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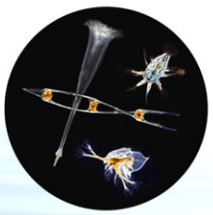

Featured Article

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Seed bank and seasonal patterns of the eukaryotic SAR (Stramenopila, Alveolata and Rhizaria) clade in a New England vernal pool

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Vernal pools are dynamic freshwater ecosystems that dry during the summer. These unique habitats are vital to a number of well-studied animal species but there is little documentation of the diversity of the SAR—Stramenopila, Alveolata and Rhizaria—clade in vernal pools. Here, we characterize the protist community over a portion of the hydroperiod as the vernal pool transitions from its winter stage through its drying out in late summer. Our study focuses on the SAR clade, which encompasses a broad range of morphological diversity and a variety of trophic modes within the microbial food web. Using high-throughput sequencing, we investigate the total community (DNA) and the active (RNA) members on a temporal scale. These molecular data reveal seasonality within microbial communities, suggesting a larger community of autotrophs in the winter followed by an increase in heterotrophs in the summer. Our analysis also suggests the presence of a microbial seed bank, a collection of encysted protists, in the sediments below the pool. We hypothesize the seed bank allows for community turnover: taxa encyst in the sediment in poor environmental conditions and exit their cysts when favorable conditions occur. We also observe seasonal preference and partitioning of the environment within clades of close relatives, including taxa closely related to the ciliate *Halteria* and the oomycete *Haptoglossa*. These data provide insights into the seasonal patterns of a frequently overlooked group of organisms in this unusual environment.

KEYWORDS: microbial eukaryotes; freshwater; high throughput sequencing; temporary forest pond

INTRODUCTION

Vernal pools are shallow freshwater ecosystems that undergo cyclic periods of dryness and are commonly found within temperate forests (Brooks, 2000). They are isolated pools that range in depth from a few centimeters to a few meters and are primarily fed by rainfall, rising groundwater and snowmelt (Brooks, 2000; Burne, 2001). These protected wetlands host a variety of diverse organisms from fairy shrimp to salamanders (Wiggins *et al.*, 1980; Burne, 2001). Vernal pools are subject to rapid changes in abiotic parameters due to their shallow depths and lack of consistent inputs (Bamforth, 1958; Bonner *et al.*, 1997; Carrino-Kyker and Swanson, 2008; Carrino-Kyker *et al.*, 2013; Simon *et al.*, 2016), which forces inhabitants to adapt to the routine period of dryness.

Studies that focus on the community of protists (microbial eukaryotes) in vernal pools are sparse (Stout, 1984; McGrady-Steed and Morin, 1996; Carrino-Kyker and Swanson, 2008; Simon *et al.*, 2015) despite their documented importance in aquatic food webs (Downing, 2010; Jardillier *et al.*, 2010; Zinger *et al.*, 2012). Protists are involved in carbon, nitrogen and silica cycling in aquatic habitats (Sherr and Sherr, 2002; Jardillier *et al.*, 2010; Foulquier *et al.*, 2014; Carrino-Kyker *et al.*, 2016), but their patterns of diversity remain to be elucidated on a broad scale. Existing studies of freshwater protist diversity focus primarily on lakes and other freshwater habitats (Müller *et al.*, 1991; Richards *et al.*, 2005; Lara *et al.*, 2011; Triadó-Margarit and Casamayor, 2012; Debroas *et al.*, 2015; Simon *et al.*, 2016) to the neglect of vernal pools, despite their status as nutrient-cycling hotspots (Carrino-Kyker *et al.*, 2011; Capps *et al.*, 2014).

Thus far, protist diversity in vernal pools has been almost entirely categorized by morphology (McGrady-Steed and Morin, 1996; Bonner *et al.*, 1997; Kuppers and Claps, 2012), leaving a disparity between taxonomic classification by morphology and estimates from molecular data (Savin *et al.*, 2004; McManus and Katz, 2009; Hirst *et al.*, 2011; Monchy *et al.*, 2012). Though powerful in identifying many groups, methods for morphological analyses are time consuming, need a high level of taxonomic expertise, and, nevertheless, can fail to distinguish cryptic species (Amann *et al.*, 1995; Šlapeta *et al.*, 2005; Rossi *et al.*, 2016), and often lack the resolving power for small and rare taxa (Amann *et al.*, 1995; Sogin *et al.*, 2006; Hajibabaei *et al.*, 2011; Zinger *et al.*, 2012; Debroas *et al.*, 2015; Simon *et al.*, 2015a, 2015b). Such limitations can be addressed by molecular analyses (Moreira *et al.*, 2002; Hajibabaei *et al.*, 2011; Mahé *et al.*, 2015; Hu *et al.*, 2016), yet molecular assessments of microbial communities in vernal pools have thus far been biased towards fungi and bacteria, and overlook the diversity of protists (Carrino-Kyker and Swanson, 2008). The SAR (Stramenopila, Alveolata and Rhizaria) clade, the focus of this study, represents a diverse range of eukaryotic microbes including influential and cosmopolitan clades such as ciliates (alveolates), diatoms (stramenopiles) and Cercozoa (Rhizaria) with numerous life strategies including autotrophy, heterotrophy, mixotrophy and parasitism of animals and plants (Burki *et al.*, 2007; Grattepanche *et al.*, 2018). By focusing on this clade with high-throughput sequencing (HTS) and morphological assessment of the three most abundant and diverse groups within SAR, we capture a broad range of ecological diversity to better understand community dynamics within the vernal pool.

Given their isolation and small sizes, vernal pools are subject to extreme abiotic changes (Bonner *et al.*, 1997; Carrino-Kyker and Swanson, 2008). This may lead to an emergence of encysted taxa from the seed bank and a resulting change in community dynamics (Galotti *et al.*, 2014). Inhabitants of vernal pools, protist or otherwise, have adapted to the routine environmental disruptions characteristic of the habitat. Many clades of protists are known to encyst, or enter a metabolically dormant state, in unfavorable environments while retaining the ability to "resurrect" upon the return of hospitable conditions (Fenchel *et al.*, 1997; Jones and Lennon, 2010; Lennon and Jones, 2011; Simon *et al.*, 2016). This process creates a "microbial seed bank" in the soil: a reserve of past genetic variants that can reappear and alter community dynamics when living conditions in the pool are favorable (Moon-van der Staay *et al.*, 2006; Lennon and Jones, 2011; Galotti *et al.*, 2014).

Here, we aim to assess the SAR community in a New England vernal pool as it transitions from its winter phase to its summer phase using HTS of amplicons generated with a SAR-specific primer that targets the V3 region of the small ribosomal subunit (SSU-rRNA). We also include a survey of easily recognizable and abundant morphotypes as a means of evaluating our HTS data. We consider DNA amplicons to represent the entire community, including the active members and the quiescent encysted seed bank. In contrast, RNA amplicons represent the metabolically active community. The coupling of these two types of amplicons gives insight to activity levels of individual taxa, and allows assessment of the presence of a microbial seed bank, as evidenced by quiescent taxa present in DNA but not RNA amplicons. Here we hypothesize that SAR taxa show (i) a seasonal succession related to the environmental conditions; (ii) rapid turnover of the

community including switch among clades of closely related taxa; and (iii) that part of the diversity from the water column is present in the soil (seed bank) at the end of the hydroperiod.

