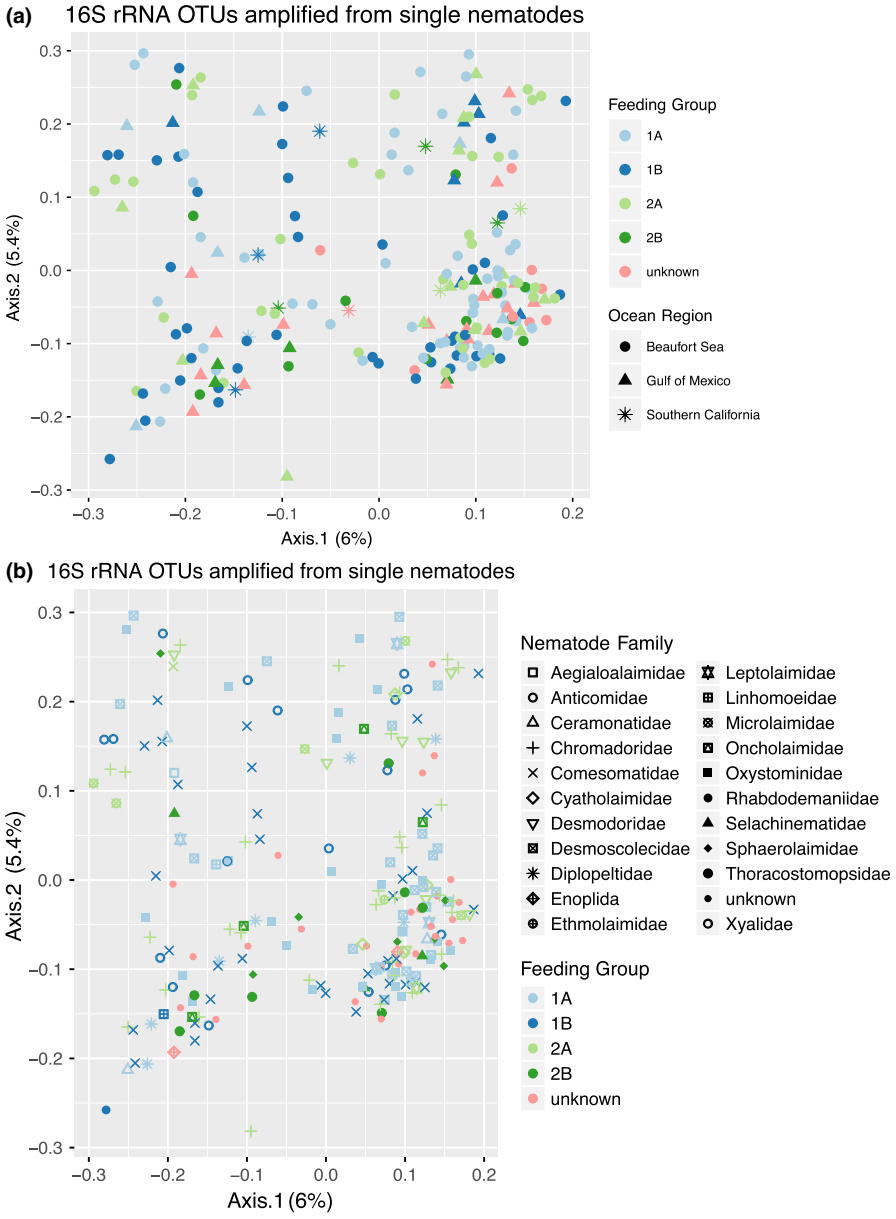

FIGURE 3 Unweighted UniFrac PCoAs generated from bacterial/archaeal 16S rRNA OTUs amplified from individual nematode specimens. No discernible clustering patterns were observed according to feeding group or ocean region (a) or attributed to morphological taxonomy at the family level or below (b). Raw sequence reads were clustered at 97% sequence identity and all OTUs present in blank and mock community samples were subtracted from the data set (taxonomy blanks, kit control blanks and mock community PCR controls). PCoAs were generated using rarefaction values of 500 sequences per sample [Colour figure can be viewed at wileyonlinelibrary.com]

the four sites with the highest number of samples in our data set (Arctic samples A5.20, B1.200A, B1.200B and B1.200C). No further within-site groupings were evident for nematode feeding groups or genus ID at any site (Figure S3). Filtered OTU tables were subjected to further beta-diversity analyses in QIIME, using Bray–Curtis (to test for patterns in abundant species), Canberra (a metric which focuses on rare species) and Jaccard (the simplest shared OTU index) metrics on both bacterial and eukaryotic data sets, rarefied at 100, 500 and 1,000 sequences per sample. However, no other distinct clustering patterns were observed in any of these community similarity analyses (data not shown).

For further confirmation of these results, we carried out an extended analysis of bacterial/archaeal 16S rRNA amplicons focused on six Arctic sediment sites, representing both single-worm microbiome profiles and microbial assemblages recovered from the surrounding (unprocessed) sediments obtained from the same core samples. The combined 16S data set (raw reads from nematode microbiomes and unprocessed sediments) was re-analysed using the same 97% open-reference OTU picking workflow and QIIME parameters (see Section 2). In unweighted UniFrac PCoAs (rarefied at 1,000 sequences per sample; Figure 5) and Bray–Curtis analysis (data not shown), sediment microbial assemblages grouped strongly and separately from nematode microbiome profiles, indicating that bacterial/archaeal taxa within nematode mouths and gut tracts do not represent the same community assemblage that is present in the surrounding benthic habitat. Furthermore, in these PCoAs we continued to observe an overall lack of patterns or grouping among single-nematode samples (no clustering of samples according to nematode feeding group or sampling location; Figure 5a,b, respectively).

