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Identifying protist consumers of photosynthetic picoeukaryotes in the surface ocean using stable isotope probing

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Summary

Photosynthetic picoeukaryotes contribute a significant fraction of primary production in the upper ocean. *Micromonas pusilla* is an ecologically relevant photosynthetic picoeukaryote, abundantly and widely distributed in marine waters. Grazing by protists may control the abundance of picoeukaryotes such as *M. pusilla*, but the diversity of the responsible grazers is poorly understood. To identify protists consuming photosynthetic picoeukaryotes in a productive North Pacific Ocean region, we amended seawater with living ^{15}N, ^{13}C-labelled *M. pusilla* cells in a 24-h replicated bottle experiment. DNA stable isotope probing, combined with high-throughput sequencing of V4 hypervariable regions from 18S rRNA gene amplicons (Tag-SIP), identified 19 operational taxonomic units (OTUs) of microbial eukaryotes that consumed *M. pusilla*. These OTUs were distantly related to cultured taxa within the dinoflagellates, ciliates, stramenopiles (MAST-1C and MAST-3 clades) and *Telonema* flagellates, thus, far known only from their environmental 18S rRNA gene sequences. Our discovery of eukaryotic prey consumption by MAST cells confirms that their trophic role in marine microbial food webs includes grazing upon picoeukaryotes. Our study provides new experimental evidence directly linking the genetic identity of diverse uncultivated microbial eukaryotes to the consumption of picoeukaryotic phytoplankton in the upper ocean.

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Introduction

Picoeukaryotes are single-celled eukaryotes representing the smallest size class of protists (0.2–2 μm) (Knight-Jones and Walne, 1951; Butcher, 1952l; Worden and Not, 2008). Picoeukaryotes are diverse (Not *et al.*, 2009; Massana, 2011) and photosynthetic picoeukaryotes can contribute an important fraction of primary production in the photic zone (Agawin *et al.*, 2000; Worden *et al.*, 2015). *Micromonas* is an ecologically relevant model taxon for picoeukaryotic phytoplankton (Worden *et al.*, 2009) because it is widely distributed in both coastal and oceanic marine waters (Cuvelier *et al.*, 2008; Treusch *et al.*, 2012; Lopes Dos Santos *et al.*, 2016; Simmons *et al.*, 2016). Marine viruses and grazing by protists are considered to be important factors influencing the population dynamics of this species (Evans *et al.*, 2003; Lønborg *et al.*, 2013).

Picoeukaryote biomass often represents a significant portion of the microbial ecosystem in upper ocean seawater. In oligotrophic waters their abundances are often between 500 to 1000 cells ml^{-1} whereas in coastal regions their abundance ranges between approximately 5000 cells ml^{-1} to upwards of 20 000 cells ml^{-1} (Worden and Not, 2008). Protist grazing is an important source of mortality for picoeukaryotes in general (Landry *et al.*, 2011; Pasulka *et al.*, 2015), but the diversity and identity of mixotrophic

and heterotrophic protists responsible for phagotrophic consumption (i.e., grazing) of picoeukaryotes is poorly understood. For example, the majority of algal production in the upper ocean is often consumed by protist grazers (Schmoker *et al.*, 2013; and references therein), but grazing measurements generally reflect bulk rates without information on the specific protistan groups responsible (Worden *et al.*, 2015).

Because grazing by protists is responsible for rapid removal of fresh primary production in the surface ocean, it is important to better understand the taxonomic and functional diversity of protists that underlie this component of the marine carbon cycle (Strom, 2008). Many phagotrophic protists are mixotrophs that also can perform photosynthesis (Sanders *et al.*, 2000; Simon *et al.*, 2015; Stoecker *et al.*, 2016) and these mixotrophs may play an important role in the flux of carbon from the surface ocean to the deep sea on a global scale (Ward and Follows, 2016). Amplicon-based studies to examine protistan diversity in the ocean have revealed an enormous diversity of marine microbial eukaryotes (Epstein and López-García, 2008; de Vargas *et al.*, 2015), but the specific trophic roles of many microbial eukaryotes still remains unclear. DNA and RNA stable isotope probing are cultivation independent methods that can identify the protists responsible for phagotrophic consumption of a specific prey group, and provide insights into a putative trophic mode for uncultivated taxa (Lueders *et al.*, 2004; Frias-Lopez *et al.*, 2009; Glaubitz *et al.*, 2009). For example, using living ^{13}C-labelled *Prochlorococcus* and *Synechococcus* cells, RNA-SIP identified heterotrophic and mixotrophic protists consuming these cyanobacteria in the open ocean at Station ALOHA (Frias-Lopez *et al.*, 2009).

Here, we combined high-throughput sequencing of 18S rRNA V4 amplicons and DNA-SIP, hereafter referred to as 'Tag-SIP' (Morando and Capone, 2016; Orsi *et al.*, 2016) using live ^{15}N, ^{13}C-labelled *M. pusilla* as a model for picoeukaryotic phytoplankton. This allowed us to identify protist consumers of this important group of the phytoplankton. We sampled euphotic zone ocean waters of the central California Current System (CCS), where *M. pusilla* is known to be a resident of the phytoplankton community (Simmons *et al.*, 2016). Tag-SIP identified 19 OTUs of uncultivated microbial eukaryotes that were active in the predation of *M. pusilla*. Most of these OTUs were related to protists known only by environmental 18S rRNA gene sequences, thus, our research provides new experimental evidence of their ecological roles in the marine microbial food web.

Results

After a 24 h incubation (T_{24}), DNA from the $>$ 3 μm size fraction was extracted from seawater that had been incubated with either stable isotope labelled or unlabelled living *M. pusilla* CCMP1545 cells (Fig. 1A). Density gradient centrifugation of the bulk DNA shows a clear shift in peak density of 0.009 g ml^{-1} between labelled and unlabelled treatments, with minimal variability between replicates ($\pm$0.03 g ml^{-1}; Fig. 2). In order to identify which protist taxa were isotopically enriched (and thereby the grazers of *M. pusilla*), DNA from 12 density gradient fractions with buoyant densities spanning 1.685–1.735 g ml^{-1} from each bottle were selected for barcoded 18S rRNA gene V4 hypervariable region amplicon sequencing on the Illumina MiSeq platform (Supporting Information Fig. S1).