METHOD

Sampling

We collected water from two sites within Massachusetts Certified Vernal Pool #3391 (42°26′59′′N, 72°41′2′′W) on the Smith College MacLeish Field Station property (Whately, MA, USA). The vernal pool is about 60 m in diameter and made up of two basins, one smaller and more shaded during periods of foliage (Spring and Summer) and another larger with greater sun exposure, referred to as Sites 1 and 2, respectively. The sites are connected during the winter, but become separated by an internal wall in early summer as the pool dries. We collected two 4 L containers of water per site each sampling trip, roughly once a month over the span of 5 months until the pool began drying more rapidly in which sampling frequency increased (Table I). We adjusted our sampling procedure based on environmental conditions of the pool. In winter, we sampled the water column with a PVC pipe from beneath a sheet of ice to capture the whole water column (diameter of the pipe 10 cm). After the ice melted, we sampled water from the deepest portion of the pool. We prescreened the samples on-site using an 80 μm mesh to remove polymerase chain reaction (PCR) inhibitors and predators of protist. We recognize that our methods only permit us to capture organisms smaller than 80 μm, but we are also aware that some organisms, such as oblong or flexible organisms, are able to slip through the 80 μm pores. After the pools dried, we collected 25 g of soil material from sites within and between the basins. Air temperature and water temperature were recorded using a mercury thermometer.

Serial filtration

We filtered 4 L of water per site using a peristaltic pump, collecting organisms on 10 μm and 2 μm Nanopore™ polycarbonate filters [Millipore, MA, USA] to assess micro- and nanosize fractions, respectively. The water was filtered within 2–3 h of collection to best capture the *in situ* community. If the filters clogged with cells or sediment, we replaced the filter no more than twice. We divided the filters roughly in half based on the visual distribution of organic matter on the filter. Duplicate filters were collected for the 3 June and 10 June sampling trips in order to test the validity of the filter cut. One half was stored in 0.5 mL DNA preparation buffer [5.0 M NaCl, 10 mM Tris, 25 mM EDTA, 0.5% SDS, water, (Grattepanche *et al.*, 2014, 2016)] for DNA extraction and the other in 0.6 mL RLT Buffer [Qiagen] with β-mercaptoethanol for RNA extraction and subsequent cDNA synthesis. The DNA samples were stored at 4°C until their extraction, and the RNA samples were immediately stored at −80°C. For soil samples, we collected ~1 g of surface sediments and added 0.6 mL DNA preparation buffer and vortexed tubes to distribute the buffer throughout the sample.

Reverse filtration and enumeration

We used reverse filtration to concentrate cells for inverted microscopic enumeration to confirm the presence of the most common operational taxonomic units (OTUs) present in our molecular data. We concentrated ~2 L of prescreened water per site to roughly 35 mL using a 1 μm mesh to remove water. The samples were fixed with Lugol's fixative and stored at 4°C in the dark. We then used an Utermöhl Chamber to settle between 10 and 25 mL of fixed sample (Utermöhl, 1958). We divided the Utermöhl plate into eighths and counted morphotypes for roughly one-fourth of the plate. For a morphotype (dinoflagellates) that was too abundant to count efficiently, we enumerated two transects on the Utermöhl plate. We then converted the raw data into abundance (cells per liter). Morphotypes were categorized into three unambiguously discernable groups: ciliates, diatoms and dinoflagellates. Lugol's fix is known to produce artifacts that alter the morphology of many protists, and thus we were not able to perform a comprehensive morphological assessment at this time (Leakey *et al.*, 1994; Montagnes *et al.*, 1994; Stoecker *et al.*, 1994; Zarauz and Irigoien, 2008).

DNA, RNA extraction and cDNA synthesis

We extracted whole community DNA and RNA using the ZR Soil Microbe DNA MiniPrep™ extraction kit [Zymo Research, CA, USA], and the Qiagen RNEasy Mini kit [Qiagen], respectively, each following the manufacturer's protocol. We removed DNA from the extracted RNA with the TURBO DNA-free™ Kit [Invitrogen, CA, USA], and then generated single-strand cDNA using the SuperScript® III First-Strand Synthesis System [Invitrogen, CA, USA] with random hexamer primers [thermofisher, USA] to allow us to analyze total RNA.

Table I: Sampling and environmental parameters show substantial changes over the course of the study

Sample	Date	Site	GPS	Type	Season	Air (°C)	Water (°C)	DNA	RNA
Feb10_S1	2/10/16	S1	42°26′59″N 72°41′2″W	Water	Winter	3	0	✓	
Feb10_S2	2/10/16	S2	42°26′58″N 72°41′1″W	Water	Winter	3	1	✓	
Mar9_S1	3/9/16	S1	42°26′59″N 72°41′2″W	Water	Winter	15	4	✓	✓
Mar9_S2	3/9/16	S2	42°26′58″N 72°41′1″W	Water	Winter	15	4	✓	✓
Apr17_S1	4/17/16	S1	42°26′59″N 72°41′2″W	Water	Winter	14	4	✓	✓
Apr17_S2	4/17/16	S2	42°26′58″N 72°41′1″W	Water	Winter	14	4	✓	✓
May18_S1	5/18/16	S1	42°26′59″N 72°41′2″W	Water	Spring	15	12	✓	✓
May18_S2	5/18/16	S2	42°26′58″N 72°41′1″W	Water	Spring	15	12	✓	✓
Jun3_S1	6/3/16	S1	42°26′59″N 72°41′2″W	Water	Summer	21	17	✓	✓
Jun3_S2	6/3/16	S2	42°26′58″N 72°41′1″W	Water	Summer	21	16	✓	✓
Jun10_S1	6/10/16	S1	42°26′59″N 72°41′2″W	Water	Summer	19	13	✓	✓
Jun10_S2	6/10/16	S2	42°26′58″N 72°41′1″W	Water	Summer	19	13	✓	✓
Jun17_1	6/17/16	S1	42°26′59″N 72°41′2″W	Soil		26	n/a	✓	
Jun17_2	6/17/16	M	42°26′58″N 72°41′2″W	Soil		26	n/a	✓	
Jun17_3	6/17/16	S2	42°26′59″N 72°41′2″W	Soil		26	n/a	✓	
Jun17_4	6/17/16	S2	42°26′58″N 72°41′2″W	Soil		26	n/a	✓	
Jun17_5	6/17/16	S2	42°26′57″N 72°41′2″W	Soil		26	n/a	✓	

S1 = site 1, S2 = site 2, M = median between S1 and S2. n/a = not applicable.