In the search of finer-scale patterns among nematode-associated microbes, the filtered 16S rRNA OTU table was also explored through multivariate analyses including nonmetric multidimensional

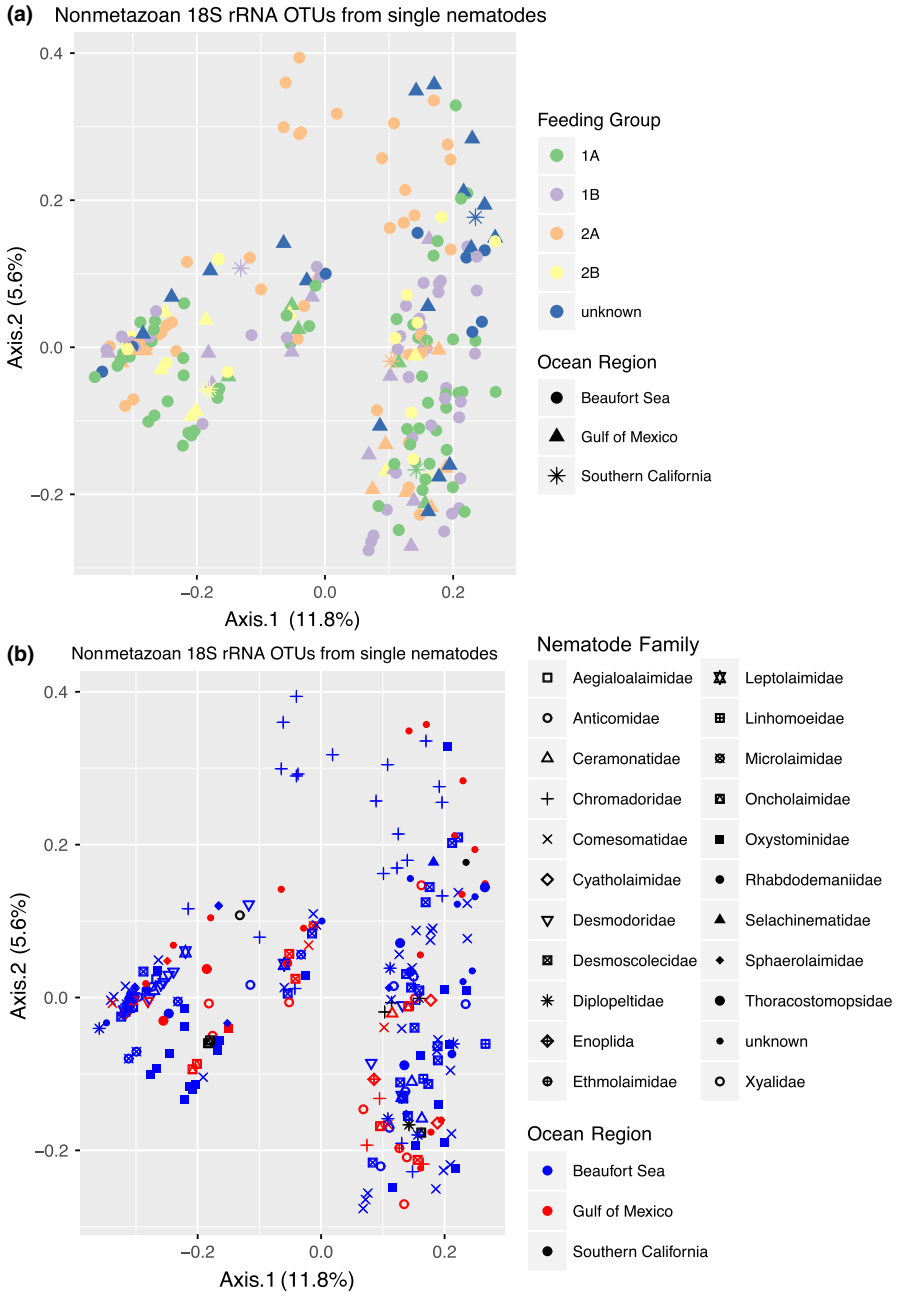

FIGURE 4 Unweighted UniFrac PCoAs generated from nonmetazoan 18S rRNA OTUs amplified from individual nematode specimens. No discernible clustering patterns were observed according to feeding group or ocean region (a) or attributed to morphological taxonomy at the family level or below (b). Raw sequence reads were clustered at 99% sequence identity and all OTUs present in blank and mock community samples were subtracted from the data set (taxonomy blanks, kit control blanks and mock community PCR controls). All OTUs with assignments to metazoan taxa were additionally removed from the data set before UniFrac analyses. PCoAs were generated using rarefaction values of 500 sequences per sample [Colour figure can be viewed at wileyonlinelibrary.com]

scaling (nMDS) and analysis of similarity (ANOSIM) in PRIMER version 6.1.6 (Clarke & Gorley, 2006). The final 16S rRNA data set (with blank/control OTUs removed) was converted into a sample/species matrix based on the taxonomic assignments (i.e., family rank) obtained through QIIME. The number of reads for each taxon was standardized (i.e., transformed to relative abundance) and log (x + 1)-transformed prior to the building of a resemblance matrix using Bray–Curtis index. ANOSIM was used to test for significant differences among ocean regions (i.e., Gulf of Mexico, Beaufort Sea and Southern California), among samples of the sample ocean region (i.e., Beaufort samples), among samples representing a depth transect in the Beaufort Sea (i.e., B1.200A, B.1.500A and B1.1000), among different habitats in the Gulf of Mexico (i.e., methane seep, nonseep and *Lophelia* sites) and among samples representing the most common genera isolated in this study (e.g., *Cervonema*, *Desmoscolex* and *Sabatieria*). Similarly, analysis was performed on the filtered 16S rRNA OTU table (but considering the species rank) using the functions adonis and betadisper within the vegan package (Oksanen et al., 2017) in the R environment (R Core Team 2017). Resemblance matrices were built using the Bray–Curtis index and multiple comparisons (same as for ANOSIM) tested with function permutest (pairwise = TRUE) in vegan. Overall, no clear pattern was observed when samples were explored through the nMDS (Figures S4 and S5). Whether samples are grouped by ocean region, habitat (i.e., within Gulf of Mexico) or depth (i.e., within Beaufort Sea), their spatial distribution in the nMDS plot was often clumped and exhibited a large

