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Linking bacterivory and phyletic diversity of protists with a marker gene survey and experimental feeding with BrdU-labeled bacteria

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ABSTRACT: Over the last few decades, molecular methods have vastly improved our ability to study the diversity of microbial communities. In molecular diversity surveys, the function of protists is often inferred from phylogeny. Yet these surveys are unable to distinguish between different trophic modes among closely related taxa. Here we present results from a culture-independent study linking bacterivory to the diversity of pelagic protists from 3 depths of a stratified mesotrophic lake. Bacteria were labeled with bromodeoxyuridine (BrdU) and added to lakewater samples; after incubation, total DNA was extracted from filtered samples. Part of the DNA extract was subjected to immunoprecipitation with anti-BrdU antibodies, and then both whole DNA and BrdU-labeled samples were analyzed using 454-pyrosequencing of the v9 region of 18S small subunit rRNA gene amplicons. The results show that a different community of protists exists at each depth, with limited overlap of taxonomic composition between depths. The community of BrdU-labeled protists, deemed putative bacterivores, is largely a subset of the community found in the whole DNA samples. Many of these BrdU-labeled taxa are poorly represented in GenBank and thus are probably rarely isolated and/or uncultured species. Several of the taxa identified as bacterivores are also phototrophs, highlighting the important role of mixotrophy among eukaryotic microbes. Definitive identity of functional traits among taxa requires careful experimentation, yet this method allows a first-pass assay of the trophic role of microbial eukaryotes from environmental samples.

KEY WORDS: Molecular methods · Microbial community · Mixotrophy · Bromodeoxyuridine · Culture-independent · Eukaryotic microbes · Pyrosequencing · Lake microbes

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INTRODUCTION

Microorganisms are crucial to the function of all ecosystems and biogeochemical cycles. In aquatic ecosystems, microbes typically make up the majority of biomass, carbon fixation, and nutrient cycling (Azam et al. 1983). Due to their extremely diverse metabolic pathways, prokaryotic microbes are major contributors to many of these biogeochemical processes (Newman & Banfield 2002). Eukaryotic microbes (protists) also play major roles in decomposition, nutrient recycling, and ecosystem energy flow, including carbon fixation (Paul 2007). Bacterivorous protists remineralize nutrients otherwise tied up in bacterial biomass, are the link between prokaryotic microbes and higher trophic levels, and are a major factor in regulating bacterial population size and community structure in aquatic ecosystems (Sherr & Sherr 2002).

The use of molecular tools in microbial ecology has bloomed over the last 25 yr, allowing the discovery of diversity, taxonomic affinity, and ecology of many unculturable or morphologically indistinct prokary-

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otes and protists (Olsen et al. 1986). Pyrosequencing has further improved our ability to rapidly characterize whole microbial communities and identify rare lineages (Sogin et al. 2006). Increasingly these marker gene surveys, such as amplicon pyrosequencing of a community's rRNA genes, have been applied to examine eukaryotic microbial communities (Stoeck et al. 2010, Bik et al. 2012). Although amplicon pyrosequencing has limited utility in determining relative abundance between species because of gene copy number variation and non-linear PCR amplification (Medinger et al. 2010), next-generation amplicon sequencing is effective for rapidly analyzing microbial taxon richness and community structure from environmental samples. However, the ability of marker gene surveys to discern the function of a particular microbial taxon typically depends only on the taxon's phylogenetic affinity with well-studied organisms, i.e. those in culture.

Culture-independent methods such as stable isotope probing can be combined with marker gene surveys to more directly infer the function of recovered taxa (Gutierrez-Zamora & Manefield 2010). Another related method uses bromodeoxyuridine (BrdU), a thymidine analog commonly applied in molecular and cell biology studies of cell proliferation, which becomes incorporated as a cell undergoes DNA synthesis. In microbial ecology, BrdU has been used to identify metabolically active microbes and determine phylotype-specific growth rates from environmental samples (Urbach et al. 1999). In these methods, BrdU is added to environmental samples, where the compound is taken up by active microbes and incorporated into their DNA. By immunoprecipitation (IP), BrdU-labeled DNA is separated from a subsample of the whole DNA extract, preparing for further analysis by standard molecular ecology techniques. This method has also been used to study bacterivory: organisms that ingest bacteria labeled with BrdU themselves become labeled (Randa 2007).

We coupled the ability to trace the transfer of BrdU-labeled bacterial DNA into bacterivores (Randa 2007) with environmental marker gene survey pyrosequencing to investigate protistan bacterivore diversity at 3 distinct depth layers (epilimnion, metalimnion, and hypolimnion) in a thermally stratified lake. We compared these data with overall microbial eukaryote diversity at the same depths. The communities of bacterivorous protists were less diverse, but largely overlapped with the whole community of protists in the thermally stratified lake, with the greatest diversity of both groups in the mid-depth metalimnion.

MATERIALS AND METHODS

Preparation of BrdU-labeled bacteria

Cultures of *Pasteurella* sp. (isolated from Ice House Pond, Massachusetts, USA, size 0.6–0.7 × 1.2 µm) and *Planococcus* sp. (isolated from Barnegat Bay, New Jersey, USA, diameter ≤1 µm) were maintained in 1% yeast extract (YE) solution and 0.1% YE solution in 32 psu artificial sea water, respectively (Kemp et al. 1993). For BrdU-labeling experiments, 5 ml of YE supplemented with 20 µM 5-bromo-2′-deoxyuridine (BrdU, Sigma B5002) were inoculated with bacteria and incubated for 36 h at room temperature (RT) in the dark. BrdU-labeled bacterial stocks were enumerated using epifluorescence microscopy from samples collected onto black 0.2 µm polycarbonate filters (Millipore GTBP02500) and stained with DAPI-Vectashield (Vector Labs H-1200). BrdU-labeled *Planococcus* sp. bacteria were used for assessment of IP efficiency as described below. The BrdU-labeled *Pasteurella* sp. were prepared for environmental lakewater experiments and were harvested by centrifugation (3000 × *g*, 10 min), washed 3 times, and dispersed and resuspended by pipetting with cold phosphate-buffered saline.

DNA extraction

A hot detergent, bead homogenization protocol adapted by Gast et al. (2004) from Kuske et al. (1998) was used for all DNA extractions. Bacterial cultures were harvested by centrifugation, and DNA was directly extracted from cell pellets. Organisms in environmental samples and the field experiment were collected by vacuum filtration on 47 mm polycarbonate 0.8 µm (Millipore ATTP04700) filters.