A total of 2.1 million reads were obtained after amplicon sequencing from the 48 fractions (4 bottles, 12 fractions each), as well as the starting (T_0) and end (T_{24}) samples that were not subject to density gradient fractionation (Fig. 1A). Sequencing depth for samples with the lowest and highest number of raw sequences were 17 934 and 129 133 respectively. After quality control and rarefaction analysis to account for uneven sequencing depth between samples, a total of 8958 reads remained for each sample. After *de novo* OTU clustering, a total of 397 OTUs were formed at 95% identity. This number of OTUs is unusually low for protist diversity in seawater and is likely due to our selection of only the $>$ 3 μm size fraction, together with the removal of low abundance OTUs as a part of the Tag-SIP quality control measures (see Experimental Procedures).

After the 24h incubation, the relative sequence abundance of dinoflagellates decreased 18% compared to T_0, whereas the relative abundance of 18S rRNA gene sequences affiliated with the Syndiniales, Rhizaria and Chlorophyta all increased slightly (5%–10% relative to T_0) (Fig. 2A). The 10 OTUs with highest rank (i.e., abundance relative to other OTUs) at T_0 were affiliated mostly with dinoflagellates (Fig. 2B). With the exception of the 9th and the 10th most abundant T_0 OTUs (OTU1143: *Torodinium teredo*, OTU3619: *Gyrodinium cf. gutrula*), the eight top ranked OTUs at T_0 were still represented within the top eight after 24 h (Fig. 2B). After 24 h, OTU1143 (top BLAST hit Dinophyceae; *Torodinium teredo*) and OTU3619 (top BLAST hit Dinophyceae;*Gyrodinium cf. gutrula*) decreased to the 12th and 14th most abundant OTUs, respectively. The experimental conditions appear to not have had a marked effect on the relative abundance of the dominant taxa because (1) all of the top ten OTUs at T_0 were represented in the top 14 OTUs at T_{24}, and (2) no OTU changed rank more than four positions (Fig. 2B).

We recovered a single OTU related to *M. pusilla* CCMP1545 that displayed a large (0.024 g ml^{-1}) increase in DNA buoyant density, corresponding to our added labelled prey (Supporting Information Fig. S1). This OTU was represented by 40 sequences, corresponding to $<$ 0.1% of the total sequences recovered. Of the 397 OTUs passing our quality control criteria, 19 met our

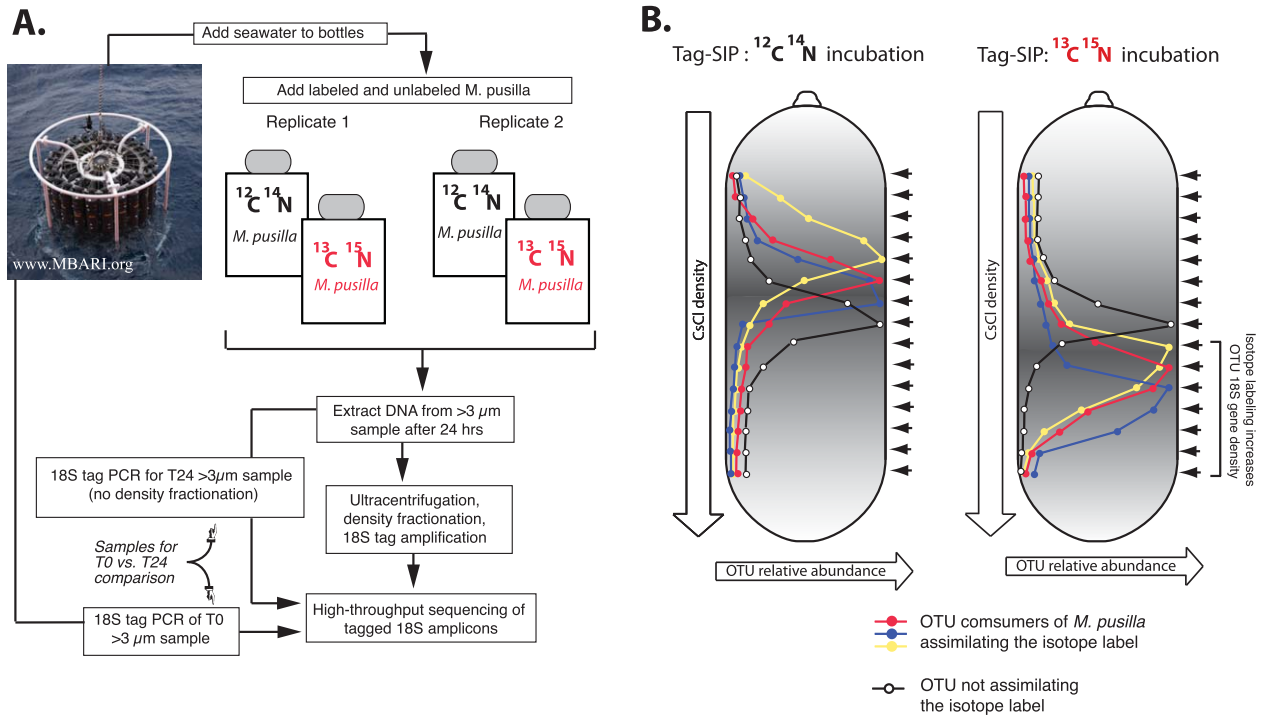

Fig. 1. A. Flow chart demonstrating the experimental setup. To specifically target the protistan predators, DNA was extracted only from the $>$ 3 μm fraction. The changes in community composition over time (e.g., those shown in Fig. 2 and Table 1) were assessed by comparing relative sequence abundance (rank) from a T_0 sample to an unfractionated (no density gradient fractionation) T_{24} sample.
B. Schematic overview of Tag-SIP, where actively growing microbes assimilate an isotopically labelled substrate into their DNA during DNA repair and replication. DNA is extracted and separated based on density in tubes containing a CsCl gradient after ultracentrifugation. The grey smearing in the tubes represents the DNA distribution in the gradient, which derives from diverse communities, where some populations did, or did not, assimilate the substrate. Arrows indicate sections of the gradient that are fractionated and separately subjected to rRNA gene tag sequencing. In this cartoon example three OTUs utilized the substrate (filled circles), whereas a fourth (open circle) did not. Cartoon is modelled after Orsi *et al.* (2016).

criteria for isotopic incorporation (see Experimental Procedures) in both of the replicated experimental incubations (Table 1). These OTUs displayed an increase in 18S rRNA gene buoyant density in the labelled bottles relative to the controls (Supporting Information Fig. S1). Between replicate bottles, there was variability in terms of the amount of isotopic enrichment observed for each OTU (Supporting Information Fig. S1, 3C). For example, OTU #9 that was related to *Telonema antarcticum* (Fig. 3A) exhibited an increase in buoyant density of 0.012 g ml^{-1} in replicate 1, and 0.036 g ml^{-1} in replicate bottle 2 (Fig. 3C). Although some OTUs consuming *M. pusilla* exhibited variability in the degree of isotopic enrichment in the replicate bottles (Supporting Information Fig. S1 and Fig. 3C), the observation that all 19 OTUs exhibited a shift in buoyant density relative to controls in both replicate bottles strongly indicates that these organisms were consuming *M. pusilla*.