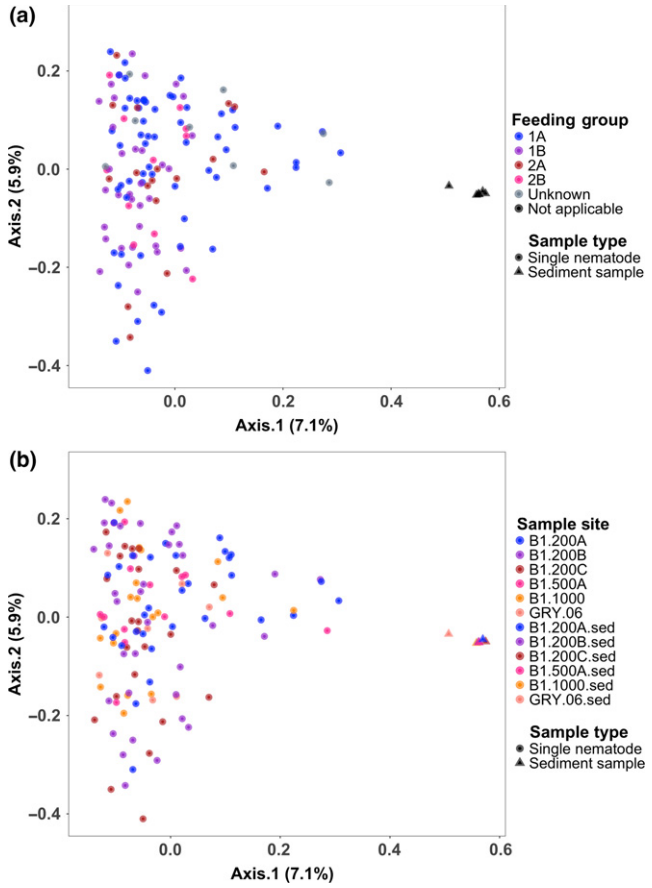

FIGURE 5 Comparison of nematode microbiome profiles and sediment microbial assemblages. Unweighted UniFrac PCoAs (rarefied at 1,000 sequences per sample) generated from 16S rRNA OTUs recovered from six Arctic sites, representing both single-nematode microbiome profiles (circles) and the bacterial/archaeal assemblages present in the unprocessed sediment derived from the same core sample (triangles). While sediments and nematode microbiomes showed strong and distinct grouping, no visible clustering patterns were observed within nematode microbiome profiles according to feeding group (a) or sampling location (b) [Colour figure can be viewed at wileyonlinelibrary.com]

degree of overlap. Although the ANOSIM detected significant differences ($p < .05$) among some of the comparisons (Table S2), their respective values of R were usually low (including Global R) thus suggesting that variations among groups are also low and might be a result of differences in sample size. The results from the PERMANOVA showed significant differences in most of the comparisons (Table S2) and sometimes differed from those of the ANOSIM. The differences between ANOSIM and PERMANOVA outcomes may be due to the taxonomic rank/resolution (i.e., family and species) used in these analyses. Although a lower taxonomic rank such as the one used in the PERMANOVA can increase resolution and more easily distinguish groups, the homogeneity dispersion test (betadisper) showed that our groups do not have the same dispersions. Thus, PERMANOVA results seem to be heavily influenced by differences in composition within groups (i.e., heterogeneous dispersion) and should therefore be interpreted with caution. Among the most common nematode genera, the 16S nematode microbiome profiles associated with the genus *Desmoscolex* seemed to group (partially) according to sampling site (Figure S4), a variation also detected by the ANOSIM (Table S2). However, additional specimens from this genus and increased statistical power will be required to confirm whether these loose groupings are biologically valid.

3.5 | Alpha diversity and fine-scale taxonomy of nematode microbiomes

The microbiome profiles associated with single nematodes were also explored at the level of the OTU, to summarize the most abundant taxonomic groups and assess any fine-scale patterns that may emerge from the exploration of specific taxa recovered from single worms. Nematode specimens were grouped by morphological taxonomy (order, family or genus ID) and microbiome profiles were visualized using taxonomy bar charts to display relative abundance of host-associated taxa (16S data; Figures 6, 7 and S6). For the 18S eukaryotic data set, the majority of single-nematode samples did not retain any sequence reads after the stringent filtering process whereby blank/control OTUs and nonmetazoan OTUs were removed from the data set. The subset of nematodes retaining >500 sequence reads per sample was visualized in its entirety without any further grouping according to nematode morphology (Figure 8).

In the filtered 16S data set (blank/control OTUs subtracted), the majority of host-associated microbes were from the Bacterial domain of life, however several Archaeal taxa were recovered from single-nematode samples including *Methanobacterium* sp., *Nitrosopumilus* sp. and *Candidatus Nitrososphaera* species SCA1170 and SCA1145 (Table 3). The most abundant bacterial taxa associated with single nematodes included class Deinococci, and bacterial phyla Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Gemmatimonadetes, Planctomycetes and Proteobacteria. Within the Proteobacteria, we observed a high number of OTUs assigned to the marine genus *Alteromonas* (López-Pérez & Rodriguez-Valera, 2016), the polar cold-tolerant genus *Glaciecola* (Van Trappen, Tan, Yang, Mergaert, & Swings, 2004), benthic members of the family Colwelliaceae including *Thalassomonas sediminis* (Xu, Lu, Liang, Chen, & Du, 2016), marine Gammaproteobacteria from the OM60 clade (Yan et al., 2009) as well marine bacteria from the order Oceanospirillales. In taxonomy bar charts, we observed that nematodes from disparate geographic regions and distinct phylogenetic clades could demonstrate similar microbiome profiles (e.g., MEMB.nem.193 and MEMB.nem.174, *Cervonema* sp. nematodes; Figure 6); on the other hand, nematodes from the same geographic region and falling within the same phylogenetic clade could exhibit microbiome profiles that were notably distinct (e.g., MEMB.nem.40 and MEMB.nem.220, *Cervonema* sp. nematodes; Figure 6). To test whether the observed single-nematode 16S microbiome profiles were stochastic or driven a specific biotic or abiotic factor, we subsequently carried out linear discriminant analysis (LDA) effect size (LEfSe) analysis (Segata et al., 2011) on the filtered 16S OTU table using an online Galaxy server (http://huttenhower.sph.harvard.ed