Dot blots to determine positive labeling of bacteria

Before use, bacterial strains were tested to verify incorporation of BrdU using dot blots in a protocol adapted from Ueda et al. (2005). For each sample, 10 µl of 25 ng µl^{-1} genomic DNA was denatured by incubation with 40 µl of 0.4N NaOH for 30 min at RT. Samples were then placed on ice to prevent annealing and neutralized with 50 µl cold 2M ammonium acetate. Using a microfiltration device (Bio-Rad 170-6545), samples were dot-blotted onto nitrocellulose membrane pre-wetted with 6× saline sodium citrate buffer (SSC, 0.9M NaCl, 90 mM sodium citrate, pH

7.0). Each well was then rinsed once with 2× SSC, the membrane was removed, rinsed again with 2× SSC, and baked at 80°C under vacuum for 2 h. After blocking for 30 min at RT with 1% dry nonfat milk in TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6), membranes were probed by adding monoclonal anti-BrdU mouse IgG (Sigma B-8434) at a 1:5000 dilution, washed 3× 10 min with TBS-T, incubated 1 h with anti-mouse HRP-conjugated IgG (Cell Signaling 7076), and then washed 3× 20 min with TBS-T. Blots were visualized with ECL Plus (Pierce 32132) and X-OMAT LS film (Kodak 868-9358).

Immunoprecipitation

IP of BrdU-labeled bacterial DNA was performed following the protocol published by Urbach et al. (1999). One modification was the use of unlabeled bacterial genomic DNA from *Pasteurella* sp. (instead of eukaryotic salmon sperm DNA, as used in the Urbach protocol, Urbach et al. 1999) in the blocking step. This was to prevent contamination of the samples with exogenous eukaryotic (salmon) DNA that would subsequently amplify with eukaryotic ribosomal gene primers.

qPCR assessment of IP efficiency

SYBR-based quantitative PCR (qPCR) was used to assess the efficiency of the IP protocol. DNA extracts from *Planococcus* sp., labeled and unlabeled with BrdU, underwent IP as above. Primer3 (Rozen & Skaletsky 2000) was used to design quantitative PCR primers for the 16S rRNA gene of *Planococcus* bacteria, Plano16SqPCRf, 5′-GTG TGT AGC CCA GGT CAT AAG G-3′ and Plano16SqPCRr, 5′-GAT CTT AGT TGC CAG CAT TCA GT-3′. Unknown sample and standard curve (using purified genomic *Planococcus* DNA) reactions were done in triplicate.

qPCR assessment of uptake of BrdU by protists from bacteria

Cultures of the bacterivorous protist *Paraphysomonas* sp. (Macaluso et al. 2009) were fed BrdU-labeled *Pasteurella* bacteria over a time course to examine incorporation of BrdU into the protist DNA. Uptake was determined by qPCR of *Paraphysomonas* DNA after immunoprecipitating whole DNA extracts from samples after 0, 6, 12, and 24 h. *Paraphysomonas*-specific primers were designed as above (Paraphyso_18S_qF: 5′-GCC TGC GGC TTA ATT TGA CT-3′, and Paraphyso_18S_qR: 5′-CAA CTA AGA ACG GCC ATG CA-3′), and reactions were done in triplicate.

Field site and bacterial feeding

Our field site was Lake Lacawac (41° 22.912′ N, 75° 17.543′ W) in the Pocono Mountains, Pennsylvania, USA. Lacawac is a 13 000 yr old glacial lake formed by ice scour with a maximum depth of 13 m, with surface chlorophyll ranging from 2 to 5 µg l^{-1}. This 21 ha mesotrophic lake and its watershed remain undeveloped and are protected by the Lacawac Sanctuary Foundation. The annual phytoplankton community in Lake Lacawac is dominated by chrysophycean algae (Siver & Chock 1986), at times including known mixotrophs (bacterivorous algae) like the colonial flagellates *Dinobryon* and *Uroglena*. Small mixotrophic flagellated nanophytoplankton are the dominant bacterivores in the lake during winter (Berninger et al. 1992), and occur in the lake during other seasons (R.W. Sanders pers. obs.).

On 1 October 2010, the water column oxygen and temperature profiles in the lake were characterized using a dissolved oxygen–temperature meter (YSI Model 85) to identify the depths of the mixed surface layer (epilimnion), the thermocline (metalimnion), and the deeper, cool hypolimnion (Fig. 1). Near dusk,

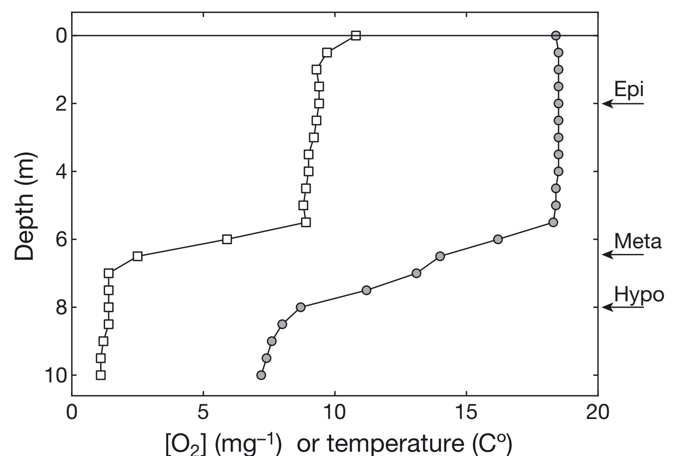

Fig. 1. Depth profile of Lake Lacawac (Pennsylvania, USA) taken on 1 October 2010, indicating thermal and chemical (O_2) stratification. Open squares represent dissolved oxygen (mg l^{-1}), and circles represent water temperature (°C). Three sampling depths are indicated: Epi: epilimnion, Meta: metalimnion, Hypo: hypolimnion

samples were taken from these distinct layers of the thermally stratified water column using a Van Dorn bottle, and were gently prefiltered through a 250 µm Nitex mesh into incubation flasks to remove the larger crustaceans that prey on the protist community. BrdU-labeled bacteria at a final concentration of 1×10^6 cells ml^{-1} (~30% of the typical native bacterial abundance) were added to the 500 ml samples, and the closed flasks were immediately returned to their respective sampled depths attached to a moored rope and incubated overnight and into the morning for a total of 16 h. Light levels were determined just prior to sample retrieval with a Li-Cor LI-250A light meter and LI-193 spherical quantum sensor; the light extinction coefficient (η) was 0.73 m^{-1}. Recovered flasks were kept on ice in the dark until filtered, within 2 h of recovery.

