ACTA
PROTOZOOLOGICA

Acta Protozool. (2015) 54: 265–273
www.ejournals.eu/Acta-Protozoologica
doi:10.4467/16890027AP.15.021.3535

Eukaryotic Microbial Communities Associated with Rock-dwelling Foliose Lichens: A Functional Morphological and Microecological Analysis

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Abstract. Lichens are widely recognized as important examples of a fungal-algal or fungal-cyanophyte symbiosis; and in some cases they are a major food source for some animal grazers such as caribou (*Rangifer tarandus*), especially in the Arctic during winter. However, relatively little is known about the ecology of their co-associated bacterial and protistan communities. This is one of the first reports of an analysis of microbial communities associated with rock-dwelling foliose lichens (*Flavoparmelia* sp.), including a more detailed analysis of the microbial communities associated with segments of the shield-like, radially arranged lobes. Samples were taken from lichens on granite boulders beneath an oak and maple tree stand on the Lamont-Doherty Earth Observatory Campus, Palisades, N.Y. The bacteria and protist members of the lichen associated microbial communities are comparable to recently reported associations for foliose lichens growing on tree bark at the same locale, including the presence of large myxomycete plasmodial amebas, heterotrophic nanoflagellates, and naked and testate amebas. To obtain evidence of possible differences in the microecology of different portions of each radial lobe, three segments of the radial lobe in the shield-like lichen were sampled: 1) inner, more mature, central segment; 2) middle section linking the central and peripheral segments; and 3) outer, peripheral, usually broader, less closely attached segment. The mean densities (number/g) and biomasses (µg/g) of bacteria and heterotrophic nanoflagellates were highest in the older central segment and lowest in the peripheral segment of the radial lobes, especially when expressed on moist weight basis. Large myxomycete plasmodial amebas were typically located in the outermost segment of the radial lobe. The proportion of vannellid amebas (*Vannella* spp. and *Cochliopodium* spp.) were significantly more abundant in the samples of the inner lobes compared to non-vannellid amebas that were more prevalent in the outer lobes. The outer segment of the thallus lobe was typically more spongiose and absorbed more water per unit weight (based on a wet/dry-weight ratio) than the innermost segment. In general, patterns of densities and taxonomic composition of bacteria and eukaryotic microbes intergraded from the inner most segment to the outer part of each lobe – indicating a possible microecological gradient, coincident with the age-related and morphological radial gradations of the lobe. Overall, the evidence shows that the radial variation in the morphology and age-related variables of the three lobe segments may affect the microenvironment of the lobe segments and hence influence the organization of the microbial communities within each segment.

Key words: Algal symbiosis, amebas, bacteria, carbon-biomass, *Flavoparmelia*, food webs, heterotrophic nanoflagellates, microbial communities.

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INTRODUCTION

Recently, there has been considerable progress in the study of microbial communities associated with lichens, particularly the bacteria, including molecular genetic analyses (e.g. Farrar 1976; States *et al*. 2001; Lawrey and Diederich 2003; Cardinale *et al*. 2006; Arnold *et al*. 2009; Grube *et al*. 2009; Hodkinson and Lutzoni 2009; Bates *et al*. 2011, 2012, 2012; Mushegian *et al*. 2011). However, there is less information on combined data for bacterial and eukaryotic microbial communities associated with lichens (e.g. Thompson *et al*. 1958, Beyens *et al*. 1986, Roberts and Zimmer 1990, Anderson 2014, Bates *et al*. 2012, Wilkinson *et al*. 2014). Lichens are of general ecological interest because they represent an interesting symbiosis between a fungus and usually an algal or cyanophyte symbiont, and in some cases both a cyanophyte and algal photobiont (Nash 2008a). Moreover, in some terrestrial locations they can account for a significant amount of biomass (e.g. McCune 1993, McCune *et al.* 1997, Clement and Shaw 1999, Berryman and McCune 2006), and in arctic locations they are a major winter food source for grazers such as caribou (*Rangifer tarandus*) (Edwards 1960). Lichens are categorized into three major groups: 1) crustose, that form thin, crust-like patches on the substratum; 2) foliose, that are broad, usually radially lobate and with a shield-like pattern of growth; and 3) fruticose, more twig-like and erect in growth. Some lichens are epiphytes on trees forming a wispy, filamentous growth.