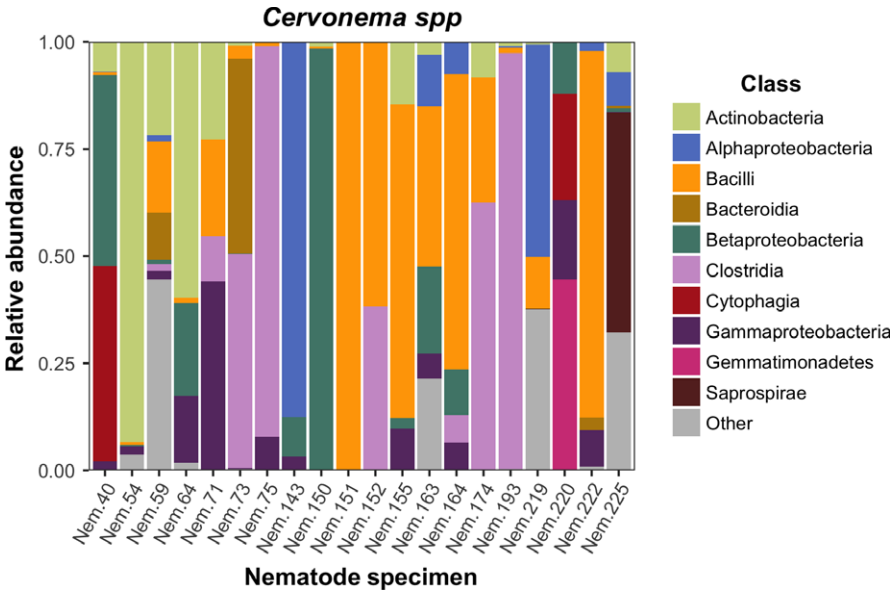

FIGURE 6 Alpha diversity of bacteria/archaea associated with *Cervonema* spp. nematodes (feeding group 1B). Taxonomy bar chart showing relative abundances of bacterial classes observed in the filtered 16S rRNA data set clustered at 97% identity in QIIME (blank/control OTUs subtracted). Each bar represents the microbiome profile associated with a single worm, with sample IDs listed on the *x*-axis. Chart displays the ten most abundant bacterial classes, with remaining taxa collapsed under "Other" [Colour figure can be viewed at wileyonlinelibrary.com]

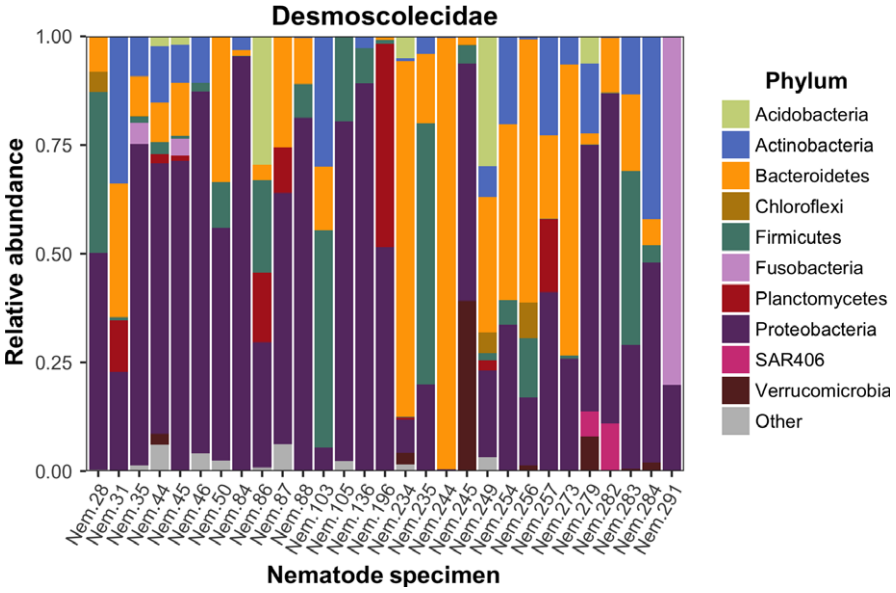

FIGURE 7 Alpha diversity of bacteria/archaea associated with Desmoscolecidae nematodes (feeding group 1A). Taxonomy bar chart showing relative abundances of bacterial phyla observed in the filtered 16S rRNA data set clustered at 97% identity in QIIME (blank/control OTUs subtracted). Each bar represents the microbiome profile associated with a single worm, with sample IDs listed on the *x*-axis. Chart displays the ten most abundant bacterial phyla, with remaining taxa collapsed under "Other" [Colour figure can be viewed at wileyonlinelibrary.com]

u/galaxy/). LEfSe analysis is designed to identify microbial biomarkers across sample metadata categories (i.e., enrichment or depletion of specific OTUs or microbial taxa). Per-sample normalization was carried out on all input OTU tables, and LDA effect size was calculated using the strict "all-against-all" strategy for multiclass analysis, using default parameters and thresholds. No significant enrichment or depletion of 16S OTUs was found when samples were categorized by feeding group or morphological classification (order-, family- or genus-level categories). However, LEfSe analysis showed significant enrichment of 16S OTUs belonging to the cyanobacteria order Synechococcales in nematode samples isolated from Southern California (e.g., the photosynthetic genus *Synechococcus* commonly found in marine surface waters; Figure S7). This enrichment is unsurprising as light-dependent *Synechococcus* cells are likely to be much more readily available as a food source in marine surface waters (e.g., for nematode specimens isolated from kelp holdfasts) compared to deep-sea sediments (all other samples).

In the filtered 18S data set (blank/control OTUs subtracted and nonmetazoan taxa removed), the most abundant nematode-associated eukaryote groups were reported as Alveolata, Apusomonadidae, Chloroplastida, Discosea, Holozoa, LEMD255, Nucletmycea (fungi), Rhizaria and Stramenopiles. Nematode-associated eukaryotes most likely represent food sources and gut contents, as the OTU taxonomy indicates a strong signature of photosynthetic organisms (e.g., OTU hits to "Chloroplastida" are typically indicative of rRNA primers amplifying chloroplast gene regions), as well as planktonic taxa that have likely fallen to the seafloor and been ingested by marine nematodes. For example, Alveolate OTUs indicate a number of planktonic ciliates such as *Cyclotrichium cyclokaryon* (described from Arctic waters (Meunier, 1910; Xu, Song, & Hu, 2005)) as well as planktonic dinoflagellate genera such as *Amphidinium* and *Alexandrium* (Taylor, Hoppenrath, & Saldarriaga, 2008). A diversity of single-celled eukaryotes were also recovered from single-nematode samples, including a number of distinct Cercozoan protist lineages within the Alveolata OTUs (Table 3),