From each incubation flask, 250 ml were filtered onto a 0.8 µm polycarbonate filter, DNA was isolated, and a 2 µg subsample of DNA from each whole DNA sample was used for BrdU-IP as described above. Thus 6 DNA samples were obtained for downstream analyses: 'whole DNA' (i.e. not treated by BrdU-IP) and 'BrdU-IP' from each of the 3 depths: epilimnion, metalimnion, and hypolimnion (Fig. 1).

Denaturing gel gradient electrophoresis (DGGE)

Before proceeding with the pyrosequencing run, DGGE was used to examine how the BrdU-IP community profiles compared to those from the whole DNA sample. Protocol was followed as published by Gast et al. (2004).

Primers, PCR amplicon preparation, and pyrosequencing

We incorporated forward PCR primer 1391F (Stoeck et al. 2010) and reverse primer 1510R (Amaral-Zettler et al. 2009) into fusion primers with Lib-L 1-way read sequencing adaptors and keys as described by 454 Life Sciences Corp. (2010). Forward Primer A 1391F incorporated 6 unique 10 bp Multiplex IDs (MIDs) to distinguish the 6 different amplicon libraries. We chose these primers because, among all of the possible pairs of small subunit (SSU) rRNA V9 region eukaryotic universal primers reported by Amaral-Zettler et al. (2009) and Stoeck et al. (2010), this pair, 1391F (5′-CCA TCT CAT CCC TGC GTG TCT CCG AC-TCA G-[MID]-GTA CAC ACC GCC CGT C-3′) and 1510R (5′-CCT ATC CCC TGT GTG CCT TGG CAG TC-TCA G-CCT TCY GCA GGT TCA CCT AC-3′), has the greatest coverage against a UCLUST 70% clustered subset (consisting of 3164 centroids) of the SILVA eukaryotic SSU rRNA database.

For each sample, we performed 3 PCR reactions, each at a volume of 50 µl that contained 0.2 µM of each primer, 1× reaction buffer, 200 µM each dNTP, and 0.5U Phusion DNA Polymerase (New England Biolabs F-553). Cycling conditions were as follows: 98°C for 120 s; 10 cycles of 98°C for 15 s, 67°C decremented by 1°C $cycle^{-1}$ for 20 s, and 72°C for 15 s; 25 cycles of 98°C for 15 s, 57°C for 20 s, and 72°C for 15 s; and a final extension step of 72°C for 120 s. Each sample was purified with AMPure XP beads (Beckman Coulter A63880) following the manufacturer's protocol. The University of Pennsylvania DNA Sequencing Facility performed the sequencing reaction using a '1-way read' approach to amplicon pyrosequencing with the GS Junior Titanium emPCR Lib-L kit, as recommended by 454 Life Sciences (2010). The resulting read data and our associated analytical results were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number SRA048528.

Pyrosequencing marker gene survey data analysis

We removed barcodes, primers, and low-quality sequences and de-multiplexed the data using QIIME (Caporaso et al. 2010). Sequences were de-noised using the algorithm of Reeder & Knight (2010) implemented in QIIME. Chimeras were removed by de novo detection using UCHIME, and operational taxonomic units (OTUs) were clustered at 95% identity using UCLUST, both implemented in USEARCH (Edgar 2010). It is critical to implement an analytical pipeline that filters out pseudodiversity, especially from PCR chimeras (Behnke et al. 2011). A second de novo check for chimeras was performed after the final list of OTUs was established. Many of the chimeras may have been removed earlier in the process of clustering the OTUs at 95% identity and removing taxa that did not identify as 'eukaryote' against the Ribosomal Database Project (RDP) database. Linux bash shell scripts were written when necessary to prepare data for different applications. QIIME was used to generate rarefaction plots, and OTU tables were exported to PRIMER 6 (Clarke & Warwick 2001) for analysis of community similarity using a variety of metrics. To incorporate phylogenetic signal in our analyses, we used UniFrac

(Lozupone et al. 2007) implemented in QIIME. OTUs were assigned as putative bacterivores if >70% of the total sequence reads for a given OTU were found in the BrdU-IP treatments; only those OTUs with >0.05% sequence read abundance, i.e. >38 reads total, were considered.

RESULTS AND DISCUSSION

Effectiveness of labeling and precipitation

Not all strains of bacteria will take up and incorporate thymidine or its analog BrdU into DNA (Hamasaki et al. 2007). Consequently, it is important to test experimental bacteria to ensure that sufficient labeling will occur. Anti-BrdU dot blots demonstrated that DNA of both *Pasteurella* and *Planococcus* was effectively labeled when grown in media supplemented with BrdU.

Quantitative PCR was used to verify that IP effectively isolated BrdU-labeled DNA. IP of BrdU-labeled DNA from *Planococcus* bacteria yielded 13.0 ± 0.7 ng μl^{-1} (95% CI) of *Planococcus* DNA, while IP of unlabeled control DNA from *Planococcus* bacteria yielded only 53 ± 6.6 pg μl^{-1} (95% CI) This represents a 245-fold enrichment of labeled over unlabeled DNA. The time course of a bacterivorous protist, *Paraphysomonas*, feeding on BrdU-labeled bacteria demonstrated that these protists take up BrdU from their food (Fig. 2). In laboratory experiments, Randa (2007) also found that a marine strain of *Paraphysomonas* and a marine ciliate, *Uronema* sp., ingested BrdU-labeled bacteria and incorporated the label into their genomic DNA in laboratory experiments.

Analysis of the DGGE gels showed that the BrdU-IP samples in our field experiment had fewer total bands than the whole DNA samples. This was expected since the putative bacterivores (BrdU-labeled taxa, BrdU-IP) should be a subset of the whole community of microbial eukaryotes (whole DNA untreated with IP). While some of the DGGE bands appearing in the BrdU-IP samples were unique, most were shared with the whole DNA samples. In these instances of shared band presence, the BrdU-IP bands were often brighter, suggesting greater representation in that recovered DNA pool.