This is an analysis of microbial communities, including bacteria and protists, associated with a rock-dwelling, foliose lichen (*Flavoparmelia* sp.) growing on the surface of boulders beneath the canopy of a stand of oak and maple trees in northeastern U.S.A. Lichens as miniature ecosystems have been recognized for sometime (e.g. Farrar 1976, Seaward 1988, Nash 2008b), especially in relation to the variety of photobionts, mycobionts, and invertebrates dwelling in the symbiotic association. Previous research at broadly different global geographic locations, including this one on the Lamont-Doherty campus of Columbia University, documented the organization of microbial eukaryotic communities associated with bark-dwelling foliose lichens, and their microecology (Anderson 2014). The results of that research showed that lichens from diverse geographic locales including Arctic, temperate North America, subtropical (Florida), northern Europe, and Australia, all exhibited similar patterns of bacterial and protistan community composition, including the presence of large plasmodial, myxomycete amebas. However, an interesting question remained to be examined, i.e. to what extent are there similar communities associated with rock-dwelling foliose lichens. The surfaces of rock clearly provide a very different substratum for lichen growth than tree bark, including surface texture, organic content, and more importantly water holding capacity, among other features. To broaden our knowledge of the possible affects of substratum on lichen microbial community composition, we have completed a study of rock-dwelling lichens comparable to that of the tree-bark study of Anderson (2014). Additionally in this study, we report the densities and composition of microbiota in three sections of each radially arranged lobe (Fig. 1): 1) inner, more mature segment; 2) middle segment; and 3) outer, never growing segment; and discuss the microbial variables in relation to the functional morphology of each segment of the lobe.

MATERIALS AND METHODS

Sample collection

Rock-dwelling foliose lichens were collected by gently cutting samples of dry thalli from rocks using a sterile razor blade. Samples were initially cut as strips of approximately 1.5 cm wide along the radial axis of each lobe. Then, each strip was further divided laterally to obtain sections from three regions of the lichen (Fig. 1): i.e. A) the well-attached inner section, B) the intermediary middle section, and C) the more loosely attached outer section. The sections were removed sequentially from the rock, beginning with the inner segment and proceeding to the outer segment to prevent possible cross-contamination of the inner lichen segments by the more active outer segments during the removal process. Ten lichen samples were collected in this way from an outcrop of large boulders, with relatively smooth surfaces and rounded contours, beneath the canopy of an oak and maple stand of trees on the Lamont-Doherty Earth Observatory campus, Palisades, New York (geographic coordinates: 41.004985, 73.907189). Samples were immediately sealed in glass vials and returned to the laboratory for analysis.

Sample analyses

Lichen taxonomy and alga symbionts. Lichen specimens were identified to genus level using an online key from the Natural History Museum, London (http://www.nhm.ac.uk/nature-online/life/plants-fungi/lichen-id-guide/index.dsml) and further confirmed by comparison to online images of each genus and in relation to published taxonomic descriptions. All of the samples were from *Flavoparmelia* spp. To identify the kind of photobiont associated with each lichen genus, a portion of the dried thallus (~5 mm^2) was placed on a microscope slide, moistened with 0.45 μm pore-size

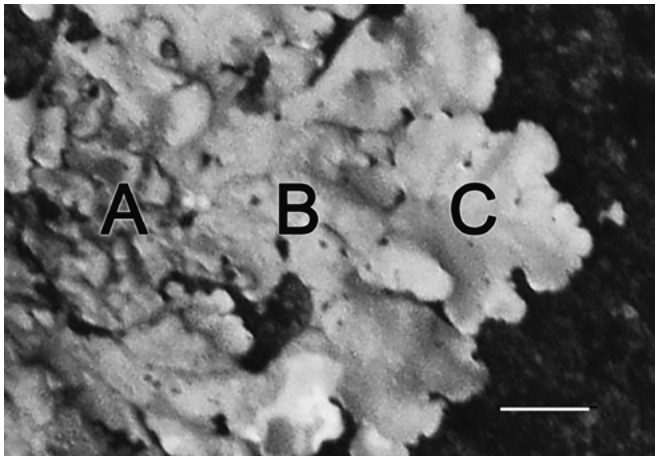

Fig. 1. Photograph of a portion of a *Flavoparmelia* thallus showing an example of a radially oriented lobe with three segments sampled in analyzing the microbial communities: A – inner, B – middle, and C – outer. Scale bar: 5 mm.