Most (13 out of 19) of the OTUs identified as consumers of *M. pusilla* are affiliated with uncultivated eukaryotes related to heterotrophic and mixotrophic dinoflagellates. Heterotrophic dinoflagellates include the genera *Protoperidinium*, *Abedinium*, *Sinophysis*, *Histioneis* and *Gyrodinium*, whereas the mixotrophic dinoflagellates identified were affiliated with species within the genera *Brachidinium*, *Gymnodinium*, *Alexandrium*, *Karenia*, *Pentapharsodinium*, *Warnowia* and *Gonyaulax* (Table 1). Other OTUs that consumed *M. pusilla* were related to taxa within the heterotrophic nanoflagellate genus *Telonema* (2 OTUs) and MAST (2 OTUs) (Table 1). Additionally, an OTU related to the mixotrophic ciliate genus *Strombidium* also consumed *M. pusilla* (Table 1). Two OTUs distantly related (83%–95% 18S rRNA gene identity) to the parasitic Syndiniales taxa *Ichthyodinium and Amoebophyra* were also labelled after the 24 h incubation.

Phylogenetic analysis of the labelled OTUs showed that most of the consumers of *M. pusilla* are distantly related to named taxa (Fig. 3A). Compared to the named taxa, the labelled OTUs tend to be more closely related to 18S rRNA gene sequences recovered from environmental surveys of seawater (Fig. 3A). During the incubation most *M. pusilla* consumers affiliated with ciliates and dinoflagellates increased in rank, whereas the MAST and *Telonema*-related consumers decreased in rank (Fig. 3B). Out of all the OTUs that consumed *M. pusilla*, the two MAST OTUs

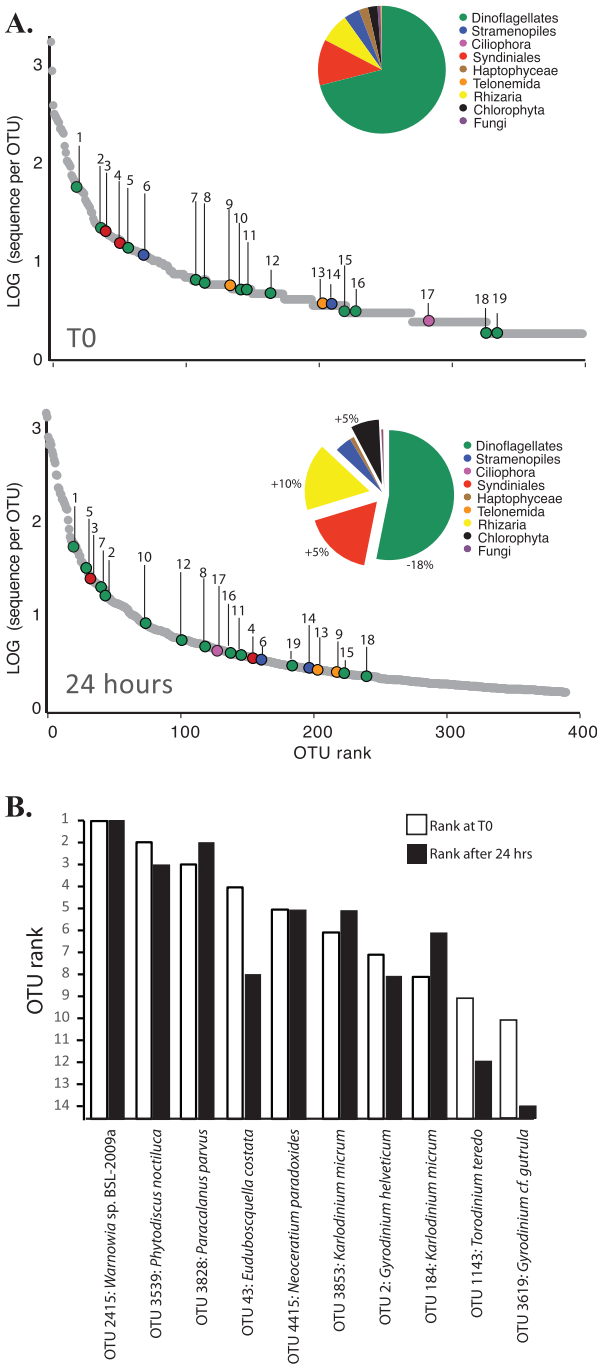

Fig. 2. Changes in community composition over time assessed from the T_0 compared to the unfractionated (no density gradient fractionation) T_{24} sample (Fig. 1A).
A. Rank abundance curves for OTUs at T_0 and T_{24} in unfractionated samples (Fig. 1A). Pie charts show the distribution of major eukaryotic groups at the two time points, $<$ 1% of 18S rRNA gene sequences were unable to be assigned to a eukaryotic Phylum. The T_{24} chart shows percentage change for taxa that changed $>$ 1% compared to their abundance at T_0. For example, dinoflagellates decreased 18% relative to T_0 whereas Syndiniales increased 5%. OTUs that were found to consume *M. pusilla* in the two replicated DNA-SIP incubations are highlighted in colors according to taxonomic affiliation, the numbers correspond to specific OTUs (see Table 1, and Fig. 3).
B. Change in OTU rank of the ten most abundant OTUs at T_0. Note that all of the top ten most abundant OTUs at T_0 were also in the top 14 OTUs at T_{24}, and that no OTU changed rank more than four positions.

related to the MAST-1C and MAST-3 clades exhibited the highest degree of isotopic enrichment with buoyant density shifts of 0.18–0.36 g ml^{-1} (Fig. 3C). For both of these MAST OTUs, a high level of isotopic enrichment was consistent between both replicate bottles (Fig. 3C).

Discussion

Our results broaden the understanding of the phagotrophic protists responsible for consuming picoeukaryotic phytoplankton in the surface ocean. Our study reports new information linking the grazing activity of uncultivated protists to the consumption of *M. pusilla*, an ecologically relevant model picoeukaryote (Worden *et al.*, 2009) with a global distribution (Lopes Dos Santos *et al.*, 2016; Monier *et al.*, 2016; Simmons *et al.*, 2016). The protists identified as consuming *M. pusilla* derive from uncultivated microbial eukaryotes from multiple lineages on the eukaryotic tree (Fig. 3A), that affiliate with described protists known to exhibit mixotrophic and heterotrophic lifestyles (Table 1).