Diversity of protists

After initial quality filtering, which removed 12.6% of sequences, clustering OTUs at 95% identity yielded 581 OTUs. Ten of these (1.7%) were identified as chimeras and removed. After assigning taxonomy using the RDP database, we identified a total of 312 protist OTUs in the combined data set. Rarefaction curves from the pyrosequencing data showed that taxonomic sampling was more complete for the BrdU-IP samples than for the whole DNA samples at all depths (Fig. 3). This confirmed the expectation that the protists actively grazing bacteria represented a subset of the total microbial community. The majority of the OTUs recovered from each of the 'BrdU-IP' samples were also found in their respective 'whole DNA' samples (Fig. 4A), including all of the OTUs with high sequence abundance (>0.1% of the total) from the BrdU-IP samples (Table 1). The rarefaction curves (Fig. 3) also predict that the ranking of diversity for both BrdU-IP and whole DNA samples will remain the same with deeper sequencing.

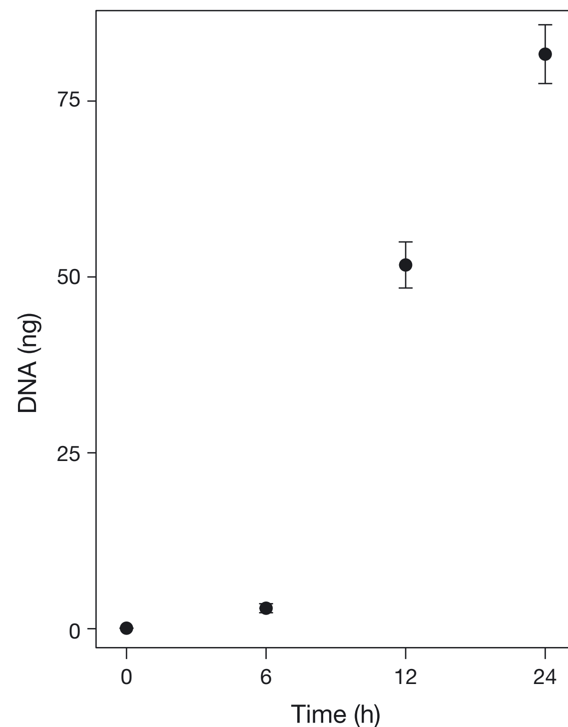

Fig. 2. Quantitative PCR results of bromodeoxyuridine (BrdU)-immunoprecipitated DNA from *Paraphysomonas* cultures fed BrdU-labeled bacteria, with primers specific to *Paraphysomonas* 18S rRNA sequence. Error bars represent 1 SE of the mean

The greatest diversity was found in the metalimnion and least diversity in the hypolimnion (Figs. 3 & 4A). Studies of protistan communities in a stratified anoxic fjord also found increased diversity at an intermediate depth within the pycnocline where density and chemistry was changing rapidly with

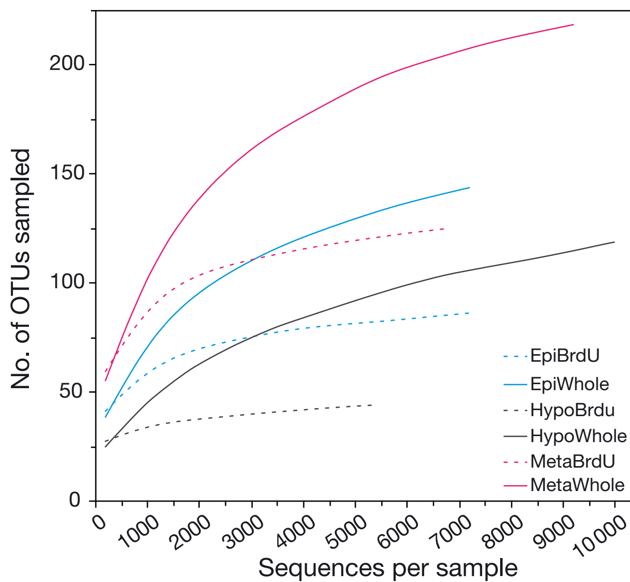

Fig. 3. Rarefaction curves of the 6 samples (mean of 20 values per step), showing the diversity of operational taxonomic units (OTUs). Data were sampled at 200 sequence increments up to 10^4 sequences. EpiBrdU: epilimnion bromodeoxyuridine immunoprecipitated (BrdU-IP), EpiWhole: epilimnion whole DNA, HypoBrdU: hypolimnion BrdU-IP, HypoWhole: hypolimnion whole DNA, MetaBrdU: metalimnion BrdU-IP, MetaWhole: metalimnion whole DNA

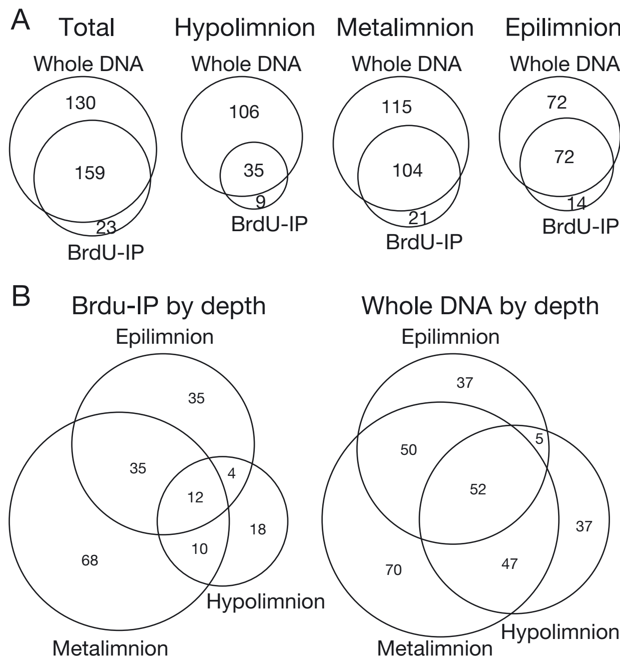

Fig. 4. Overlap in recovered operational taxonomic units (OTUs) between samples. (A) Comparison of OTUs identified from whole DNA samples and bromodeoxyuridine immunoprecipitated (BrdU-IP) samples for each depth sampled and for the combined lake community (total). (B) Overlap OTUs identified from all depths for BrdU and whole DNA samples