Amplicon generation and HTS

We generated DNA and cDNA amplicons by PCR using Q5® Hot Start High-Fidelity DNA Polymerase [NEB, MA, USA]. For each reaction, we used 19 μL of master mix [13 μL H_20, 4 μL Q5 5× buffer [NEB, MA], 1 μL 10 mM bovine serum albumin, 0.4 μL dNTPs, 0.2 μL of each primer and 0.2 μL Q5 Hot Start High Fidelity polymerase [NEB, MA]] and 1 μL sample DNA. We used PCR primers that we designed to target a 150 bp hyper-variable V3 region of SSU rDNA of SAR lineages, with adapters for Illumina MiSeq High-Throughput Sequencing (Forward primer: 5′-TCGTCGGCAGCGTCAGATGTGTATAAG AGACA GAYTCAGGGAGGTAGTGACAAG-3′; Reverse primer 5′-GTCTCGTGGGCTCGGAG ATGTGTAT AAGAGACAGRACTACGAGCTTTTTAACTGC-3′; see supplementary methods for more details; Fig. S1). The PCR cycling conditions began with an initial denaturation step at 98°C for 2 min, followed by 28–37 cycles of 98°C for 15 s, 67°C for 15 s, 72°C for 30 s and a final extension at 72°C for 2 min. We used ~0.1 ng/μL of sample DNA as estimated by Qubit Fluorometric High-Sensitivity Range Quantitation (Thermofisher). We conducted each PCR in triplicate and pooled the PCR products to reduce PCR bias (Lahr and Katz, 2009; Jung *et al.*, 2012). We cleaned the pooled amplicons with Agencourt AmPure XP Beads (Beckman Coulter) to remove primer dimer. The University of Rhode Island performed Illumina MiSeq High-Throughput Sequencing on the 61 generated amplicons: 30 aquatic DNA amplicons, 26 aquatic RNA amplicons and 5 soil DNA amplicons (Table S1). The 30 aquatic DNA and 26 RNA amplicons include replicates for two size fractions and two sites sampled on five dates. The sequencing yielded 7 620 778 reads, with individual samples ranging from 54 351 to 194 936 reads.

OTU libraries

We merged paired-end reads using PEAR (Zhang *et al.*, 2013) with a 33 quality cutoff and minimum overlap of at least 120 bp. Reads with ambiguous bases were discarded. We built the OTU library with SWARM (Mahe *et al.*, 2015) at a distance of 1. OTUs that only had one read, chimeric sequences (uchime_denovo implemented in Usearch; (Edgar *et al.*, 2011)) and OTUs that were too dissimilar (<65% similar to the most abundant OTU using Water Local Pairwise Alignment implemented in EMBOSS package) were removed to reduce noise produced by HTS. We removed non-SAR sequences using a phylogenetic approach: OTUs were aligned within a curated full-length SSU rDNA SAR alignment (Grattepanche *et al.*, 2018) using MAFFT (Katoh and Standley, 2013) and the "add fragment" parameter. To build the phylogenetic tree and run the evolutionary placement algorithm (EPA; (Berger *et al.*, 2011)), a guide tree with the curated full-length SSU rDNA SAR alignment was built using RAxML (Stamatakis, 2014) with the GTRGAMMI parameter on CIPRES REST (Miller *et al.*, 2010). After removing outgroup sequences, we normalized each amplicon by rarefying at 61 000 reads. Cleaned amplicons with less than 61 000 reads are considered in their entirety; only two samples had fewer reads than our rarefying threshold (Mar9_S1_10R and May18_S1_10R with 43 962 and 30 717 reads, respectively). Our approach resulted in 10 131 OTUs with 2880 678 total reads over 61 amplicons.

Data analyses

The diversity of each sample is estimated using OTU richness, Chao1 index (to estimate the total diversity) and Shannon diversity index (to estimate the number of major contributors of the community; (Shannon, 1948)), and Faith's Phylogenetic Diversity Index (Faith, 1992) using an even subsample of 61 000 reads. For each OTU, we assigned taxonomy using two approaches: (i) a BLAST approach against the curated full-length SSU rDNA SAR database of 3460 full-length SSU sequences and (ii) a phylogenetic approach, where the OTUs were assigned to their closest sister on the tree. We used MEGA7 (Kumar *et al.*, 2016) to assign pairwise distance values to calculate similarity between the OTUs and their closest sister in our phylogeny. The OTUs with a pairwise distance greater than four (i.e. more than four substitutions excluding gaps, four has been defined arbitrarily) are referred to as "sister" to the reference taxa. For OTUs with smaller pairwise distances (i.e. ≤4 substitutions), we report the taxon name and number of substitutions.

To estimate the difference among our samples, we used Bray–Curtis (Bray and Curtis, 1957) and Unifrac dissimilarity indices (Hamady *et al.*, 2010; Lozupone *et al.*, 2011). The difference between Bray–Curtis and Unifrac dissimilarity indices is that the Unifrac dissimilarity index considers the phylogenetic relationship between OTUs in addition to abundance of each OTU. We assessed seasonality through principal coordinate analysis (PCoA) and canonical correspondence analysis (CCA), multivariate analyses that allow relation between community divergence (PCoA and CCA) and the species that drive the distinction between community clusters (CCA). The analyses were performed in R using the Phyloseq (McMurdie and Holmes, 2013) and vegan packages (Oksanen *et al.*, 2007). For these analyses, we considered two datasets: all OTUs for PCoA, and OTUs with more than 5000 reads total from the subsampled amplicons for CCA. We also assess the turnover (OTUs replacement over time) vs. nestedness (OTUs loss over time) of our data using the betapart package in R (Baselga, 2010; Baselga *et al.*, 2012).

We assess broad trends in diversity of major SAR clades by using read distribution as an approximation for species abundance. Read numbers do not reflect an absolute number of individual organisms, as many SAR protists (such as ciliates) are known to differentially amplify their genomes, particularly their SSU-rRNA genes (Baird and Klobutcher, 1991; DeBolt, 2010; Bellec and Katz, 2012; Gong *et al.*, 2013; Huang and Katz, 2014). By comparing the relative number of reads in a subsampled amplicon to that of other amplicons, we are able to observe changes in community structure and infer fluctuations in OTU abundance.

RESULTS

Environmental conditions

The physical characteristics of the pool varied through the season; the size and depth of the pool diminished noticeably with each sampling trip from roughly 30 cm in February to 0 cm on 17 June. Water temperatures ranged from 0 to 17°C (Table I). The physical properties of the pool also changed throughout the year: during the February and March sampling trips (Feb10 and Mar9, respectively) an 8–13 cm layer of ice covered the surface of the pool; by April (Apr17), the ice had thawed, though water temperature remained consistent with the previous sampling trips in March (4°C, Table I). By 3 June, the pool had split into two distinct lobes as water levels dropped and an elevated region of the forest floor (median, M) separated Site 1 (S1) from Site 2 (S2). By mid-June (Jun17), the pool was nearly dry, so we sampled dry soils and still-damp sediments from Site 2.

Diversity and community composition

The community diversity shows a complex pattern, with a greater number of major contributors in April and the soils, as assessed by Shannon index. No clear patterns are observed between nucleic acid type (DNA vs. RNA), size fraction (nanosize vs. microsize), and sampling site. The OTU richness ranges from 205 to 757 OTUs (average 445 ± 151 OTUs) and the total diversity estimated from abundance (Chao1 index) from 247 to 864 OTUs (average 518 ± 169 OTUs), except in the RNA samples at Site 1 for both size fractions in April (samples Apr17_S1_10R and Apr17_S1_2R), which show an OTU richness and total diversity two times higher (1693 and 1641, 1757 and 1715, respectively; Fig. 1, Table S1). Shannon indices, which ranged from 1.7 to 4.6, (average 3.2 ± 0.7) show greater values in April and in the soil samples, suggesting a higher OTU diversity (Fig. 1). Faith's index, a measure of phylogenetic diversity, adheres to the same pattern as the Shannon indices. The Faith's index ranges from 20 to 263 (average 56 ± 40). Much like what can be observed from the Shannon index, the Faith's index spikes in April and is higher in soil samples than aquatic samples. The cell abundance ranged from 500 to almost 18.10^3 cells per liter (Table II).