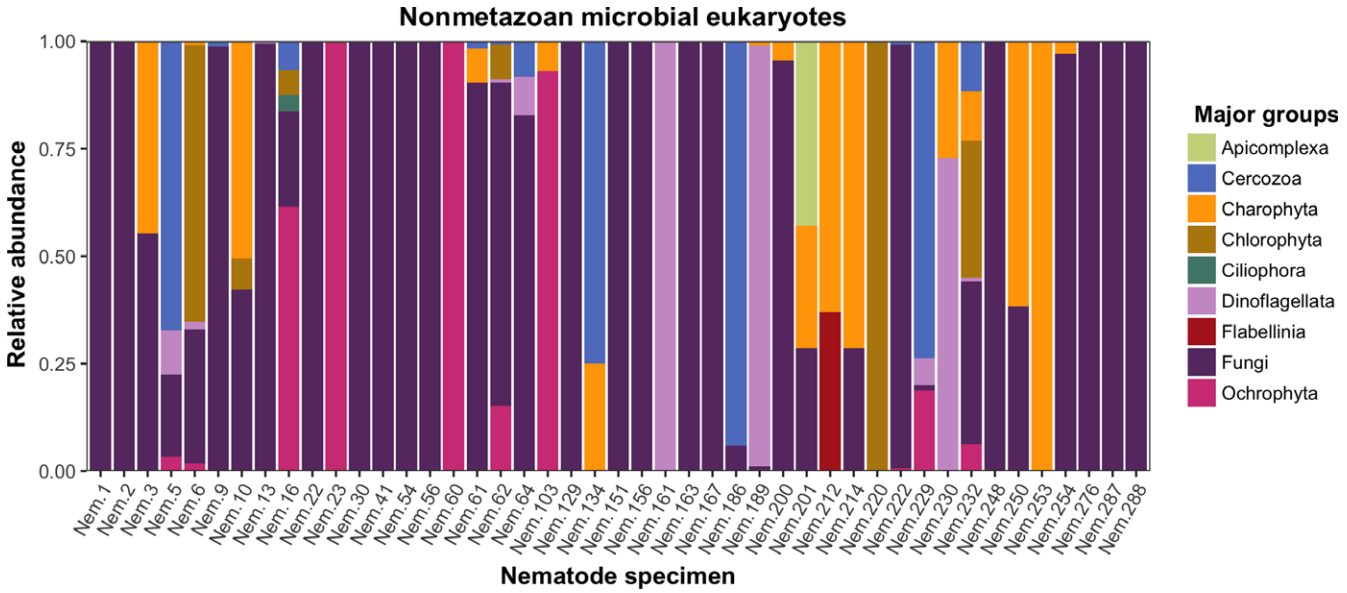

FIGURE 8 Alpha diversity of nonmetazoan microbial eukaryotes associated with single marine nematodes (all feeding groups). Taxonomy bar chart showing relative abundances of major nonmetazoan groups observed in the 18S rRNA data set clustered at 99% identity in QIIME (blank/control OTUs subtracted and metazoan OTUs removed). Each bar represents the microbiome profile associated with a single worm, with sample IDs listed on the *x*-axis. Only nematode specimens with >500 reads remaining after OTU/taxonomy filtering are shown [Colour figure can be viewed at wileyonlinelibrary.com]

including such known parasitoid/predatory heterotrophic nanoflagellates such as *Cryothecomonas* (previously described from Arctic regions; Thaler & Lovejoy, 2012). Intriguingly, eukaryotic data sets also recovered a number of marine parasite reads from single-nematode DNA extracts, with OTUs assigned to the Ascetosporea [a known parasitic group of marine invertebrates such as polychaete worms and crabs (von Vaupel Klein, 2015)], *Paradinium poucheti* [a copepod parasite (Skovgaard & Daugbjerg, 2008)] and Labyrinthulomycetes [marine slime net fungi that exhibit both parasitic and commensal relationships with marine invertebrates, including genus *Aplanochytrium*, an algal-associated taxon recovered in this study (Table 3; Raghukumar, 2002; Raghukumar & Damare, 2011)]. Typically these parasitic and host-associated taxa were represented by low numbers of reads (tens to hundreds in filtered 18S OTU tables) and recovered from only a few isolated nematode specimens, so it is unclear whether the nematodes themselves were being parasitized, or alternatively, whether these parasitic taxa were affiliated with larger food sources of nematodes (e.g., algae, copepods or polychaetes ingested as prey, all of which were represented by hundreds of OTUs in the unfiltered 18S data set).

4 | DISCUSSION

To date, microbiome studies of nematodes have largely focused on one or a few closely related species isolated from a limited geographic range (Baquiran et al., 2013; Cheng et al., 2013; Derycke et al., 2016; Dirksen et al., 2016). Thus, the present study aimed to broadly expand the taxonomic and geographic representation of nematode microbiome studies, by characterizing host-associated microbial communities in the major marine nematode groups recovered from distinct habitat types (e.g., intertidal sediments, methane seeps, deep-sea) in disparate geographic regions (the Arctic, Southern California and the Gulf of Mexico). Thus, this study aimed to investigate both global and local patterns in marine nematode microbiomes, including (i) the existence of species-specific microbiome assemblages and potential co-evolutionary signatures with nematode hosts, (ii) specialist vs. generalist feeding strategies across the four nematode feeding groups defined by Wieser (1953), including evidence of selective feeding and distinct gut contents within each sample site, and (iii) fine-scale ecological interactions across different marine habitat types, such as nematode–microbe symbioses and ingested prey species.

In the present study, we used two rRNA genes to evaluate nematode–microbiome associations (i.e., 16S for bacteria/archaea and 18S for other microbial eukaryotes). Hypervariable rRNA gene regions have been broadly used for microbiome investigations, with previous applications in nematodes showing specific host–microbiome patterns (Derycke et al., 2016; Dirksen et al., 2016; Elhady et al., 2017). However, despite strict laboratory and bioinformatics protocols that were designed to maximize the recovery of nematode microbiomes, our results from free-living marine species showed no clear patterns in host-associated prokaryotic or eukaryotic taxa corresponding to nematode morphology, ocean region or feeding ecology (Figures 4 and 5). These results suggest that in their natural habitats, nematodes may be more generalist feeders than previously acknowledged (Moens, Yeates, & De Ley, 2004). The historical classification of marine nematodes into four discrete feeding groups proposed by Wieser (1953) was strictly based on the visual morphology of nematode mouth parts and did not take into account ecological considerations such as local food availability, energetic constraints of foraging or the existence of both

TABLE 3 Prokaryotic and eukaryotic OTUs associated with single-nematode specimens