depth (Behnke et al. 2006). The metalimnion is a layer of high biomass and fine-scale habitat differentiation, and is therefore expected to have high biodiversity (Finlay et al. 1997). Additionally, suspended organic macroaggregates that are numerically enriched in both heterotrophic and phototrophic protists are common in the metalimnion of this lake (Caron 1991) and offer a different habitat from the surrounding water. The epilimnion is a mixed surface layer, without the gradient of nutrients, temperature, and oxygen typical of the metalimnion, and thus may have reduced range of habitat type. Finding the lowest diversity in the hypolimnion was expected due to its more 'extreme' environmental conditions with low oxygen (Fig. 1) and higher hydrogen sulfide as evidenced by its distinct odor (S.A. Fay pers. obs.). Photosynthetic protists also would be more rare in the low light hypolimnion (1.0 µmol s^{-1} m^{-2}). Despite the lower diversity in the hypolimnion, there was considerable overlap of OTUs identified from the layers in both BrdU-IP and whole DNA samples (Fig. 4B); <7 % and <21 % of the BrdU-IP and whole DNA OTUs, respectively, were unique to the hypolimnion. The proportion of OTUs shared between layers was always greater for the whole DNA than for BrdU-IP samples, regardless of which layers were compared (Fig. 4B). This likely reflects DNA from metabolically inactive cells or cysts that could sink through the water column that would have been identified in whole DNA samples, but not in samples labeled by feeding on BrdU-labeled bacteria.

Community composition

An analysis of community composition differentiating between the 6 samples using non-metric multidimensional scaling (NMDS) shows that the communities of eukaryotic microbes in the BrdU-IP samples are more similar to the communities of their respective whole DNA samples than they are to each other (Fig. 5). This pattern holds true using a variety of beta-diversity distance metrics available in PRIMER 6, including ones that disregard relative abundances (Jaccard), incorporate joint absences (Euclidean), and incorporate phylogenetic signal (Unifrac, both weighted and unweighted for relative abundances). The relative sequence abundance of higher taxonomic categories among the samples (Fig. 6) is consistent with the NMDS analyses. The finding of different protist communities at different depths is not unique to Lake Lacawac and in fact is likely a widespread phenome-

Table 1. Operational taxonomic units (OTUs) with the highest total number of sequences recovered. Protist taxa are ordered by the total number of sequences recovered. Included are taxa with >0.075% of total sequences recovered. Epi: epilimnion, Meta: metalimnion, Hypo: hypolimnion, Whole: whole DNA sample, BrdU-IP: bromodeoxyuridine immunoprecipitated sample, RDP: Ribosomal Database Project

No. of sequences recovered								Closest GenBank BLAST hit with >85% identity	BLAST identity (%)	RDP consensus lineage
Epi		Hypo		Meta		Sum				
BrdU-IP	Whole	BrdU-IP	Whole	BrdU-IP	Whole	BrdU-IP	Whole			
3	65	1831	10110	146	1281	1980	11456	*Cryptomonas*	100	Eukaryota
2452	4936	117	123	261	735	2830	5794	*Chrysosphaerella*	99	Eukaryota; stramenopiles; Synurophyceae; Synurales; Synuraceae
5	8	961	2535	10	52	976	2595	*Pelagothrix*	100	Eukaryota; Alveolata; Ciliophora; Intramacronucleata; Prostomatea
1196	212	1	10	791	1561	1988	1783	*Synura*	93	Eukaryota; stramenopiles
0	2	166	1365	18	32	184	1399	*Cryptomonas*	98	Eukaryota
6	5	0	66	27	715	33	786	Uncult. alveolate	100	Eukaryota; Alveolata
0	1	309	664	8	14	317	679	*Monosiga*	93	Eukaryota
0	0	0	0	959	539	959	539	*Uroglena*	93	Eukaryota; stramenopiles
5	32	0	4	291	454	296	490	Uncult. ciliate	98	Eukaryota
0	0	104	473	17	2	121	475	*Histiobalantium*	100	Eukaryota; Alveolata; Ciliophora; Intramacronucleata; Oligohymenophorea
112	20	5	92	164	301	281	413	Uncult. eukaryote	90	Eukaryota; Rhizaria
179	119	13	8	110	265	302	392	*Oikomonas*	91	Eukaryota; stramenopiles; Chrysophyceae
3	8	254	306	72	68	329	382	Ciliate	96	Eukaryota; Alveolata; Ciliophora; Intramacronucleata
453	151	0	21	899	209	1352	381	*Gonyostomum*	100	Eukaryota; stramenopiles; Raphidophyceae; Chattonellales; Vacuolariaceae
83	54	21	39	66	260	170	353	*Strombidium*	95	Eukaryota
186	48	54	287	0	8	240	343	*Arcuospathidium*	99	Eukaryota; Alveolata; Ciliophora; Intramacronucleata; Litostomatea
184	72	0	51	35	176	219	299	*Oikomonas*	89	Eukaryota; stramenopiles
0	0	174	252	23	2	197	254	*Athalamea*	89	Eukaryota; Rhizaria
89	163	1	2	1	74	91	239	*Chromulina*	89	Eukaryota; stramenopiles
0	7	51	223	1	4	52	234	Uncult. eukaryote	100	Eukaryota; Alveolata; Ciliophora; Intramacronucleata
8	6	0	3	101	218	109	227	*Monosiga*	87	Eukaryota
0	0	147	206	17	5	164	211	*Cryptomonas*	100	Eukaryota; Cryptophyta; Cryptomonadales; Cryptomonadaceae; Cryptomonas
179	184	0	1	1	18	180	203	*Uroleptus*	100	Eukaryota; Alveolata; Ciliophora; Intramacronucleata; Spirotrichea
142	17	0	2	322	175	464	194	Uncult. freshwater eukaryote	100	Eukaryota
0	4	27	85	49	79	76	168	Uncult. cryptophyte	98	Eukaryota
45	19	0	1	103	139	148	159	Uncult. lake picoplankton	100	Eukaryota
98	121	0	1	21	14	119	136	*Mallomonas*	100	Eukaryota; stramenopiles; Synurophyceae; Synurales
0	1	1	6	18	128	19	135	Uncult. ciliate	100	Eukaryota; Alveolata
205	90	0	3	31	24	236	117	*Peridinium*	99	Eukaryota; Alveolata; Dinophyceae
0	2	0	0	84	102	84	104	*Paraphysomonas*	99	Eukaryota; stramenopiles
0	6	1	0	1	95	2	101	Uncult. Katablepharidaceae	100	Eukaryota
0	0	42	97	0	1	42	98	*Antrodia*	81	Eukaryota
0	0	0	0	265	96	265	96	*Spongomonas*	88	Eukaryota
0	0	37	63	0	29	37	92	*Stokesia*	92	Eukaryota; Alveolata
14	3	270	76	0	5	284	84	Chlorellaceae	99	Eukaryota; Viridiplantae; Chlorophyta; Trebouxiophyceae
3	1	55	46	72	21	130	68	*Nolandia*	94	Eukaryota; Alveolata; Ciliophora; Intramacronucleata