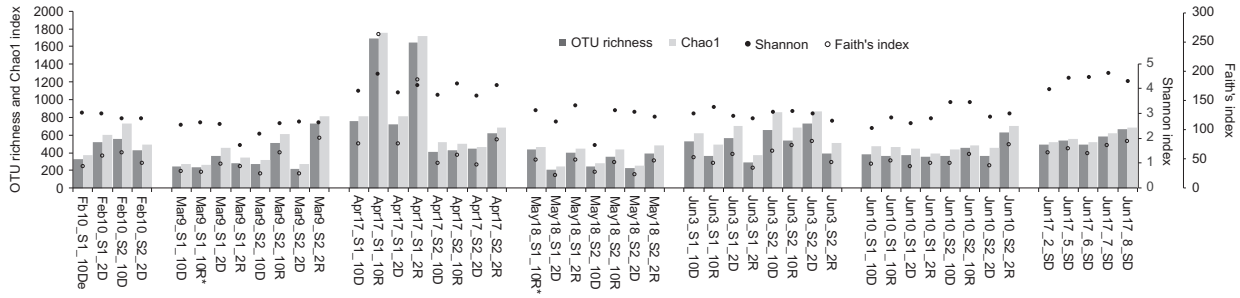

Fig. 1. Diversity pattern measured by OTU richness, Chao1, Shannon and Faith's indices indicates a relatively stable number of OTUs present at any given time. Samples marked with an asterisk (*) had fewer reads than our rarefying threshold (61 000) and are considered in their entirety.

Table II: Estimated abundance (cell L^{-1}) of major clades from morphological enumeration shows an increase of diatoms from winter to summer, a bloom of ciliates in May and a bloom of dinoflagellates in June

	Ciliate excluding *Halteria*	*Halteria*	Diatom	Dinoflagellate
Mar9_S1	79	201	153	106
Mar9_S2	3958	132	1041	5
Apr17_S1	80	161	1285	0
Apr17_S2	201	60	1667	0
May18_S1	133	592	2352	0
May18_S2	1078	219	762	0
Jun3_S1	30	148	533	473
Jun3_S2	244	266	1132	0
Jun10_S1	36	185	170	17503*
Jun10_S2	752	1	1182	0

Ciliate, *Halteria* and diatom categories were counted by enumerating one-eighth of the plate twice (total of one-fourth of an Utermöhl chamber). Dinoflagellate counts for Jun10 were estimated by raw counts from two diameters of an Utermöhl chamber converted into cells per liter (see Methods section).

By assessing changes in rarified read abundance, we infer that Stramenopila (48% of the total read number) and Alveolata (38%) are far more abundant than Rhizaria (14%) across all samples (Fig. 2, Fig. S1). Within Alveolata, ciliates within the Spirotrichea, Litostomatea and Colpodea classes are the most dominant groups throughout the course of the hydroperiod (Fig. 2). Oomycetes, Chrysophyceae and Synurophyceae dominate the stramenopiles (Fig. 2). Rhizaria are almost only composed of cercozoans (Fig. 2). The two size fractions, the two sites and the RNA and DNA show no clear particular pattern in read composition, except in the June water samples (particularly in 10 June samples) where Alveolata dominated Site 1, while Stramenopila dominate Site 2 (Fig. 2).

We find that some OTUs are present during the whole sampling period (i.e. are found at least once for each time point), suggesting that these species comprise a "common community" (Fig. 3 marked by *, Fig. S2). Examples of these OTUs include the stramenopiles OTU1 (2 substitutions from *Haptoglossa zoospora* KT257318) and OTU29 (sister to diatom *Asterionella glacialis* AY485447), the alveolates OTU3 (sister to *Halteria* sp. LN869995) and OTU13 (sister to *Playtophorya spumacola* KJ873051) and a rhizarian OTU53 (sister to cercozoan *Orciraptor agilis* KF207873; Fig. 2).

Seasonality

Aquatic vernal pool SAR communities exhibit clear patterns of seasonality. On a broad taxonomic scale, stramenopiles are more abundant during the winter and alveolates during the spring/summer (Fig. 2, Fig. S2). Within alveolates, while Spirotrichea are abundant during the whole study, the read distribution shows a switch from the dominance of Spirotrichea class during the winter and spring months to a dominance of Colpodea and Litostomatea classes in June (3 and 10 June; Fig. 2). We observe a similar composition change for the stramenopiles; Chrysophyceae dominate the winter and spring months while oomycetes are the most abundant Stramenopiles in summer months (Fig. 2). Based on CCA (Fig. 4A) and PCoA (Fig. S3), community samples cluster into distinct seasonal groups: winter (Feb10, Mar9, Apr17) and summer (Jun3, Jun10), with a transitional spring community (May18) falling between (Fig. 4A). Site 1 and 2 samples collected on the same date consistently cluster together until Jun10, when the two lobes of the pool have been separated for ~2 weeks (Fig. 4A). After this date, communities at Sites 1 and 2 are more distinct from one another (Fig. 4A, Fig. S3). DNA and RNA amplicons from the same samples also tend to cluster together (Fig. 4A, Fig. S3). The soil samples cluster separately from the water samples and exhibit the most divergence from one another, with Jun17_3_SD, a sample taken from the wet Site 2 sediments on our last sampling trip, falling closer to the winter aquatic cluster than any other sediment sample (Fig. 4A). PCoAs conducted with the Bray–Curtis dissimilarity index show similar patterns to the analyses done