All of the top ten most abundant OTUs at T_0 were also represented in the top 14 OTUs at T_{24}, and no OTU changed rank more than four positions (Fig. 2B). This shows that the experimental conditions did not have a marked effect on the relative abundance of the dominant *in situ* protist taxa. Only a single OTU of *M. pusilla* was recovered in our dataset that was present at very low relative abundance (40 total sequences, $<$ 0.1% total sequences), which is likely due to the small size of these cells ($<$ 2 μm) and the fact that we analyzed the $>$ 3 μm fraction of seawater to focus our dataset towards larger phagotrophic protists. However, the same species (*M. pusilla*, Clade D *Micromonas*) is present at other times in the CCS (Simmons *et al.*, 2016), and thus, it is possible that this single OTU recovered represents a mixture of added labelled cells and native cells of this same strain that were already present in the environment at the time of sampling. Additionally, dinoflagellate and ciliate sequences were abundant in our samples and these taxa are known to contain multiple copies of 18S rRNA genes (Vd'acny *et al.*, 2012; Guo *et al.*, 2016) which will reduce representation of taxa with fewer 18S copy numbers such as *M. pusilla*, which has two copies. Several OTUs that increased markedly in rank over the course of the incubation were relatively low in rank at T_0 (Table 1), suggesting that the dark experimental condition may have impacted the protist predators ingesting *M pusilla*. As observed in other

Table 1. The taxonomic affiliations and change in rank for the OTUs that consumed *M. pusilla* cells.

Putative trophic mode	Labelled OTU #	T_0 rank	T_{24} rank	Change in rank	Closest described species	Group	Percent identity (V4)	Accession number	Evidence for nutritional mode
Heterotrophic	19	321	185	+136	*Protoperidinium bipes*	Dinoflagellata	98	AB284159	Phagotrophic, lacks chloroplasts (Jeong *et al*., 2010, and references therein)
	5	63	31	+32	*Abedinium dasypus*	Dinoflagellata	100	GU355679	Phagotrophic, lacks chloroplasts (Saburova *et al*., 2013)
	3	56	33	+23	*Ichthyodinium chabelardi*	Dinoflagellata	83	FJ440623	Heterotrophic parasite, lacks chloroplasts (Hollande and Cachon, 1952)
	1	22	21	+1	*Gyrodinium cf. spirale*	Dinoflagellata	97	KP790157	Phagotrophic, lacks chloroplasts (Jeong *et al*., 2010, and references therein)
	11	137	140	−3	*Sinophysis stenosoma*	Dinoflagellata	93	JQ996381	Phagotrophic, lacks chloroplasts (Hoppenrath *et al*., 2014)
	15	208	217	−9	*Histioneis longicollis*	Dinoflagellata	95	HM853804	Phagotrophic, forms symbioses with cyanobacteria (Tarangkoon *et al*., 2010)
	14	181	197	−16	*Pirsonia guinardiae*	Stramenopiles	90	AJ561112	Parasitic flagellate of diatoms, lacks chloroplasts (Schnepf *et al*., 1990)
	13	180	200	−20	*Telonema antarcticum*	Telonemia	96	AJ564773	Phagotrophic, lacks chloroplasts (Klaveness *et al*., 2005)
	6	77	153	−76	*Solenicola setigera*	Stramenopiles	99	HM163290	Phagotrophic, lacks chloroplasts (Gomez *et a*l., 2011)
	4	61	143	−82	*Amoebophrya sp. ex Cochlodinium polykrikoides*	Dinoflagellata	94	KF791347	Parasites of dinoflagellates, lack chloroplasts (Kim and Park, 2014)
	9	126	216	−90	*Telonema antarcticum*	Telonemia	95	AJ564773	Phagotrophic, lacks chloroplasts (Klaveness *et al*., 2005)
Mixotrophic	17	263	123	+140	*Strombidium cf. basimorphum*	Ciliophora	99	JF791016	Many species in the genus are mixotrophs (Sanders, 1991),
	16	233	139	+94	*Brachidinium capitatum*	Dinoflagellata	97	HM066998	Photosynthetic (Taylor, 1963)
	18	320	237	+83	*Gymnodinium aureolum*	Dinoflagellata	97	KR362891	Photosynthetic, exhibits phagocytosis (Jeong *et al*., 2010, and references therein)
	7	106	42	+64	*Alexandrium ostenfeldii*	Dinoflagellata	100	KJ361999	Photosynthetic, observation of food vacuoles (Stoecker, 1999, and references therein)
	12	162	104	+58	*Karenia papilionacea*	Dinoflagellata	96	HM067005	Photosynthetic, exhibits phagocytosis (Jeong *et al*., 2010, and references therein)
	10	127	77	+50	*Pentapharsodinium* sp.	Dinoflagellata	98	AF274270	Contain plastids (Balech, 1980; Montresor *et al*., 1993)
	2	51	47	+4	*Warnowia* sp. BSL-2009a	Dinoflagellata	95	FJ947040	Contains plastids (Gavelis *et al*., 2015)
	8	109	120	−11	*Gonyaulax spinifera*	Dinoflagellata	94	AF022155	Photosynthetic, observation of food vacuoles (Stoecker, 1999, and references therein)

OTUs are grouped into putative trophic modes (heterotrophic and mixotrophic), based on the existing evidence in the literature for either heterotrophy or mixotrophy from the closest related cultivated protist taxon. Changes in rank derive from the comparison of the T_0 sample to the unfractionated (no density gradient fractionation) T_{24} sample (Fig. 1A).

experiments (Agatha *et al*., 2005) lack of light may have affected the grazing rates of mixotrophs in particular, and hence the rate of label incorporation by these predators.