filtered distilled water and thoroughly macerated with a razor blade to expose and disperse the medullary hyphae and photobionts. The wet-mount slide, prepared with a type 0 cover glass, was observed with a Leitz Laborlux 11® compound microscope (Leica Microsystems, Inc., Buffalo Grove, IL), fitted with oil immersion optics (1,000 ×), using bright field. They were also examined by UV-fluorescence microscopy using the same microscope to better visualize the photobiont plastids by UV-excited red fluorescence. The size of the photobionts was measured with a reticule divided into 0.5 µm units. The symbionts were all green algae with size and appearance characteristic of *Trebouxia* sp.

Microbial densities and C-biomass. For enumeration and C-biomass estimates of the lichen-associated microbiota, each sample segment from the thallus while dry was weighed (~0.04–0.14 g dw), then moistened with a small aliquot of 0.22-µm filtered pond water, sufficient to fully wet the lichen sample. Each moistened sample was maintained at 25°C for 3 days to rehydrate the lichen and promote active microbial communities as per our standard procedure (Anderson 2014). Micropore-filtered pond water is used as a standard part of our laboratory observation methods to ensure that the water has sufficient natural mineral content to support the subsequent steps in the culture-based methods. The same source of pond water (Carolina Biological, Burlington, NC) was used for all preparations. After taking wet weights, the inner, middle, and outer segments of each sample were transferred to separate 15-ml conical centrifuge tubes and brought to 3-ml volume with 0.22-µm filtered water. The samples were macerated using the narrow portion of a flattened blade of a stainless steel spatula and suspended by gentle vortexing (e.g. Anderson 2014, Wilkinson *et al.* 2014). Two ml of the suspension was removed from each sample and fixed in 2% TEM-grade glutaraldehyde (Ladd Research Industries, Williston, VT) and placed in the refrigerator for subsequent bacterial and HNF enumeration. The remaining unpreserved portion was prepared for living ameba enumeration and C-biomass analysis using our standard microscopic culture observation method (COM) (e.g. Anderson 2002, 2008). In the COM method, a 20-µl aliquot of the suspended sample is placed in each well of a 24-well, sterile plastic culture dish, where each well contains 2 ml of 0.22-µm filtered pond water emended with a small cube of malt-yeast agar as a food source to support ameba proliferation. After 10–14 days, each well was observed using a Nikon Diaphot inverted microscope (Morrell Instruments Co., Melville, N.Y.) with phase contrast optics. The observation of a morphotype in each well indicated that at least one individual of that morphotype was present in the 20-µl aliquot deposited in the well. All 20 wells were examined and the number of each morphotype observed in the 20 wells (400 µl of total inoculum) was used to calculate how many would be contained in 1 ml of the original suspension. The number ml^{-1} was converted to density, i.e. number g^{-1} lichen dry weight (dw) and number g^{-1} lichen moist weight (mw). Each ameba was also sized using an objective reticule divided into 3 µm units. Bacteria and heterotrophic nanoflagellates (HNF), typically in a size range of 3–5 µm, were enumerated by fluorescent microscopy using acridine orange stain and an epiflourescence microscope with and oil immersion optics (1000 ×) and UV illumination (Anderson *et al*. 2001). Densities of the microbiota were expressed as number g^{-1} lichen dw and mw. The C-content of each group of microbes (amebas, bacteria, and HNF) was estimated using size-based regression equations as previously published (Pelegri *et al*. 1999; Anderson 2006, 2008). Biomass was expressed as µg g^{-1} dw and mw. To obtain moist weights, the sample of hydrated lichen was blotted using bibulous paper to remove all surface-absorbed water before weighing (Jahns 1984, Anderson 2014). In addition to the five samples used to enumerate microbiota, five additional samples of thalli (10 all-totaled) were taken for the purpose of investigating the relationship between moist weight and dry weight among the three different sections. The ratio of mw to dw for each section was used to estimate the mass of water absorbed, and expressed as (g mw – g dw) g dw^{-1}, i.e. gain in weight upon hydration in grams divided by the gram dry weight of the sample. Because the density of water is 1.0, we also were able to estimate the volume of the water absorbed as cm^3 g^{-1} dw, i.e. each g of water absorbed is equivalent to 1 cm^3 of water volume.