Sample ID	Nematode ID	Feeding group	Ocean region	Habitat	Sample site	Associated taxa	References
MEMB.nem.2	Nematoda	no_data	Gulf of Mexico	Seep	GC852.16	*Rickettsiella* (intracellular bacterial pathogen of arthropods), Candidatus Nitrososphaera (ammonia-oxidizing archaea)	Leclerque (2008), Zhalnina et al. (2014)
MEMB.nem.27	Litinium	1A	Beaufort Sea	Arctic	B1.200A	Rickettsiales (bacterial pathogens or endosymbionts of eukaryotic cells)	Yu & Walker (2006)
MEMB.nem.28	Greeffiella	1A	Beaufort Sea	Arctic	B1.200A	Parvarchaea order WCHD3-30 (ultrasmall archaea <500 nm in diameter); **Polychaeta (annelid worm)**	Xia et al. (2017)
MEMB.nem.38	Halichoanolaimus	2B	Beaufort Sea	Arctic	B1.200A	Miscellaneous Crenarchaeotal Group (abundant and diverse marine archaea)	Kubo et al. (2012)
MEMB.nem.62	Campylaimus	1A	Beaufort Sea	Arctic	B1.500A	Coxiellaceae (Gammaproteobacteria species that include intracellular parasites in diverse taxa such as insects and mammals and bacterial symbionts of amoebae)	Tsao et al. (2017), Lory (2014)
MEMB.nem.87	Desmoscolex_sp2	1A	Beaufort Sea	Arctic	B1.1000	*Nitrosopumilus sp.* (ammonia-oxidizing archaea)	Bayer et al. (2016)
MEMB.nem.98	Daptonema	1B	Beaufort Sea	Arctic	GRY.06	**Thecofilosea (free-living Cercozoan protist)**	Cavalier-Smith & Chao (2003)
MEMB.nem.103	Desmoscolex_sp1	1A	Gulf of Mexico	Not Seep	AT340.209	**Ochrophyta (mostly photosynthetic heterokonts)**	Riisberg et al. (2009)
MEMB.nem.113	Mesacanthion	2B	Gulf of Mexico	Lophelia Site	VK826.236	Pirellulaceae (ammonia-oxidizing bacteria)	Mohamed et al. (2010)
MEMB.nem.122	Microlaimidae	2A	Gulf of Mexico	Lophelia Site	VK826.236	Candidatus Nitrososphaera (ammonia-oxidizing archaea)	Zhalnina et al. (2014)
MEMB.nem.129	Terschellingia	1B	Gulf of Mexico	Seep	AT340.193	**Tremellales (fungal parasites of other fungi)**	Findley et al. (2009)
MEMB.nem.132	Amphimonhystrella	1B	Gulf of Mexico	Seep	AT340.193	**Polychaeta (annelid worm)**	-
MEMB.nem.134	Cyatholaimidae	2A	Gulf of Mexico	Seep	AT340.193	Methylobacteriaceae (diverse Alphaproteobacteria family that includes methylotrophs), **Clonostachys rosea (mycoparasites of fungi and nematodes)**	Kelly et al. (2014), Toledo et al. (2006)
MEMB.nem.143	Cervonema	1B	Beaufort Sea	Arctic	A5.20	Methylocystaceae (methanotroph bacteria), **Thecofilosea (free-living Cercozoan protist)**	Bowman (2006), Cavalier-Smith & Chao (2003)
MEMB.nem.147	Spirinia	2A	Beaufort Sea	Arctic	A5.20	*Nitrosopumilus sp.* (ammonia-oxidizing archaea)	Bayer et al. (2016)
MEMB.nem.162	Desmodora	2A	Beaufort Sea	Arctic	A5.20	*Thalassomonas sediminis* (sediment bacteria)	Xu et al. (2016)
MEMB.nem.194	Oxystomina	1A	Gulf of Mexico	Lophelia Site	VK826.244	Pirellulaceae (ammonia-oxidizing bacteria)	Mohamed et al. (2010)

(Continues)

TABLE 3 (Continued)

Sample ID	Nematode ID	Feeding group	Ocean region	Habitat	Sample site	Associated taxa	References
MEMB.nem.196	Desmoscolex_sp1	1A	Gulf of Mexico	Lophelia Site	VK826.244	Pirellulaceae (ammonia-oxidizing bacteria), **Polychaeta (annelid worm)**	Mohamed et al. (2010)
MEMB.nem.209	Pontonema	2B	Southern California	Holdfast Intertidal	LAG01	Pirellulaceae (ammonia-oxidizing bacteria)	Mohamed et al. (2010)
MEMB.nem.212	Euchromadora	2A	Southern California	Holdfast Intertidal	LAG01	**Thraustochytriaceae/ *Aplanochytrium* (parasitic or commensal Labyrinthulomycota)**	Moro et al. (2003)
MEMB.nem.238	Thalassoalaimus	1A	Beaufort Sea	Arctic	B1.200B	**Thecofilosea (free-living Cercozoan protist)**	Cavalier-Smith & Chao (2003)
MEMB.nem.242	Nemanema	1A	Beaufort Sea	Arctic	B1.200B	*Methanobacterium sp.* (methanogenic archaea)	Wasserfallen et al. (2000)
MEMB.nem.244	Desmoscolex_sp2	1A	Beaufort Sea	Arctic	B1.200B	**Thecofilosea (free-living Cercozoan protist)**	Cavalier-Smith & Chao (2003)
MEMB.nem.250	Antomicron	1A	Beaufort Sea	Arctic	B1.200B	Rhodothermaceae (bacterial family containing many thermophilic and halophilic species); **Charophyta (green algae)**	Park et al. (2014), Leliaert et al. (2012)
MEMB.nem.255	Shiphonolaimidae	2B	Beaufort Sea	Arctic	B1.200B	Desulfobulbaceae (sulphate-reducing bacteria)	Blanco et al. (2014)
MEMB.nem.283	Desmoscolex_sp1	1A	Beaufort Sea	Arctic	B1.200C	**Polychaeta (annelid worm)**	–

Putative ecological associations, parasitic taxa and prey items are reported for a selected subset of marine nematodes analysed in this study. Taxonomic assignments for all OTUs derived from lowest hierarchical level assigned using the Greengenes (16S rRNA) or SILVA (18S rRNA) databases and RDP classifier in QIIME. Explanatory annotations in parentheses for each taxon are derived from accompanying references. Taxa in bold are eukaryotic species derived from 18S rRNA data; all other reported taxa are bacterial or archaeal species derived from 16S rRNA data.