It is not likely that the labelled OTUs acquired the label after remineralized C and N passed through an inorganic dissolved phase (as dissolved inorganic carbon [DIC] or NO_3^-). The $\delta^{13}C$ of DIC at the end of the experiment was only enriched 1.1‰ relative to the controls (3.4 ± 0.04 ‰ vs. 2.5 ± 0.06 ‰). Such a low level of enrichment would be insufficient to produce detectable labelling, which requires

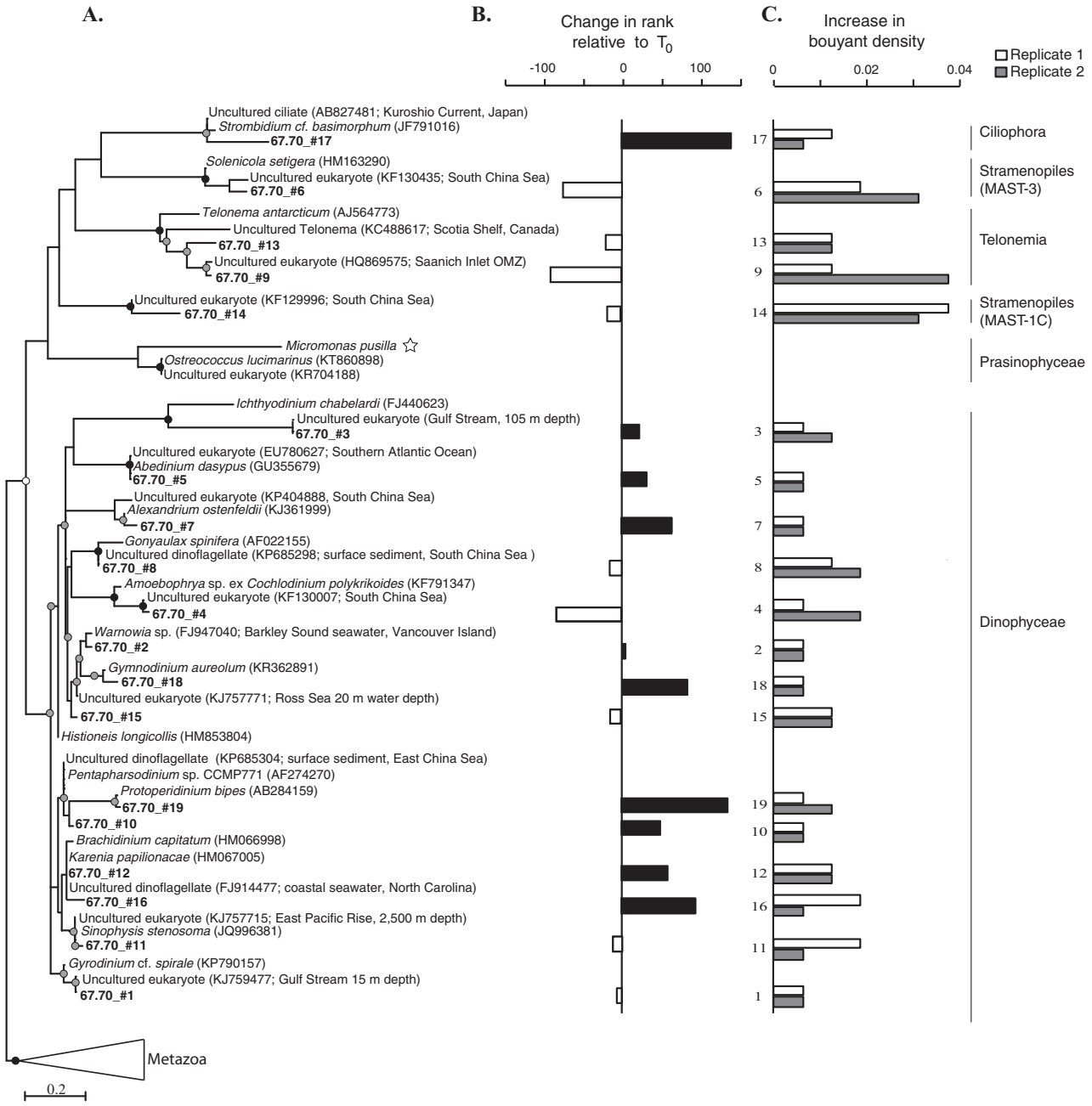

Fig. 3. A. Analysis of 18S V4 rRNA amplicon sequences from the OTUs identified as grazers of *M. pusilla* (bold font). The tree was built using RAxML on an alignment of 220 nucleotides with 1000 bootstrap replicates (clear circles: > 50% bootstrap support, grey circles: > 75% bootstrap support, black circles: > 90% bootstrap support). The number of each OTU identified as a grazer by Tag-SIP (Table 1) is shown in the branch. For example, 67.70_#13 corresponds to OTU #13 in Fig. 2A and Table 1.
B. Change in rank of the OTUs that consumed *M. pusilla*.
C. Increase in buoyant density (defined as difference between the max peak heights) for each OTU consuming *M. pusilla* in replicate incubations. The number at the base of the histograms refers to the SIP labeled OTU in Table 1.

a final enrichment in DNA of approximately 20 atm% (Lueders, 2015). For similar reasons, it is unlikely that ^{15}N labelling occurred via regenerated $^{15}NH_4^+$ resulting from prey digestion. Because the starting ^{15}N enrichment of the cells was 42 atm%, the maximum shift in DNA buoyant density possible due to $^{15}NH_4^+$ incorporation would be 0.008 g ml^{-1} (Buckley *et al.*, 2007). This would require that all N contained in labelled *M. pusilla* cells was remineralized to $^{15}NH_4^+$ during the experiment, either through their consumption or lysis. Our data show that this was not the

case because isotopically labelled *M. pusilla* cells were still recovered at the end of the experiment (Supporting Information Fig. S1B), which means that a marked proportion of the isotopically labelled biomass was not remineralized. Thus, it is highly unlikely that OTUs were labelled solely through assimilation of regenerated $^{15}NH_4^+$.

Microbial eukaryotes tend to have multiple copies of the 18S rRNA gene, and this can influence the interpretation of relative changes in marine protist communities over time (Orsi *et al*., 2011; Orsi *et al*., 2012). For this reason, in our analysis of change in relative abundance between T_0 and T_{24} we decided against basing the change in OTU relative abundance on the percent sequence representation in the entire sample. Instead, to minimize the influence of variable 18S rRNA gene copies between taxa, rank changes (e.g., whether the OTU was the first, second, third, etc., most abundant OTU) between T_0 and T_{24} only within the same OTUs were used as the proxy for their relative change in abundance. This approach assumes that each OTU whose members all share at least 95% sequence identity of its 18S rRNA genes corresponds to a taxon with roughly a species level distinction (Caron *et al*., 2009), which has minimal variability in its 18S rRNA gene copy number. Furthermore, the GC content variability, which is known to influence DNA-SIP results (Buckley *et al*., 2007), within genomes encoded by each OTU should be minimal (Orsi *et al*., 2016). Because our Tag-SIP analysis focuses only on specific OTUs, variability in GC values should not influence the analysis performed here.

The isotopically labelled OTU exhibiting the greatest increase in rank over the incubation was related to S*trombidium basimorphum* (Table 1, Fig. 3A). To our knowledge mixotrophy by this species has never been experimentally verified, but most species in the genus *Strombidium* contain plastids and are mixotrophic (Sanders, 1991). Indeed, *S. biarmatum* can be grown for a limited time under dark conditions on *M. pusilla* as a sole carbon and energy source (Agatha *et al*., 2005). Our results show that *M. pusilla* is also a preferred carbon and or nitrogen source for *S. basimorphum* in the upper ocean.