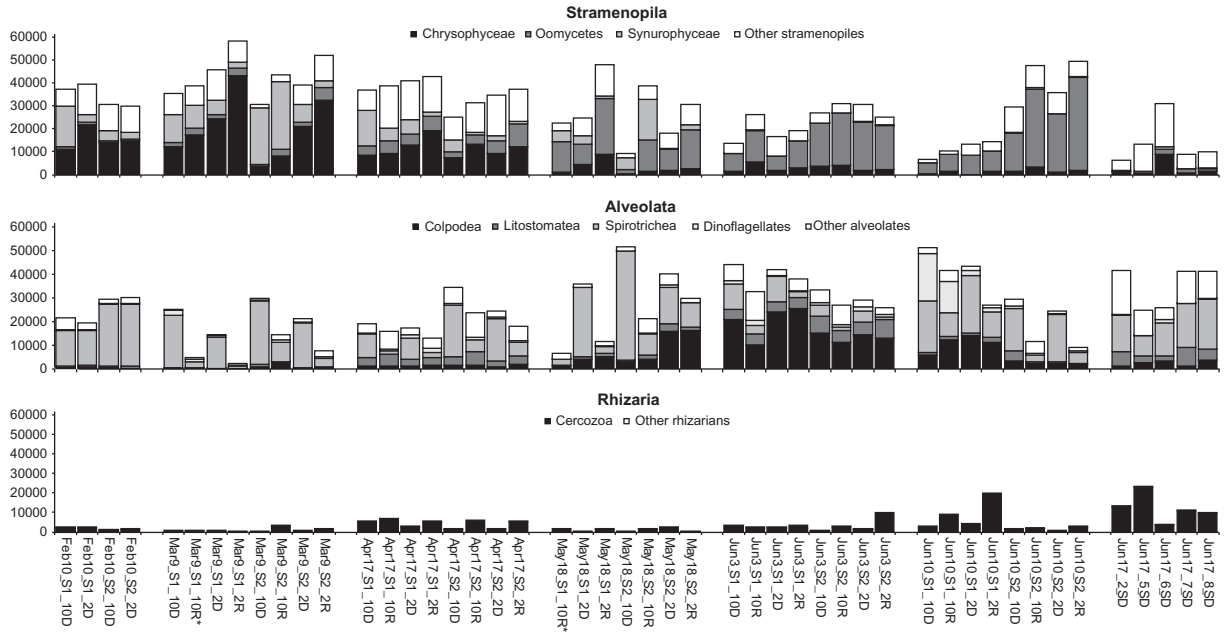

Fig. 2. Community composition patterns show seasonality as changes in rarified read abundance across the SAR lineages and within SAR during the sampling periods. Samples marked with an asterisk (*) had fewer reads than our rarefying threshold (61 000) and are considered in their entirety.

with Unifrac dissimilarity index: distinct winter and summer clusters, a transitional spring, and highly divergent soils (Fig. S3), with the Jun17_3_SD sample clustering with aquatic samples rather than other soils.

Analysis of CCA reveals the taxa that drive community patterns (Fig. 4B). Soil samples are largely influenced by three lineages: OTU73, OTU49 and OTU56, which fall sister to an early diverging stramenopile *Platysulcus tardus* (LC028904), and the ciliates *Coleps amphacanthus* (KU525296) and *Brachonella spiralis* (KF607090), respectively (Fig. 4B, Table S2). Drivers of the winter cluster include the ciliate OTU26 (two substitutions from an uncultured Strombidiidae EU024994), stramenopile OTU50 (sister to stramenopile *Xanthonema sessile* AM490818) and diatom OTU64 (sister to *Actinocyclus* sp. AY485506) among others (Fig. 4B). Influential summer taxa include OTU21 (one substitution from freshwater dinoflagellate *Tovellia aveirensis* KU359052), rhizarian OTU8 (sister to *Cercomonas alexieffi* AF411267) and the stramenopile OTU35 (sister to the diatom *Talaroneis posidoniae* AY216905). Two OTUs, OTU21 and OTU35, are nearly absent in the amplicons from winter and spring but abundant in summer (Figs 3 and 4B).

Seasonal preference in closely related lineages

A number of closely related OTUs show patterns that suggest seasonal preference between intraspecific variants or sister species that differ by 1–4 substitutions (Table S2, Table S3). A range of 1–4 substitutions was selected to account for variation within closely related lineages. For example, three abundant OTUs (OTU3, OTU4 and OTU25) that are closely related to the ciliate *Halteria* sp. (4, 3 and 1 substitutions from LN869995, respectively) show distinct seasonal patterns. OTU3 and OTU4, which differ from one another by 2 bp, are present over the course of the sampling period, but exhibit a seasonal preference as evidenced by CCA and read distribution: OTU3 is more abundant in the winter, whereas OTU4 is most abundant in the summer samples (Figs 4 and 5, Figs S4 and S5). Read distribution shows that OTU4 replaces OTU3 as the most common *Halteria* variant over the hydroperiod (Fig. 5, Fig. S5). OTU25 is markedly less abundant than either OTU3 or OTU4, but is most abundant during the spring compared to either winter or summer (Fig. 5, Figs S2 and S4). Similarly, three OTUs (OTU1, OTU5 and OTU24) that are closely related to oomycete *Haptoglossa zoospora* (3, 2 and 1 substitutions from KT257318, respectively) also exhibit seasonal patterns. All three OTUs are present throughout the hydroperiod (Figs 3 and 5, Figs S4 and S5), though differences in read distribution suggest a turnover of most common species variants: OTU5 in spring, OTU1 in summer and OTU24 almost exclusively in late summer (Fig. 5, Fig. S5). Our data suggest a turnover of the SAR community as opposed to nestedness, as variants replace one another as the most abundant OTUs.