primary and facultative feeding strategies (Moens et al., 2004). Later studies, however, showed that marine nematodes can exhibit complex feeding behaviours and Wieser's (1953) classification is likely too simplistic to represent the diversity of foraging strategies seen sediment habitats (Jensen, 1987; Moens & Vincx, 1997). "Omnivorous" nematodes that ingest prey at multiple tropic levels (e.g., feeding on both bacteria and microalgae) appear to be common across all four of Wieser's feeding groups, and nematode feeding behaviour can be additionally influenced by life history stage, density and activity of prey populations, sediment texture and pore size between grains, and the physical mechanics of prey ingestion (mouth vs. prey size) (Moens et al., 2004). For example, in laboratory experiments Moens, Verbeeck, and Vincx (1999) and Moens, Herman, Verbeeck, Steyaert, and Vincx (2000) demonstrated differential feeding strategies in two group 2B marine nematode species, *Enoploides longispiculosus* and *Adoncholaimus fuscus*. Whereas *E. longispiculosus* was shown to be a strict predator, *A. fuscus* uses additional foraging strategies (e.g., scavenging on macrofauna carcasses) to obtain sources of carbon. Similarly, nematode species in the genus *Daptonema* are considered deposit feeders [feeding group 1B; sensu Wieser (1953)], but can occasionally act as predators (Moens & Vincx, 1997). Therefore, variation in feeding strategies (i.e., flexible and generalist feeding behaviour, including both primary and facultative foraging) appears to be widespread across many marine nematode species, including shifts according to developmental stage [e.g., juveniles of *Enoplus brevis* have a distinct diet compared to adults (Hellwig-Armonies, Armonies, & Lorenzen, 1991)]. Furthermore, a growing body of evidence suggests that many nematode species can utilize Dissolved Organic Matter (DOM) as another source of nutrition, including refractory compounds such as fulvic acid (Moens et al., 2004); DOM may thus contribute to nematode growth and reproduction and serve to reduce local foraging pressure, potentially resulting in less competition for nutritional resources typically gained through predation and feeding. This apparent complexity of nematode feeding strategies and foraging behaviour will have an obvious impact on host-associated microbiome profiles, as prey species, gut contents and transient/resident bacterial taxa are likely to vary significantly across space and time, and microbiome profiles could routinely shift according to as-yet-undetermined environmental factors.

In marine nematodes, Derycke et al. (2016) showed clear differences in the microbiome associated with different lineages of

Litoditis marina, suggesting niche partitioning and/or resource allocation among sympatric cryptic species. Moreover, the authors emphasized that such differences in feeding preferences may facilitate the coexistence of cryptic species in their habitats due to reduced intraspecific competition. Conversely, niches and/or feeding preferences might overlap more often among species not living in sympatry, as may be the case for the marine nematode species we isolated in the present study. Indeed, Derycke et al.' s results suggest increased overlap in the microbiome profiles and prey items of cryptic species that rarely co-occur in their natural habitat (species Pm1 and Pm3; Derycke et al., 2016). Furthermore, it is possible that our study did not capture local environmental factors that may play a large role in shaping nematode feeding behaviours and target prey species (e.g., density and activity of bacterial prey, sediment pore size and texture; Moens et al., 2004). Among plant parasitic nematodes, Elhady et al. (2017) found not only that the microbiome of *Meloidogyne incognita* and *Pratylenchus penetrans* differed, but also that the soil types nematode species were exposed to had a significant effect on structuring their microbiomes. In natural habitats on Réunion Island, Meyer et al. (2017) showed that the microbiome of the nematode *Pristionchus pacificus*, which is found in association with scarab beetles, did not vary among contrasting environments (e.g., decaying soil, decaying beetle, pure soil) even when considering the temporal scale (with samples being collected across two consecutive years). Similarly, Koneru, Salinas, Flores, and Hong (2016) showed that entomophilic nematode microbiomes do not vary among species or when grouped according to their respective insect hosts (i.e., different habitats); surprisingly, these insect hosts (i.e., different beetle species) do carry a species-specific microbial community, thus suggesting that entomophilic nematodes lack core bacteria. Taken together, these studies indicate that patterns (or lack thereof) in nematode microbiomes can be vastly different across species and habitat. It remains to be seen whether specific microbiome patterns and feeding strategies observed under laboratory conditions [e.g., for co-occurring cryptic species complexes (Derycke et al., 2016)] are broadly applicable to the large diversity of nematode species inhabiting natural ecosystems where local habitats are more biologically complex and temporally/spatially variable.

Microbiome studies are difficult because of the technical challenges related to sequencing low amounts of DNA, including the persistent threat of laboratory contamination (easily amplified with taxonomically broad rRNA primers) and "kit microbiome" taxa which are inherently introduced during routine laboratory work protocols (Salter et al., 2014). These challenges are only exacerbated during studies of small unculturable species such as free-living marine nematodes and other microbial metazoa, which must be physically manipulated to some degree. In the present study, we aimed for sterile, contaminant-free conditions whenever possible, using disposable equipment and including a large number of blank samples designed to capture the most likely inputs of laboratory and reagent contamination. From a bioinformatic perspective our protocols were equally strict; we stringently filtered our 18S and 16S data sets to remove any OTUs present in our blank samples and used these filtered OTU tables to carry out all downstream ecological analyses and statistical tests. Manual exploration of filtered 18S and 16S OTU tables revealed a large number of OTUs which were obviously derived from marine sources and matched with the geographic locations of our sample set. These included planktonic ciliates such as *Cyclotrichium cyclokaryon* originally described from Arctic waters (Meunier, 1910; Xu et al., 2005), planktonic dinoflagellate genera such as *Amphidinium* and *Alexandrium* (Taylor et al., 2008), marine bacteria from the genus *Alteromonas* (López-Pérez & Rodriguez-Valera, 2016), the polar cold-tolerant bacterial genus *Glaciecola* (Van Trappen et al., 2004) and members of the bacterial family Colwelliaceae including sediment-dwelling *Thalassomonas sediminis* (Xu et al., 2016). In addition, we were able to manually confirm the visual morphological identifications of single-nematode specimens using 18S rRNA OTUs mined from the Illumina data set. The presence of expected marine taxa (both bacterial taxa and nonmetazoan eukaryotes), the molecular confirmation of expected nematode 18S OTUs and the generally low numbers of sequence reads reported for blank/control samples (Table S2) suggest that our observed lack of patterns in nematode microbiomes is a biologically valid result and is not a consequence of confounding artefacts or contamination.