While the physiology of MAST groups remains enigmatic, studies have shown them to be important heterotrophic bacterial grazers (Massana *et al*., 2006; 2009; Lin *et al*., 2012; Piwosz *et al*., 2013). Here, two OTUs affiliated with the stramenopile clades MAST-3 and MAST-1C were found to consume *M. pusilla*. The only cultivated representative from the MAST-3 clade is a heterotrophic nanoflagellate (Gomez *et al*., 2011) which is consistent with our identification of a new taxon in this clade that grazed the isotopically labelled picoeukaryotic prey. MAST-1 cells have also been observed to graze cyanobacteria (Frias-Lopez *et al*., 2009), and fluorescent *in situ* hybridization (FISH) was used to show previously that MAST-1C cells ingest *Micromonas* and *Ostreococcus* (Massana *et al*., 2009). Moreover, single cell genomes of MAST cells recovered after flow cytometric sorting of single cells have been observed to contain genetic material from *Micromonas*, further indicating that they graze on picoeukaryotes (Mangot *et al*., 2017). Our Tag-SIP data is confirmatory of this earlier FISH-based study showing that MAST cells phagocytose eukaryotic prey (Massana *et al*., 2009), which should be considered as a part of their functional role in marine food webs.

The two OTUs that were distantly related to *Telonema antarcticum* (Table 1) were most closely related to an 18S rRNA sequence recovered from 10 m water depth off the Scotia Shelf (Atlantic Canada; NCBI Accession KC488617) and another from the hypoxic marine water column of the Saanich Inlet, Vancouver Island (HQ869575) (Orsi *et al*., 2012). *T. antarcticum* is a heterotrophic nanoflagellate (Klaveness *et al*., 2005) with a wide geographic distribution in marine habitats (Throndsen, 1969; Vørs, 1992; Massana *et al*., 2004). Heterotrophy is the dominant trophic mode of the genus *Telonema*, although it is affiliated with the predominantly photosynthetic chromalveolate supergroup (Burki *et al*., 2009). The close relation of telonemid OTU #9 to the environmental sequence recovered from the Saanich Inlet oxygen minimum zone (HQ869575) (Orsi *et al*., 2012) indicates that this group might also be important for grazing picoplankton in oxygen depleted marine waters. Despite being heavily labelled, the MAST and *Telonema*-affiliated consumers decreased in rank. Additional experiments are required to validate these findings and identify possible causes of this decrease in rank such as dark conditions, increased grazing by predators of MAST cells, or viral lysis.

The majority of labelled OTUs were related to dinoflagellates (Table 1) indicating that these were likely the most important grazers of *M. pusilla* over the 24-h incubation. These OTUs were related to dinoflagellates with known mixotrophic and heterotrophic lifestyles (Stoecker, 1999; Jeong *et al*., 2010; Stoecker *et al*., 2016). Compared to the named species, the dinoflagellate consumers of *M. pusilla* tend to be more closely related to 18S rRNA gene sequences recovered from environmental surveys of seawater from the China Sea (Wu *et al*., 2014), Ross Sea (Lie *et al*., 2014) and North Atlantic Gulf Stream (Lie *et al*., 2014) (Fig. 3A). This indicates that the dinoflagellates responsible for consuming *M. pusilla* derive from relatively novel taxa with a wide geographic distribution.

The Syndiniales parasites related to the two labelled OTUs (Table 1, Fig. 3A) are thought to be important infective agents of dinoflagellates (Hollande and Cachon, 1952; Schnepf *et al*., 1990; Kim and Park, 2014). The labelling of these parasites thus likely derives from active parasitic infection of dinoflagellates that had phagocytosed *M. pusilla*. Active infection of dinoflagellates by Syndiniales

parasites can also explain the relative decrease in dinoflagellate sequences at T_{24} (–18%), because Syndiniales-affiliated sequences increased 5% in relative abundance during the incubation (Fig. 2A). Despite the general trend of decreasing relative abundance of dinoflagellate sequences over the 24-h incubation, most of the dinoflagellate OTUs consuming *M. pusilla* increased in relative abundance (Fig. 3B). Given the indirect uptake of label by the Syndiniales parasites, it is possible that some larger grazers (e.g., dinoflagellates or ciliates) acquired the label by grazing labelled dinoflagellates (that were currently infected with Syndiniales).

While the phylogenetic analysis of the V4 hypervariable region (Fig. 3A) is based on a shorter gene fragment compared to full length 18S rRNA gene analyses, it still resulted in consistent monophyletic grouping of the major taxonomic groups (Dinophyceae, Ciliophora, Telonema; Fig. 3A) as expected (Hu *et al*., 2015). This showed that a phylogenetic grouping of the OTUs within the existing framework of protistan taxonomy is possible with our V4 data. In our analysis, it is notable that no haptophytes were found to have consumed *M. pusilla*, despite their representation in the amplicon data (Fig. 2A). Haptophytes were represented by nine OTUs were affiliated with *Chrysochromulina* sp. RCC2300 (6 OTUs), *C. parva* (1 OTU) and *Phaeocystis globosa* (2 OTUs). This result is surprising because haptophytes are known to be important mixotrophic grazers of bacterioplankton in the surface ocean (Unrein *et al*., 2014), including the cyanobacteria *Prochlorococcus* and *Synechococcus* (Frias-Lopez *et al*., 2009). From our experiment at a single depth at one location it is unclear whether the lack of haptophyte consumption of *M. pusilla* is a reflection of their feeding habits, or rather an artefact due to insufficient sample size or the influence of seasonal and other environmental factors at the time. The low diversity of haptophytes in our dataset could also be due to the PCR primers used, which have a bias against haptophytes (Balzano *et al*., 2015). The lack of labelled haptophytes might also be a reflection of a different metabolism, for example if they do not recycle food into DNA as quickly as dinoflagellates or Stramenopiles, or exhibit lower growth efficiencies. Haptophytes also did not increase in relative abundance over the incubation (Fig. 2A), and so a potentially slower growth rate or lower growth efficiency compared to other protists indeed may explain their lack of detection with Tag-SIP. It could also be explained by the dark conditions of our incubation, since mixotrophy in haptophytes is (in addition to nutrient availability) related to light availability (Unrein *et al*., 2014). Given these points, we are unable to reliably rule out haptophytes as grazers of picoeukaryotes with our data.