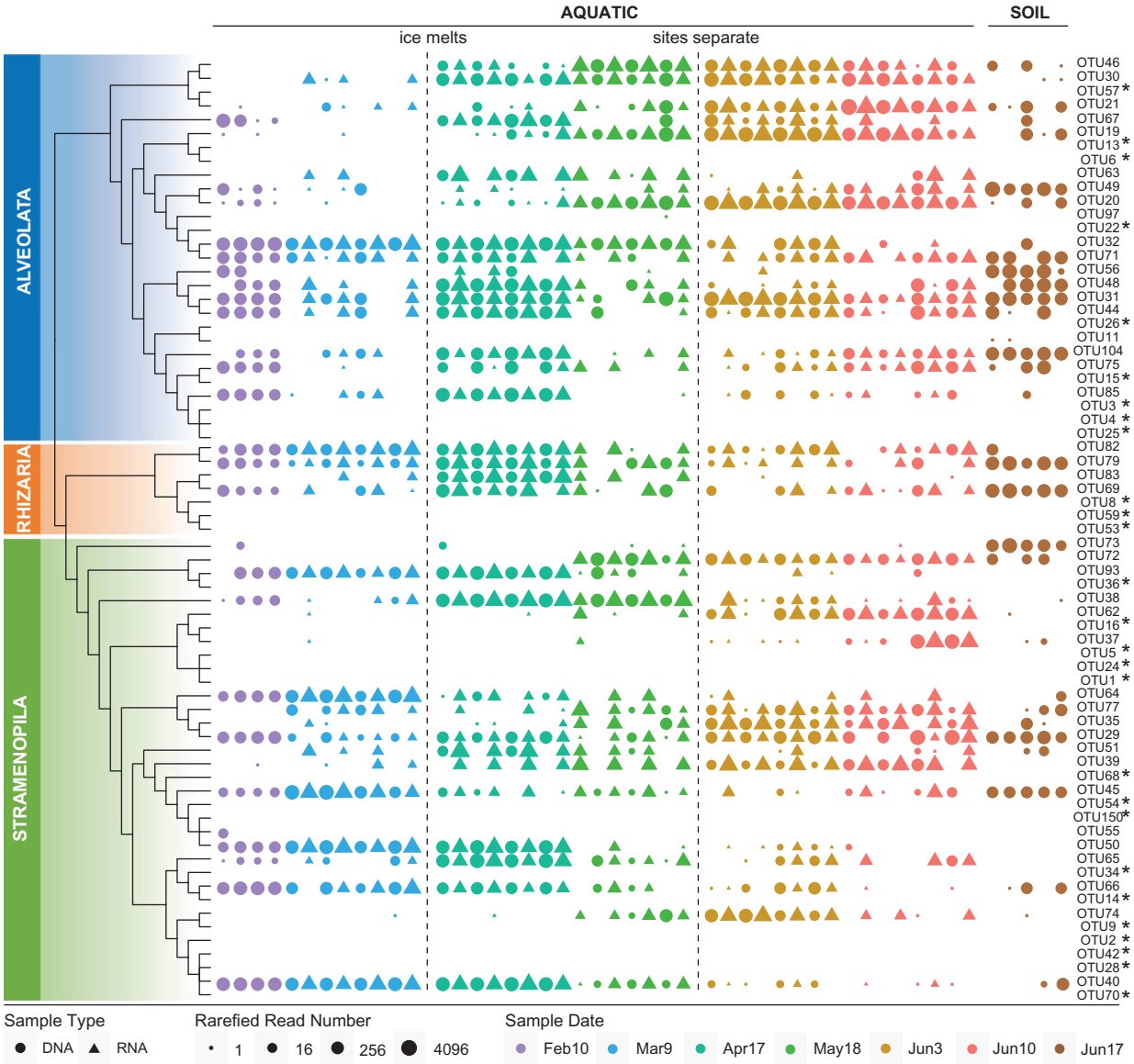

Fig. 3. Distribution of abundant OTUs reveals distinct seasonality in some lineages. The OTUs included at the far right are those with greater than 5000 reads. OTUs present at each sampling time are marked by * and we removed symbols for these to more clearly visualize seasonal patterns for the remaining OTUs (see Fig. S3 for full version). The presence in DNA (circle) but not RNA (triangle) suggests encystment (e.g. OTU75 on Apr17); conversely the presence in RNA but not DNA suggests high activity and rarity (e.g. OTU63, OTU39 from Mar9–May18). Dotted lines represent visible environmental changes to the vernal pool landscape: the melting of the ice layer and the separation of the lobes that make up Sites 1 and 2 by a small internal wall that appeared after drying.

Patterns in soils

Soil amplicons, which we sampled only for DNA, contain species that are unique to soils as well as those present in the aquatic samples. Some OTUs are abundant year-round in aquatic samples as well as soil samples (e.g. OTU1, OTU3 and OTU53); other OTUs are only present in winter and spring and then later reappear in the soil samples (e.g. OTU56, a ciliate sister to *Brachonella spiralis* KF607090, and OTU69, a cercozoan, sister to *Sphenoderia lenta* KF529410; Fig. 3). Other OTUs, such as the ciliate OTU48 (two substitutions from *Dileptus mucronatus* HM581675) occur sporadically during summer and winter but are abundant during particular months (Mar9) and in the soil (Fig. 3). Others appear to be "soil-specific" (e.g. stramenopile OTU73), in that they are nearly absent in the aquatic samples but abundant across all soil samples (Fig. 3). Rhizaria are more consistently present in soil amplicons than either alveolates or stramenopiles (Fig. 3).

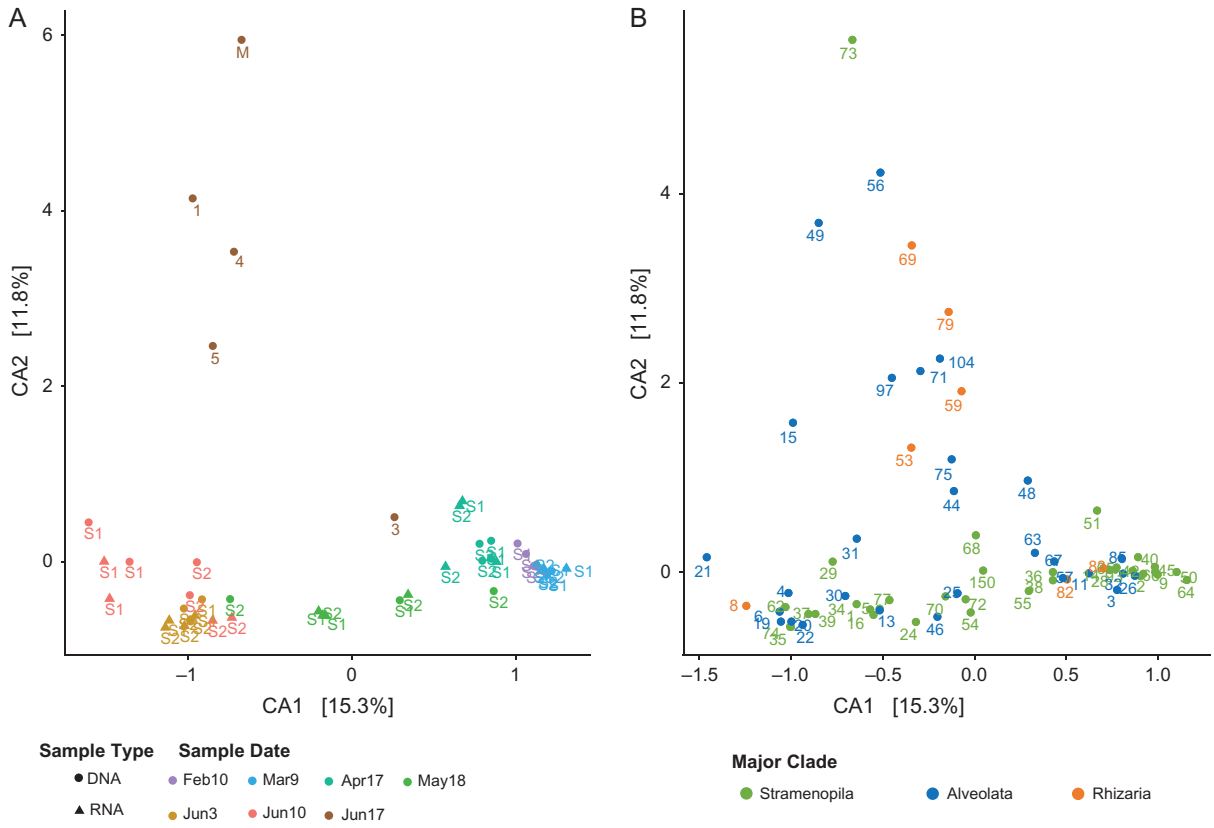

Fig. 4. CCA shows (**A**) seasonality in vernal pools and (**B**) the OTUs that drive seasonal distinction. Panel A represents communities, which cluster by season (winter, summer) with spring bridging between the two main clusters and soils being highly divergent. Panel B shows OTUs that drive community clustering, colored by major clade, with the bulk of stramenopiles (green) in appearing in water samples while rhizarians (orange) and alveolates (blue) present in both water and soil samples.

DISCUSSION

This study provides a number of insights into the composition of the microbial communities within an ephemeral environment: (i) the SAR community, as estimated by HTS sequencing, exhibits seasonal patterns in a New England vernal pool that are likely due to the rapid environmental changes associated with the transient nature of vernal pools; (ii) within these communities, some lineages exhibit seasonal variation; and (iii) combining these data with sediments sampled after the pool dried allow us to speculate on the presence of a SAR seed bank that may be able to sustain the SAR community and account for seasonal turnover, or replacement of OTUs.