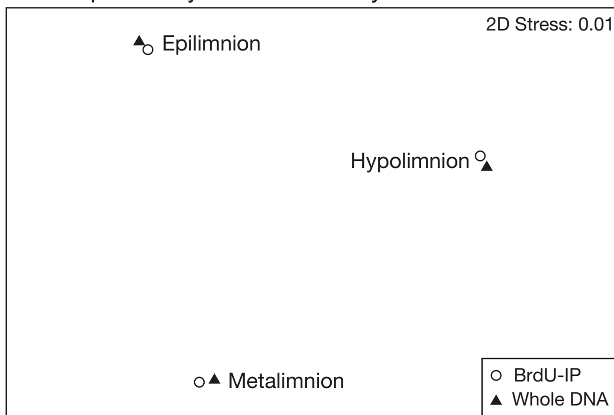

Fig. 5. Analysis of community similarity, showing non-metric multidimensional scaling (NMDS) 2D ordination of a Bray-Curtis community dissimilarity matrix for the 6 samples. Close proximity of data points indicate high similarity

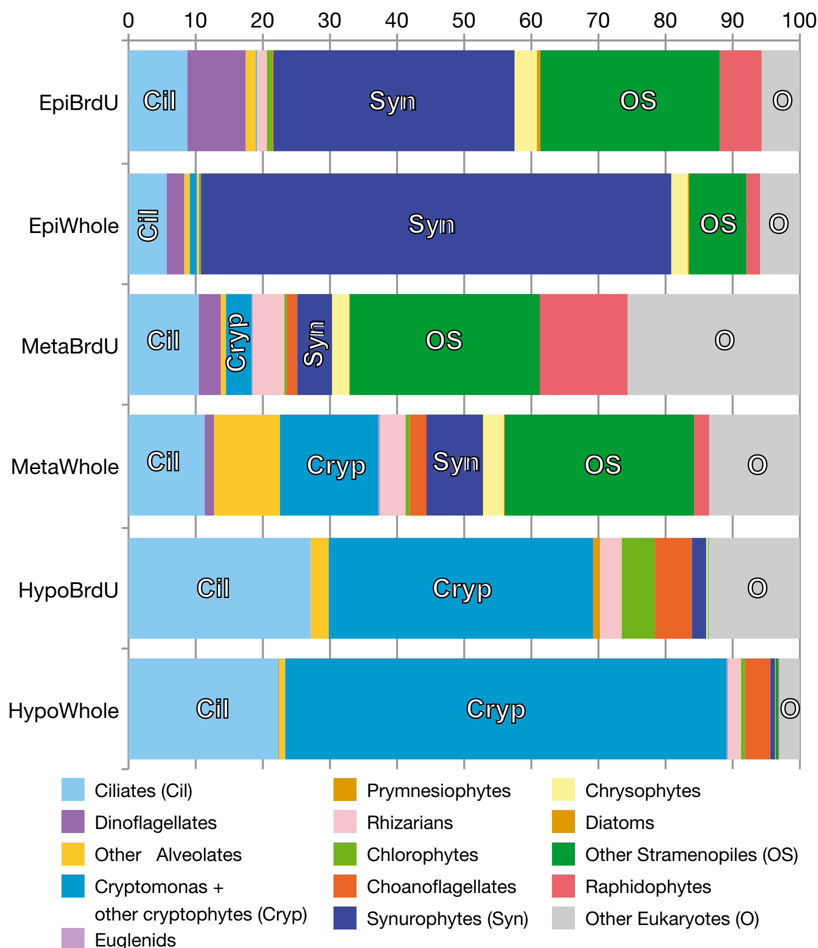

Fig. 6. Taxon plots, where bars show the relative abundance of sequences recovered from each higher-level taxon within each sample. EpiBrdU: epilimnion bromodeoxyuridine immunoprecipitated (brdU-IP), EpiWhole: epilimnion whole DNA, MetaBrdU: metalimnion BrdU-IP, MetaWhole: metalimnion whole DNA, HypoBrdU: hypolimnion BrdU-IP, HypoWhole: hypolimnion whole DNA

non, having been noted in both shallow ponds and deep lakes (Finlay et al. 1988, Müller et al. 1991).

The most abundant OTUs from whole DNA (Table 1) and those with >70% enrichment in the BrdU-IP samples (Table 2) come from a broad phyletic diversity of eukaryotes, including stramenopiles (particularly chrysophytes and synurophytes), alveolates (particularly ciliates and dinoflagellates), rhizarians, green algae, cryptophytes, and haptophytes. As indicated above, some groups showed especially large differences in their relative abundance at different depths (Fig. 6). Synurophytes, all of which have chloroplasts, are most common in the surface waters, while cryptomonads are more common in the hypolimnion (Fig. 6).

In Lake Lacawac, 9 of the 36 (25%) most abundant OTUs in whole DNA samples and 13 of the 32 (41%) most abundant OTUs in the BrdU-IP samples are most similar to uncultured organisms from environmental clone libraries or do not have any close homolog (≥85% sequence identity) in GenBank. This breadth of higher-level taxa from a single sample is known for several other planktonic systems, and a large proportion of the OTUs identified with molecular methods are often most similar to uncultured organisms (Luo et al. 2010). Note that close homologs to our sequences may have been recovered from other eukaryotic pyrosequencing projects, but tag sequences <200 bp unfortunately cannot be deposited in GenBank as per NCBI rules; unlike GenBank, the NCBI SRA can currently be searched only by project. Randa (2007) used a cloning procedure to identify bacterivores from 5 eukaryotic lineages (cercozoans, alveolates, stramenophiles, metazoans, and cryptophytes) that ingested BrdU-labeled bacteria in a coastal marine system. The majority of phylotypes labeled with BrdU in those experiments were also not closely related to previously identified protist species (Randa 2007).