Our examination of fine-scale patterns in nematode microbiomes revealed an unexpected diversity of putative species interactions (Tables 3 and S3), including potential nematode–fungal associations, parasites of nematodes or their ingested prey, and novel bacterial and archaeal prey (or symbiont) species that included ammonia oxidizers, methanogens, methanotrophs and sulphate reducers. Nematode–fungal associations have been previously reported from high-throughput and Sanger sequencing data sets obtained from shallow water and deep-sea sites (Bhadury et al., 2011). The routine co-amplification of 18S sequences from marine fungi and nematodes suggest that these associations may be ubiquitous and ecologically important in marine environments; however, further work is needed to determine whether these reported nematode–fungal associations are reflective of symbiosis or parasitism or simply reflect partially digested food present in the nematode gut. Putative parasite species included bacterial taxa such as Rickettsiales (pathogens or endosymbionts of eukaryotic cells; Leclerque, 2008; Zhalnina et al., 2014), *Rickettsiella* (intracellular pathogen of arthropods; Yu & Walker, 2006), Coxiellaceae (Gammaproteobacteria species that include intracellular parasites in diverse taxa such as insects and mammals and bacterial symbionts of amoebae; Lory, 2014; Tsao et al., 2017), fungal taxa such as Tremellales (fungal parasites of other fungi; Findley et al., 2009), Clonostachys rosea (mycoparasites of fungi and nematodes; Toledo, Virla, Humber, Paradell, & Lastra, 2006) and enigmatic eukaryote lineages whose phylogenetic placement has not been fully resolved such as Thraustochytriaceae/Aplanochytrium (parasitic or commensal taxa within the Labyrinthulomycota; Moro, Negrisolo, Callegaro, & Andreoli, 2003). None of these putative parasites had previously been reported from nematode hosts, with the exception of *Clonostachys rosea* which is a known pathogen of terrestrial nematodes such as *Heterodera* spp. and *Globodera* spp. (Toledo et al., 2006). However, some of these reported taxa are known to parasitize a variety of marine

invertebrate species (e.g., Coxiellaceae and Labyrinthulomycetes; Lory, 2014; Raghukumar, 2002; Raghukumar & Damare, 2011). Interestingly, the three bacterial groups *Rickettsiella*, Rickettsiales and Coxiellaceae appear to represent intracellular pathogens and endosymbionts of eukaryotic cells (previously reported from arthropods and amoebae; Leclerque, 2008; Tsao et al., 2017; Yu & Walker, 2006; Zhalnina et al., 2014); it is unclear whether these intracellular pathogens/symbionts were derived from prey species or nematode host cells themselves. Overall, our data emphasizes the sheer paucity of knowledge regarding the breadth of parasitic interactions and symbiotic/commensal relationships in marine sediment habitats.

From a nutrient cycling perspective, nematodes appear to ingest (or maintain other associations with) a metabolically diverse array of bacterial taxa that play key roles in carbon pathways and organic matter degradation. Nematode microbiome profiles included members of the ammonia-oxidizing archaea (AOA; including Candidatus Nitrososphaera, *Nitrosopumilus* sp.), ammonia-oxidizing bacteria (AOB; including Pirellulaceae), methylotrophs (Alphaproteobacteria within the family Methylobacteriaceae), methanotroph bacteria (Proteobacteria within the family Methylocystaceae), methanogenic archaea (*Methanobacterium* sp.) and sulphate-reducing bacteria (Deltaproteobacteria within the Desulfobulbaceae). Thus, nematodes may play a significant but unacknowledged role in global nutrient cycling of carbon, nitrogen, methane and sulphur; recovery of these metabolically important bacterial and archaeal taxa in nematode microbiome data represents a pressing area for further research. Finally, the recovery of planktonic and photosynthetic taxa within nematode microbiomes (planktonic ciliates such as *Cyclotrichium cyclokaryon* and planktonic dinoflagellate genera such as *Amphidinium* and *Alexandrium*; Meunier, 1910; Taylor et al., 2008; Xu et al., 2005) underscores the ecological importance of fresh organic matter sinking from surface ocean waters; recovery of ~400-bp 18S fragments from these planktonic species suggests that nematodes had recently ingested nondegraded planktonic cells, as ancient DNA and degraded organic matter are typically characterized by much shorter DNA fragments (<150 bp, e.g., Lejzerowicz et al., 2013).

Future work on nematode microbiomes should include an even broader range of morphological genera and geographic locations, including additional focus on samples from shallow water habitats. Our present investigation of nematode microbiomes was limited to existing sample material collected during prior projects (e.g., deep-sea samples), and opportunistic samples taken from a limited geographic area proximate to UC Riverside research laboratories in Southern California (kelp holdfast samples from Laguna Beach). We chose to use reliable, well-tested rRNA primer sets for nematode microbiome profiling in the present study; however, more in-depth investigations of host-associated microbes should be expanded to include shotgun metagenomics [e.g., computational reconstruction of bacterial genomes if possible (Sharpton, 2014)] as well as additional taxon-specific markers that can provide lower-level taxonomic resolution for eukaryotic microbes [e.g., ITS for fungi and additional protist-focused markers (De Beeck et al., 2014; Pawlowski et al., 2012)]. Deeper sequencing and broader genetic resolution could potentially reveal subtle and fine-scale patterns across nematode taxa, feeding types and geographic locations that were not possible to identify using the current study design.

Microbiome studies are critical for broadly advancing our understanding of ecology and evolution across diverse taxa and ecosystems. Insights from "neglected" phyla such as nematodes are particularly important for our understanding of ecological interactions and ecosystem function, especially in vast and unexplored habitats such as marine sediments that are known to harbour extremely high biodiversity (Ramirez-Llodra, Brandt, Danovaro, & De Mol, 2010). Characterization of host-associated microbes has potential to shed light on evolutionary relationships (of both host and microbiome taxa), niche partitioning and resource availability, as well as unveil previously uncharacterized symbiotic relationships and microbial associations (e.g., Distel et al., 2017). For groups such as nematodes with a long tradition of morphological taxonomy, there is a plethora of anecdotal evidence derived from decades of careful, observational work. Such anecdotal insights on feeding ecology, species interactions and foraging behaviour (e.g., Moens et al., 2004, 2013) warrant formal study and in-depth investigation, particularly in regard to the structure and function of host-associated microbiome assemblages in marine and terrestrial ecosystems worldwide.

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DATA ACCESSIBILITY

Raw Illumina reads generated in this study have been submitted to the NCBI Sequence Read Archive (BioProject PRJNA422296 and SRA accession SRP128131). Primer constructs, QIIME mapping files and final OTU tables have been deposited in FigShare (https://doi.org/10.6084/m9.figshare.5701090). All scripts used for processing and analysing the data are available on GitHub (https://github.com/BikLab/ME_microbiome_2017).

AUTHOR CONTRIBUTIONS

H.M.B., T.S. and T.J.P. designed and conceived the study, contributed to data analysis and wrote the manuscript. T.J.P. isolated nematode specimens and generated video capture images of nematode morphology. T.S. completed DNA extractions and 18S/16S PCR amplification and prepared libraries for Illumina sequencing. T.S. and H.M.B. processed raw sequence data and executed bioinformatics workflows. S.H. collected Arctic sediment samples and provided

intellectual contributions on data analysis results. All authors read and approved the final version of the manuscript.

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SUPPORTING INFORMATION

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