Seasonality of communities

HTS of SAR communities reveals a distinct pattern of seasonality within the vernal pool. Previous studies based on morphology suggested an increase in α-diversity of microbial eukaryotes over time in vernal pools, with species richness peaking shortly after the filling period (late spring) (Bonner *et al.*, 1997; Kueppers and Claps, 2012). In contrast, our HTS data suggest α-diversity remains relatively constant in vernal pools (Fig. 1), though the most common taxa change with the seasons (Figs 1 and 2). The difference in average water temperature between the two seasons (3°C in winter, 15°C in summer) likely contributes to the observed seasonal patterns. Temperature is known to be a driver of succession and community structure for protists, creating a "cold" (fall/winter) and a "warm" (spring/summer) split in permanent freshwater environments (Lara *et al.*, 2011). In particular, temperature has been shown to drive ciliate communities in other ephemeral bodies of water, such as ponds and streams (Bonner *et al.*, 1997; Andrushchyshyn *et al.*, 2003).

Some OTUs exhibit a strong preference for a particular season and are likely drivers of the seasonal split present in our data. For example, OTU21, an autotrophic dinoflagellate, only appears in the summer months and in great abundance in both DNA and RNA profiles (Fig. 3). The molecular data reveal an increase in read

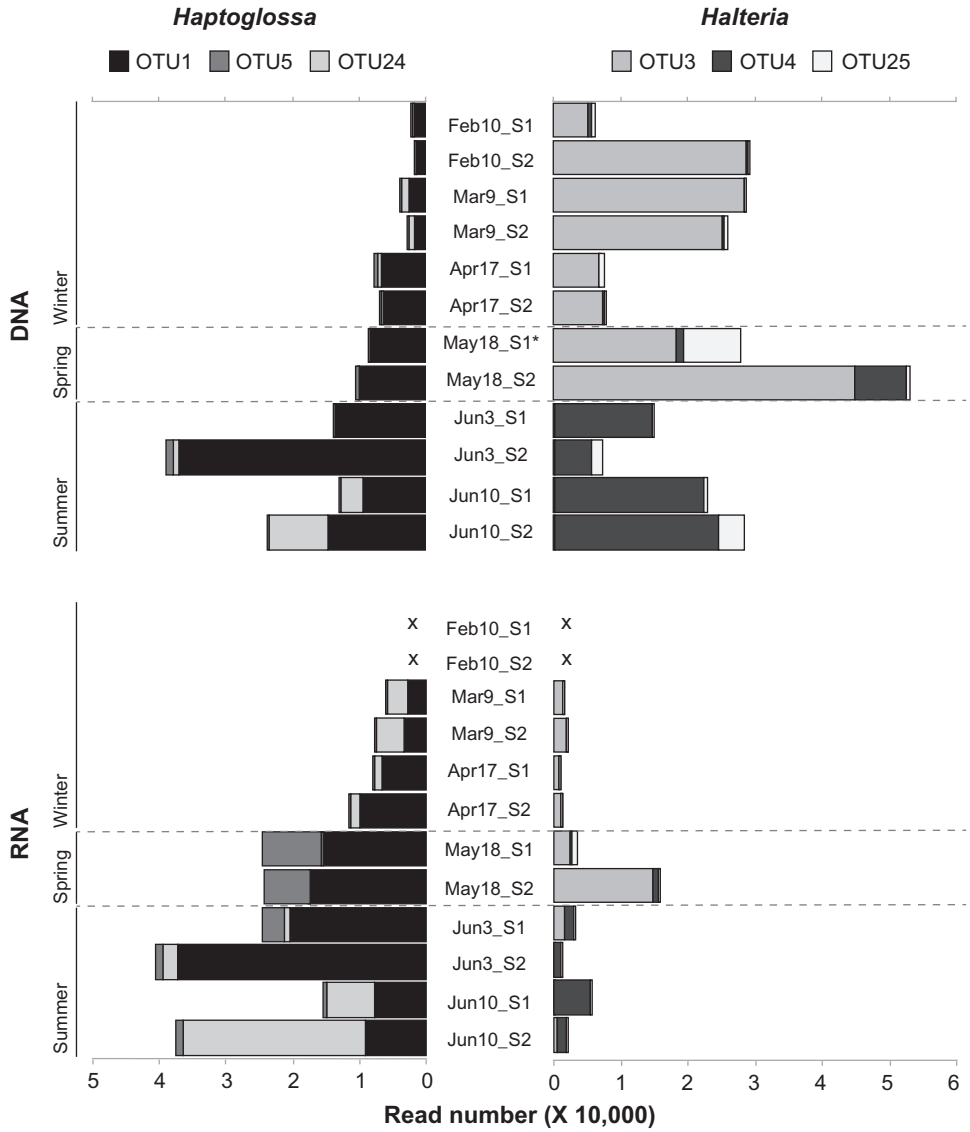

Fig. 5. Seasonal preferences of lineages closely related to the oomycete *Haptoglossa zoospora* and ciliate *Halteria* sp. Each color represents a distinct OTU, and each tick mark represents 10 000 reads. Asterisk denotes amplicons with fewer than 61 000 reads. Size fractions have been pooled. See Fig. S4 for read distribution across all samples and Table S3 for GenBank references and pairwise similarity.

number, suggesting an increase in cell count within the community. The observed molecular trend is confirmed by morphological enumeration, in which we saw a dinoflagellate bloom in the late summer samples (Table II). Conversely, OTU50, an autotrophic member of the "PX" stramenopiles clade, and OTU40, an autotrophic chrysophyte, exhibit a strong preference for winter and diminish in abundance with progression from winter to summer as evidenced by relative number of reads (Fig. 3).

Contrary to previous literature that suggested a primarily heterotrophic winter and autotrophic summer community (Bamforth, 1958; Finlay and Esteban, 1998; Debroas *et al.*, 2015; Simon *et al.*, 2015a, 2015b), we observe the inverse. Autotrophic taxa, including some chrysophytes and diatoms, are more abundant during the winter and heterotrophic taxa, such as oomycetes and ciliates belonging to the Colpodea and Litostomatea classes, are more abundant in the summer. This succession may be related to the nutrients in the winter which sustain the growth of phytoplankton even in environments with reduced light, while the temperature reduces the growth of heterotrophs. The numbers of autotrophs during the winter may act as a prolific food source for heterotrophic protists, and thus explain the relative increase in heterotrophic reads and species diversity to

increase as the season progresses. The strong contribution of heterotrophs during the summer months can also be related to the lack of metazoan predators such as copepods (Andrushchyshyn *et al.*, 2003). In the absence of many metazoan predators, ciliates may occupy this microbial niche, as lineages such as the classes Litostomatea and Colpodea are known grazers of algae and bacteria. Previous literature suggests the ciliate contribution to the microbial food web is on par with microbial metazoan predators (Beaver and Crisman, 1989; Weisse, 2006). Aside from ciliates, heterotrophic Rhizaria are also important consumers of bacteria and autotrophic protists (Simek *et al.*, 2000). In particular, the rhizarian *Orciraptor agilis* (OTU53 and OTU59) is a known grazer of green algae (Hess and Melkonian, 2013) and is present over the course of the hydroperiod (Fig. 3). The increase in heterotrophic Rhizarian reads such as OTU53 and OTU59 may reflect this predation, and thus contribute to the decrease of autotrophic lineages.