Recovery of poorly studied taxa

There are apparently many bacterivores whose SSU rRNA genes have still never been studied. Compared to the whole DNA set of protistan taxa, a greater proportion of taxa labeled with

Table 2. Subset of taxa most highly labeled with bromodeoxyuridine (BrdU). Protist taxa labeled with BrdU, ordered by total number of sequences recovered. Included are taxa with >0.05% sequence abundance (>38 sequences) with >70% of the total number of sequences coming from BrdU immunoprecipitated (BrdU-IP) samples. Sum: total number of sequences recovered. Epi: epilimnion, Meta: metalimnion, Hypo: hypolimnion, Whole: whole DNA sample, RDP: Ribosomal Database Project

No. of sequences recovered							% seqs. from BrdU-IP	Closest GenBank BLAST hit with >85% identity	BLAST identity (%)	RDP consensus lineage
Epi		Hypo		Meta		Sum				
BrdU-IP	Whole	BrdU-IP	Whole	BrdU-IP	Whole					
151	453	21	0	209	899	1733	78	*Gonyostomum*	100	Eukaryota; stramenopiles; Raphidophyceae; Chattonellales; Vacuolariaceae
17	142	2	0	175	322	658	71	Uncult. freshwater eukaryote	100	Eukaryota
3	14	76	270	5	0	368	77	Chlorellaceae	99	Eukaryota; Viridiplantae; Chlorophyta; Trebouxiophyceae
0	0	0	0	96	265	361	73	*Spongomonas*	88	Eukaryota
54	208	0	0	12	2	276	76	*Mallomonas*	92	Eukaryota; stramenopiles
1	0	8	0	45	209	263	79	None	–	Eukaryota
6	84	4	0	12	74	180	88	None	–	Eukaryota
0	0	6	33	11	82	132	87	*Cryptomonas*	97	Eukaryota; Cryptophyta; Cryptomonadales; Cryptomonadaceae; Cryptomonas
27	72	0	0	5	17	121	74	*Trachelius*	98	Eukaryota; Alveolata; Ciliophora; Intramacronucleata; Litostomatea
0	0	12	100	1	0	113	88	*Bryophyra*	91	Eukaryota; Alveolata
16	43	0	0	13	41	113	74	*Synura*	100	Eukaryota; stramenopiles; Synurophyceae; Synurales; Mallomonadaceae
3	79	1	0	2	20	105	94	*Gymnodinium*	100	Eukaryota; Alveolata; Dinophyceae
1	98	0	0	0	0	99	99	*Chrysosphaerella*	92	Eukaryota; stramenopiles
3	94	0	0	2	0	99	95	Uncult. alveolate	95	Eukaryota; Alveolata; Dinophyceae
2	94	0	0	0	0	96	98	Uncult. *Gymnodinium*	95	Eukaryota; Alveolata; Dinophyceae
1	86	0	0	0	0	87	99	*Prorocentrum*	94	Eukaryota; Alveolata; Dinophyceae
7	68	0	0	4	7	86	87	Uncult. alveolate	94	Eukaryota; Alveolata
0	16	0	0	17	44	77	78	None	–	Eukaryota
0	0	0	0	13	57	70	81	*Spongomonas*	86	Eukaryota; Rhizaria; Cercozoa
0	0	0	0	3	65	68	96	*Urocentrum*	100	Eukaryota; Alveolata; Ciliophora; Intramacronucleata
8	0	0	55	3	0	66	83	*Chrysochromulina*	99	Eukaryota; Haptophyceae; Prymnesiales; Prymnesiaceae; Imantonia
0	0	0	0	16	44	60	73	None	–	Eukaryota
0	0	0	0	4	52	56	93	*Spongomonas*	89	Eukaryota; Rhizaria; Cercozoa; Cercomonadida; Spongomonas
0	0	0	54	0	0	54	100	None	-	Eukaryota
1	41	0	0	3	9	54	93	*Chlorellidium*	89	Eukaryota; stramenopiles
1	21	1	0	4	24	51	88	None	–	Eukaryota
0	49	0	0	0	0	49	100	*Mantoniella*	97	Eukaryota; Viridiplantae; Chlorophyta; Prasinophyceae
0	0	0	0	3	45	48	94	*Dinobryon divergens*	91	Eukaryota; stramenopiles; Chrysophyceae
0	0	0	43	0	0	43	100	None	–	Eukaryota
1	42	0	0	0	0	43	98	Unclassified ciliate	93	Eukaryota; Alveolata; Ciliophora; Intramacronucleata
0	0	0	0	9	34	43	79	*Vorticella*	92	Eukaryota
0	0	7	34	0	0	41	83	None	-	Eukaryota

BrdU were poorly described or rare species. For 8 of the taxa in the list of BrdU-labeled taxa combined for all depths, we found no match to a GenBank sequence with ≥85% identity, whereas the 31 most commonly recovered taxa from the whole DNA samples all had some match within at least 85%. These OTUs with no close homolog in GenBank are likely to be uncultured taxa. They may be more difficult to culture because they are fragile, small, difficult to distinguish as distinct taxa, or have specialized metabolic requirements. The relative proportion of unidentified OTUs found in the BrdU-labeled sequences suggests that BrdU is also useful for finding additional rare protist diversity.

Identification of bacterivores

We have taken a conservative approach to estimating those taxa that are truly BrdU-labeled and thus potentially bacterivorous. Factors such as non-linear PCR amplification and contamination from unlabeled taxa are possible, so only taxa with the highest proportion of BrdU-labeled versus whole DNA sequences recovered are listed in Table 1, i.e. taxa with >0.05% sequence abundance and >70% of the total number of sequences coming from BrdU-IP samples. We identify these taxa as putative bacterivores, either pure heterotrophs or mixotrophs. Confirmation of bacterivory as a trait may require continued experimental work in culture or field experiments. For example, the prasinophyte *Mantoniella* has not previously been reported as bacterivorous, but our results (Table 1), and the fact that our Antarctic *Mantoniella* isolate has been observed to ingest particles (McKie-Krisberg et al. 2011), suggest that more work should be done to examine bacterivory in this genus. Conversely, only a few diatom OTUs were recovered. A small proportion of the diatom sequences were in the BrdU-IP samples, and it is unlikely that these were bacterivorous. We see the approach taken in this paper as a hypothesis-generating mode of research, analogous to transcriptomic assays (such as microarrays), which identify promising genes (or in this case particular taxa) for further study of function.