Because Tag-SIP gave sufficient labelling after the 24-h incubation, protist grazers may have been growing rapidly during the incubation. However, label incorporation could be due to DNA repair, or be incorporated during DNA replication prior to cell division, and thus may be due to cell maintenance processes and not necessarily recently divided cells. In either case, however, the labelled cells are metabolically active protists that have ingested labelled prey. Thus, the Tag-SIP method used here represents an alternative for researchers as a method that can be applied to identify active grazers in addition to RNA-SIP (Frias-Lopez *et al*., 2009). As with all SIP applications used to study food webs, we recommend restricting the length of incubation to as short as necessary in order to avoid cross feeding and transfer of label into an inorganic dissolved phase, and careful consideration of potential effects of light and nutrient availability.

Here, we show that several MAST groups that have previously been shown to consume bacteria also consume picoeukaryotic phytoplankton, as do a variety of larger protists some of which appear to be mixotrophs, at least based on their phylogenetic relationships to cultured taxa. Our data suggest that OTUs from uncultivated taxa within the Ciliophora, Stramenopiles, Telonemia and Dinophyceae consume picoeukaryotic phytoplankton in the eastern North Pacific. Because these OTUs are similar to environmental sequences from other ocean regions, it seems likely that they serve more generally as consumers of picophytoplankton in surface ocean environments. The taxa implicated here as predators of *M. pusilla* could be confirmed using a microscopy based approach, where ingestion of prey is unequivocally shown using FISH (Massana *et al*. 2009). Our study provides new insight into the functional ecology of several undescribed marine protistan taxa by demonstrating that they consume small eukaryotic algae. The findings expand the number of trophic linkages for several broadly distributed groups of marine heterotrophic flagellates that to date have been considered bacteriovores.

Experimental procedures

Sampling

Seawater was collected aboard the R/V *Western Flyer* during Monterey Bay Aquarium Research Institute cruise CN13ID, 7–17 October 2013, at Station 67–70 of the California Cooperative Fisheries Investigations (CalCOFI) Line 67 (36.74°N, 122.02°W) (Collins *et al*., 2003; Pennington *et al*., 2010). This station lies approximately 170 km from shore, within the coastal transition zone between the coastal upwelling and oligotrophic waters offshore (Collins *et al*., 2003). The site was selected due to the historic presence of a significant picoeukaryote population of *Micromonas* (Thomsen and Buck, 1998; Simmons *et al*., 2016). We previously documented interactions between organic matter from cultured cells of this picoeukaryote and marine bacteria and archaea (Orsi *et al*., 2015; Orsi *et al*., 2016). The seawater used in these experiments was collected from 20 m, the same water depth and at the same time, as those used in previously published Tag-SIP

experiments (Orsi *et al*., 2016). Sampling and hydrographic profiling were conducted using a conductivity-temperature-depth (CTD) rosette sampler (Sea-Bird Electronics) equipped with (12) 10 l Niskin sample bottles. The incubations were performed immediately after bringing the seawater on board, and no pre-filtration was applied to the seawater before the labelled cells were added.

Stable isotope probing incubation

Micromonas pusilla CCMP1545 was grown axenically for 10 d at 21°C under a light: dark cycle of 14h:10h using cool white light (150 μmol Q m^{-2} s^{-1}) in L1 medium (Guillard and Hargraves, 1993) based on artificial seawater amended with 2.5 mM 99 atm% ^{13}C-labelled sodium bicarbonate and 882 μM 98 atm% ^{15}N-labelled sodium nitrate (Cambridge Isotope Laboratories). Control cultures were grown under the same conditions using ^{12}C sodium bicarbonate and ^{14}N nitrate. Cells were inoculated into the respective medium at 5×10^6 cells/ml and kept in exponential growth by diluting them back to that abundance with fresh medium every other day. The growth rate was 0.47 day^{-1} (SD: 0.09). The isotopic composition of whole cells were measured at the University of California Davis Stable Isotope Facility (SIF) using on-line combustion coupled to a mass spectrometer; with measured values of 16 atm% for ^{13}C and 42 atm% for ^{15}N. The measured value for ^{13}C is potentially an underestimate because the labelled cells were not diluted with unlabelled material prior to isotopic analysis, which may result in underestimation of the actual atm% enrichment.

M. pusilla cells were added to 4 l of seawater to a final concentration of 1000 cells ml^{-1} in trace metal clean polycarbonate bottles. Before adding the cells to the bottles, they were washed with 0.2 μm filtered seawater to remove any residual ^{13}C present in the media. Bottles were amended with dual isotope labelled cells (^{13}C and ^{15}N), or unlabelled cells, in duplicate for each of these two treatments. Bottles were incubated for 24 h in the dark in coolers plumbed with flowing surface seawater to maintain *in situ* temperature. Dark conditions were chosen to reduce the activity of photoautotrophic taxa, which may have affected the grazing rates (and thus label incorporation). At the end of the incubation 1–2 l of seawater was filtered sequentially through 3 μm (Pall Versapor-3000T), 0.8 μm (Pall Supor-800) and 0.2 μm (Pall Supor-200) filters using a peristaltic pump. Filters were stored frozen at −80°C until further analysis.

DNA extraction and density gradient fractionation

DNA was extracted from $>$ 3 μm prefiltered seawater as a T_0 sample, and $>$ 3 μm prefiltered seawater from the bottles after 24 h as a T_{24} sample (Fig. 1A). At the 24 h time point, DNA was extracted for Tag-SIP from the 3 μm prefilters (the size fraction that should contain larger protistan grazers) collected from all four bottles (2 control plus 2 isotope labelled cell addition), according to the method of Santoro *et al.* (2010). As we were strictly interested in the protistan predators of *M. pusilla*, the $<$ 3 μm fraction was not analyzed for Tag-SIP. In brief, 850 μl of lysis buffer and 100 μl of 10% SDS were added to 2 ml gasketed bead-beating tubes containing the filters and 0.1 mm sterile glass beads (Biospec). Bead beating was performed for 1 min and samples heated for 2 min at 99°C. After heating, 25 μl of 20 mg ml^{-1} proteinase K was added and tubes were incubated over night at 55°C. DNA was then purified from the lysate with the DNeasy Blood and Tissue Kit (Qiagen).