Closely related lineages vary temporally

Two groups of closely related lineages, one sister to the ciliate *Halteria* sp. and the other to the oomycete *Haptoglossa zoospora*, display seasonal preferences that suggest a partitioning of changing environments. *Halteria* are globally distributed (Simek *et al.*, 2000) and known to be present in temporal ponds year-round (Kueppers and Claps, 2012). This is consistent with our morphological data; we observed a readily identifiable morphotype similar to *Halteria* across our sampled hydroperiod (Table II). We were not able to discern *Halteria*-like morphotypes beyond the genus level. Our molecular data indicate that *Halteria*-like taxa are present and active at each sampling date but with marked seasonal variation: OTU3 is dominant in winter. The most common *Halteria* OTU switches to OTU4 in summer. OTU25, though represented by fewer reads than either OTU3 or OTU4, is most abundant in spring (Fig. 5, Fig. S5). This suggests that the three closely related OTUs (Table S2) might be responding differently to abiotic conditions. OTU3 decreases in read abundance as temperatures increase and the ice covering the vernal pool melts, and is replaced by OTU4 as the dominant variant by summer (Fig. 5, Fig. S5), which suggests these variants preferentially flourish under cold and warm conditions, respectively.

Different seasonal patterns also exist for three lineages closely related to the oomycete *Haptoglossa zoospora*: OTU5, OTU24 and OTU1. OTU5 appears most abundant in spring, OTU1 and OTU24 in summer (Fig. 5, Fig. S5). *Haptoglossa zoospora* is a known endoparasite of nematodes in freshwater systems (Hakariya *et al.*, 2002), and thus we were not able to observe this particular morphotype in our enumeration samples. The seasonal variation of these OTUs may reflect seasonal changes in the abundance of the nematode hosts. Nematodes in shallow freshwater systems have been shown to exhibit a seasonal preference for summer (Beier and Traunspurger, 2003a, 2003b; Michiels and Traunspurger, 2005), which may explain the spike in abundance observed in OTU1 and OTU24 (Fig. 5, Fig. S5).

Comparing DNA and RNA

The overarching seasonal patterns in DNA and RNA profiles are similar; however, differences between rDNA (ribosomal gene in the nucleus) and rRNA (ribosomal RNAs, mostly in ribosomes) allow us to evaluate the differences between abundance and activity for individual OTUs. On a seasonal scale, there is no major difference between DNA and RNA samples: diversity indices exhibit the same patterns in DNA as in RNA (Fig. 1), and DNA and RNA amplicons from the same sampling dates consistently cluster together when analyzed with dissimilarity indices (Fig. 4). The high level of rarified RNA reads of OTU 24 (Fig. S5) indicates a high level of activity, while those of DNA for the same taxa suggest rarity in the overall community. Conversely, samples with greater DNA rarified read abundance, such as OTU3, OTU4 and OTU25, may be present as cysts rather than metabolically active members of the community (Fig. S5).

Seed bank hypothesis

Turnover in the SAR community in vernal pools may be sustained by the presence of "seed banks" (cysts or spores in sediments). Seed banks also allow taxa to endure desiccation and proliferate upon refilling of the pools. Seed banks can contribute to the aquatic community at any point of the hydroperiod, allowing taxa that prefer warmer conditions to remain encysted through the winter and providing a mechanism for cold-weather taxa to endure summer conditions. We see certain taxa appear only in summer, such as dinoflagellate OTU21, suggesting they were encysted up until spring when conditions favorable to this taxon reappear (Fig. 3). The near absence of OTU21 is corroborated by the morphological data; the morphotype does not appear until summer and then is abundant in incredible numbers (Table II). Conversely, some OTUs appear present and active in winter, as evidenced by their presence in DNA and RNA amplicons, and are present in soil amplicons even though they are absent or rare in spring and summer (Fig. 3, Fig. S4). Such OTUs include OTU79 and

OTU69 (Fig. 3). These OTUs are likely members of the microbial seed bank and may have encysted as temperatures rose and the environment became less hospitable to these taxa.

Numerous SAR lineages, including ciliates, diatoms, dinoflagellates, oomycetes and chrysophytes, form resting cysts as a mechanism for long-term survival during unfavorable conditions (Elner and Happey-Wood, 1978; Fenchel *et al.*, 1997; Hakariya *et al.*, 2002; Bravo and Figueroa, 2014; Galotti *et al.*, 2014; Pandeirada *et al.*, 2014). The formation of resting cysts is also a common strategy for taxa to cope with habitat disturbance (Wiggins *et al.*, 1980). In vernal pools, taxa are forced to encyst or migrate prior to desiccation of the pool if they are to survive. This is consistent with the PCoA based on Unifrac index, which shows that the soil and aquatic samples share some members of the community (soil samples cluster between the summer and winter aquatic clusters; Fig. S3). In contrast, the PCoA with the Bray–Curtis index shows that the soil samples contain very different communities (OTUs set) than the aquatic samples. The main difference between Unifrac and Bray–Curtis is that Unifrac considers phylogeny, while Bray–Curtis treat each OTU independently (i.e. assumes star phylogeny). This means that sediments contain lineages closely related (Unifrac) but not identical (Bray–Curtis) to those found living in the water column, suggesting that soil contains a larger "bank" of phylogenetically similar taxa than what we have observed in the water column. For example, the sediments contained two ciliates that are well documented from aquatic systems: *Coleps* (OTU 49) and *Brachonella* (OTU 56).

CONCLUSIONS

Our study demonstrates three conclusions: first, SAR communities display distinct seasonal patterns that are likely a response to changing abiotic conditions in the vernal pool; second, the presence of a microbial seed bank as evidenced by dormant (i.e. inactive) taxa permits the observed seasonal turnover by allowing organisms to encyst and re-enter the community based on favorability of environmental conditions. Third, closely related linages, such as those lineages sister to ciliate *Halteria* and parasitic stramenopile *Haptoglossa* partition the environment by exhibiting seasonal preferences. HTS data provide thorough snapshots of communities and elucidate patterns at a fine scale. Our study demonstrates HTS is an effective method for assessing community composition and patterns on a temporal scale, and is strengthened by the verification of common morphotypes by inverted light microscopy. Finally, we hope that our 1-year study of a single vernal pool inspires future work to track patterns across multiple pools and multiple years with detailed, exhaustive morphological work, allowing for more robust conclusions to be drawn about the community composition, the ecological significance of turnover, and to marry molecular and taxonomic classifications. Additionally, we hope future work records other abiotic factors such as dissolved oxygen and sulfur coupled with an extensive HTS and morphological survey to further elucidate fine-scale changes in microbial communities (Andrushchyshyn *et al.*, 2003; Carrino-Kyker *et al.*, 2013).

SUPPLEMENTARY DATA

Supplementary data is available at *Journal of Plankton Research* online.

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DATA ARCHIVING

The raw reads are available from GenBank under the BioProject PRJNA472948 and Sequence Read Archive SRP149004.

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