Among the most frequently identified BrdU-labeled protists, 3 of the 5 top hits were phytoplankton; an uncultured eukaryote and a heterotrophic flagellate rounded out the top 5 bacterivores (Table 1). The most sequences recovered in the BrdU-IP samples were for the raphidophyte *Gonyostomum*. Although mixotrophy, i.e. the combination of photosynthesis and feeding in an individual, has not been demonstrated for this genus, several species of marine raphidophytes were recently identified as mixotrophic (Jeong 2011). The 2 next most recovered algae were not previously identified as bacterivorous either, but known mixotrophic genera that were BrdU positive in these experiments included *Cryptomonas*, *Chrysosphaerella*, *Chrysochromulina*, *Uroglena,* and *Dinobryon*. These data highlight the potential importance of bacterivory as a mode of nutrition for many microalgae (Sanders 1991, Jones 2000, Zubkov & Tarran 2008). It is possible that incubating the samples overnight enhanced phagotrophy in the mixotrophs, although darkness has no effect on some common mixotroph species (Bird & Kalff 1987, Sanders et al. 2001).

Some of the BrdU-positive dinoflagellate genera identified as bacterivores contain both heterotrophic and mixotrophic species (Sanders & Porter 1988). We do not identify these specifically as mixotrophs, although mixotrophy is well known for the group (Sanders 1991). Prasinophytes, previously identified as potentially bacterivorous by González et al. (1993), Bell & Laybourn-Parry (2003), and Sanders & Gast (2012), made up from ~1 to 5% and ciliates made up ~8 to 25% of the bacterivores from all 3 depths. Heterotrophic flagellates, including choanoflagellates, also were identified with BrdU at all depths (Fig. 6). A closer look at stramenopiles shows that, within this group, the 4 OTUs with the highest sequence abundance were *Chrysosphaerella, Synura, Gonyostomum*, and *Uroglena*. Over 60% of the sequences recovered for *Uroglena* were from BrdU-IP treatments, strongly supporting its ecological role as a bacterivore. Most stramenopile taxa found in this study fall within the chrysophyte/synurophyte group (their systematics remains in flux; Andersen 2007), and Fig. 6 emphasizes the fact that this taxonomically rich group of algae holds great opportunity for the comparative study of modes of nutrition.

Certain factors may confound the use of the BrdU method by identifying 'false positive' bacterivorous taxa. False positives may occur if osmotrophs take up free BrdU, and free BrdU may result from cycling of biomolecules. In axenic culture with 1 µM BrdU, the phototrophs *Nannochloris atomus*, *Gymnodidinium sanguineum*, and *Phaeodactylum tricornutum* did not incorporate BrdU directly from the medium, but the osmotrophic labyrinthulomycete *Schizochytrium aggregatum* did become labeled (Randa 2007). Even green plants can take up free DNA (Paungfoo-Lonhienne et al. 2010). Although bacteria are better competitors for the dissolved DNA than protists (Løvdal et al. 2007) and likely for BrdU as well, we recommend low centrifugation speeds, gentle methods for

dispersion of cells, and minimization of the time between washing/centrifugation of BrdU-labeled bacteria and their addition to environmental samples to minimize potential leakage of free BrdU or free-BrdU-labeled-DNA.

Non-protistan bacterivores can also be identified by this method. Though not recorded in the tables of most commonly recovered sequences, rotifers and copepods were appropriately identified as bacterivorous in the analysis. Predators of bacterivorous protists (including other protists) at higher trophic levels might also become labeled, again generating false positives. Since this requires time for ingestion of the labeled bacterivore followed by growth and incorporation into the secondary predator's DNA, minimizing incubation times is one way to ameliorate this problem.

An important assumption in this work is that bacterivores would not be identified if they did not incorporate BrdU into their DNA after ingestion. While not all bacteria take up thymidine or BrdU, little is known about incorporation of complete nucleotides by protists. Caron et al. (1993) found that ^{3}H-thymidine from labeled bacteria did accumulate in protists, but not quantitatively over periods longer than several hours; this implies that some of the nucleotide is broken down. However, Taylor & Sullivan (1984) found that when bacteria were labeled with ^{14}C-thymidine and fed to the ciliate *Euplotes*, respiration and exudation were less than in experiments using ^{14}C-glucose, implying that a consumer is more likely to use nucleotides in anabolic processes. Protists that failed to ingest the labeled bacteria for any reason, and those with very low feeding rates, could also lead to false negatives. Size-selective feeding by protists is well known (González et al. 1990), but this is most likely to be a problem if a strain of large bacteria is used and the grazers are very small (Sanders & Gast 2012). Future work with BrdU could be directed towards labeling the natural bacterial community to reduce the effect of prey size or type, although as noted previously, not all bacteria take up BrdU or thymidine. The data presented here and by Randa (2007) show that many protists incorporate ingested nucleotides directly into their DNA, and that this method can be utilized to gain an important perspective into the link between taxonomic diversity and function.

CONCLUSION

The large phylogenetic diversity of eukaryotic microorganisms is well recognized and ascribed to a large extent to uncultured species (Medinger et al. 2010). Consequently, the ability to assign functional importance to various groups in an aquatic food web is still limited. Culture-independent molecular methods, including the BrdU-IP method used here, can partition microbial taxa by function. Most other culture-independent studies involve stable isotope probing (SIP, reviewed by Gutierrez-Zamora & Manefield 2010), whereby microbes are fed with heavy stable isotope labeled substrates. Like any technique, SIP has its drawbacks. It can be difficult to achieve sufficient incorporation of the heavy isotope, especially with *in situ* levels of the substrate (Chen & Murrell 2010). Additionally, centrifugal separation of labeled nucleic acids is not complete, and background levels of template are present in all fractions (Lueders et al. 2004).

One advantage of using halogenated nucleotides like BrdU for labeling and separation by IP is that monoclonal antibodies have very high binding affinity and will bind regardless of the molecular weight of the antigen. Our study demonstrates the utility of combining BrdU-labeling and next-generation amplicon sequencing to study microbial trophic ecology. These data show that bacterivores from a small set of lakewater samples are distributed widely across the eukaryotic tree of life. Furthermore, many of the protists identified as bacterivores are algae. The occurrence of mixotrophic algae in pelagic ecosystem nutrient flux is now well established (Sanders & Porter 1988, Jeong et al. 2010). Culture-independent methods such as BrdU-labeling add to the toolbox available for studying natural protist communities, and models of aquatic microbial food webs must continue to incorporate and expand on knowledge of the functional diversity of the many unculturable protists, including mixotrophs.

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