The protocol for density gradient centrifugation and gradient fractionation followed previously described methods for DNA-SIP (Neufeld *et al*., 2007; Dunford and Neufeld, 2010), with some minor modifications. In brief, density gradient centrifugation was performed in a TV90 vertical rotor at 20°C for 40 h at 177 000 *g* in an Optima XL-90 ultracentrifuge (Beckman Coulter). DNA was spun in 4 ml polyallomer Optiseal tubes (Beckman Coulter) in cesium chloride (CsCl) gradients with an average density of 1.725 g ml^{-1}. Centrifuged gradients were fractionated into 15 equal fractions via careful manual pipetting from the top of the polyallomer tube. DNA was precipitated with 2 volumes of polyethylene glycol for 2 h at room temperature and pelleted by centrifugation (30 min, 13 000 *g*). Pellets were washed once with 70% ethanol, resuspended in molecular grade water and quantified fluorometrically (Qubit, Life Technologies).

PCR and amplicon library preparation

18S rRNA genes from each fraction were amplified using the TAReuk454FWD1 and TAReukREV3 primers targeting the V4 hypervariable region of the eukaryotic small subunit ribosomal RNA gene (Stoeck *et al*., 2010). The primers were combined with Illumina adapter sequences, a pad, a linker as well as 12 bp Golay barcodes on the reverse primers according to the procedure of Caporaso *et al*. (2012). The barcoded PCR approach was similar to the method used previously for identification of ^{13}C-labelled 16S rRNA genes (Orsi *et al*., 2016), where each separate fraction of the CsCl gradient is PCR amplified using primers with a unique barcode. After high-throughput sequencing and OTU clustering, this approach allows for identification of individual OTUs that display a density shift in the ^{13}C-labelled treatment relative to the unlabelled treatment (Hungate *et al*., 2015). PCR reactions were carried out in 25 μl volumes for using the following thermocycling conditions: 98°C for 30 s; 10 cycles of 98°C for 10 s, 53°C for 30 s, 72°C for 30 s; 20 cycles of 98°C for 10 s, 48°C for 30 s, 72°C for 30s; with a final extension at 72°C for 5 min. Each gradient fraction was amplified using a different barcode combination and amplicons were gel extracted (QIAquick Kit, Qiagen) and pooled in equimolar concentrations prior to high-throughput paired-end Illumina sequencing (MiSeq, 2×250 bp reads) at the Sequencing and Genotyping Core Facility of the University of California Los Angeles.

Bioinformatic analysis

Processing of the paired-end MiSeq data was performed similar to Orsi *et al*. (2016), which combines the preliminary quality control steps and contig assembly described by Kozich *et al*. (Kozich *et al*., 2013) using MOTHUR version 1.37.0 (Schloss *et al*., 2009), and later the pair-wise OTU clustering was performed in QIIME version 1.8 (Caporaso *et al*., 2010). A total of 431 857 sequences were obtained that passed

through quality control and sample rarefaction. To obtain protistan OTUs roughly corresponding to species-level taxa, sequences were clustered at 95% (Caron *et al.*, 2009) sequence identity using UCLUST version 1.2 (Edgar, 2010). OTUs were identified to taxonomic groups through BLASTn (legacy BLAST version 2.2.22) searches against the GenBank nr database using the Jaguc software designed for taxonomic classification of uncultivated microbial eukaryotes from environmental samples (Nebel *et al.*, 2010; Orsi *et al.*, 2013). OTU representative sequences (used for BLAST searches, multiple sequence alignment and phylogenetic tree) were picked as centroids (using QIIME), for example, those sequences that are least dissimilar to all the other sequences within the OTU. Prior to creating the multiple sequence alignment, the closest cultivated and uncultivated relative sequences for each of the 19 OTUs (Table 1) was obtained from the NCBI nucleotide database via BLASTn searches. The V4 region of these sequences were aligned together with the 19 OTUs (Table 1) in a multiple sequence alignment using Muscle (Edgar, 2004) in the SeaView (version 4.2.11) sequence viewer (Gouy *et al.* 2010). The alignment was manually curated, and the resulting 220 aligned positions were used to construct the phylogenetic tree in SeaView (Gouy *et al.* 2010) using RAxML (GTR + I model of evolution) and 1000 bootstrap replicates.

OTU tables were rarified per size fraction, to the sample with the least number of sequences (8997 sequences). Rarified counts for each OTU in the control and SIP gradients were normalized to the maximal abundance of that OTU across density fractions in unlabelled or SIP-labelled bottles (Hungate *et al.*, 2015; Orsi *et al.*, 2016). Only OTUs with > 10 sequences that were detected in $> 50\%$ of the fractions were used in downstream analysis. Sequence data has been deposited in the NCBI database under BioProject ID PRJNA391559.

We defined Tag-SIP isotopic incorporation similar to earlier studies (Nelson and Carlson, 2012; Hungate *et al.*, 2015; Morando and Capone, 2016; Orsi *et al.*, 2016), based on a comparison of the relative distribution of an OTU across the CsCl density gradient in a control (^{12}C and ^{14}N substrate added) versus an experimental (^{13}C and ^{15}N substrate added) incubation. We defined the isotopic labelling of OTUs based on three criteria. We first screened OTUs that banded with a similar overall shape in the experimental treatment compared to the control. Second, using the density with peak OTU abundance in the control as a reference, we selected OTUs that had peak abundance in the experimental treatment at least one fraction heavier (0.006 g ml^{-1}). As a third requirement, the OTU relative abundance in the fraction immediately following its peak abundance in the experimental treatment also had to have a greater relative abundance compared to the control. This second fraction corresponds to an enrichment of 0.012 g ml^{-1}. This criteria, together with the general overlapping symmetry between OTU 'bands' (i.e., the peak density) between the label and unlabelled control is considered convincing evidence of OTU isotopic incorporation (Morando and Capone, 2016). Each fraction in our study are separated by 0.006 g ml^{-1}, and thus, a higher relative abundance in two fractions compared to the control (0.012 g ml^{-1}) roughly corresponds to an atm% enrichment of $> 20\%$ ^{13}C, which is generally considered the threshold for identifying SIP-labelled rRNA genes (Lueders, 2015). Thus, throughout the manuscript we refer to OTUs meeting the criteria defined above as those identified as consumers of *M. pusilla*.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Sup Fig. 1. (A) Distribution of DNA extracted from bottles after a 24-h incubation with ^{15}N, ^{13}C labelled (solid line) or unlabelled (dashed line) live *M. pusilla*. Error bars represent the ranges in measurements between two replicate bottles. Shading indicates fractions that were chosen for tag-amplicon sequencing of 18S rRNA genes. (B) Density gradient distribution of the *M. pusilla* OTU recovered at 24 h in the control (dashed line, circles), and isotope labelled experiment (solid line, triangles) corresponding to the prey that was added to the bottles at the beginning of the experiment. (C-U) Density gradient distribution of OTUs that consumed *M. pusilla* (control: dashed lines, labelled incubation: solid lines).