Thallus morphology and water absorption properties. To obtain a better estimate of lichen thallus functional morphology and changes during hydration of the inner and outer segment samples, in addition to visual evidence of differences in the gross morphology; the thickness of the thallus in each of these two segments was measured optically using a Nikon Diaphot inverted microscope and ocular reticule. A small section of each segment of a dry thallus was obtained by cutting a square section (ca. 5 mm on a side) from the segment using a fine razor blade. One side of the square was cut using the edge of a glass slide as a guide to ensure a clean cross-section of the thallus. The thallus section was secured perpendicularly between two glass slides resting on a large 5-cm square, no. 1 cover glass. Furthermore, the two slides securing the thallus section were supported on two other glass slides, thus providing a free space at the base of the thallus section where water could be applied freely during observations of the effects of hydration on the thallus. This assembly was viewed with the Nikon inverted microscope while illuminated from below with a high intensity fiber optic light source, so the cut surface of the lichen was clearly visible, allowing the microscopic features of the edge of the section to be examined and the thickness to be measured. It also allowed an estimation of the health

of the thallus based on the appearance of the hyphal filaments and presence of algal symbionts. The thickness of the hyphal layer and of the rhizine layer on the underside was measured separately for each one. After the dry thicknesses were obtained, a drop of ultrapure, distilled water was applied to the same lichen section, and it was observed as the water was absorbed. Changes in the thickness of the hyphal and rhizine layers were recorded when they became fully hydrated, and any changes in thickness reached a maximum value. At least ten measurements were made for inner and outer sections, and mean values of the thicknesses were obtained for the dry and the hydrated states. We particularly focused on the inner and outer segments, because they provided the most striking evidence of differences in the microbial community composition.

Statistical methods. A two-tailed t-Test was used to examine the statistical significance of differences in mean values for data comparing inner and outer segments of thalli. A paired t-Test was used to determine if there were statistically significant differences in the mean values of the dry and hydrated thallus sections, where measurements were made on the same section before and after adding water. All data used in the parametric statistical tests were examined to ensure sufficient normality of the distribution before applying the test. A chi-square test was used to determine if differences in frequencies of vannellid versus non-vannellid amebas in the inner and outer segments were statistically different. A linear Pearson correlation was used to examine the relationship between bacterial and heterotrophic nanoflagellates (HNF) densities, and bacterial and naked ameba densities. The data for these correlation analyses were examined using a scatter diagram to determine if the distribution of each pair of data was sufficiently linear to apply the Pearson correlation analysis. The critical value for all statistical tests was set at $p \leq 0.05$.

RESULTS

Microbial densities and C-biomass estimates. The mean densities and carbon biomass of bacteria, heterotrophic nanoflagellates (HNF), naked amebas, and myxomycete plasmodia from the inner, middle and outer segments expressed per g moist weight are presented in Table 1. The data are expressed as density and C-biomass per g moist weight to better represent the data under conditions where the microbes were growing while the lichen thalli were hydrated. The densities and carbon biomass of the bacteria and HNF are significantly greater in the inner segments relative to the outer segments (bacteria: $p < 0.05$, $N = 10$; HNF: $p < 0.01$, $N = 10$), with the middle segment taking an intermediate value in each case. Furthermore, myxomycete plasmodia were identified almost exclusively in the outer segments. There were insufficient numbers in the inner and middle segments to obtain a mean value (Table 1). The data for densities and C-biomasses expressed per g dry weight correspond to approximately the same set of relationships as for the moist weight, but of larger magnitude (Table 2). While no strong pattern was observed in the overall density of the naked amebas between the inner, middle, and outer segments, flattened, fan-

Table 1. Means ± S.E. for densities and C-biomass of bacteria, heterotrophic nanoflagellates (HNF), naked amebas, and myxomycete plasmodia isolated from inner, middle and outer segments of rock-dwelling foliose lichens based on moist weight of the lichen sample.[a]

Microbiota	Inner segment	Middle segment	Outer segment
Bacteria			
$\times 10^8$ g^{-1}	10.4 ± 2.1[b]	7.2 ± 2.8	3.5 ± 1.1[b]
µg C g^{-1}	27.2 ± 3.5[b]	19.0 ± 4.1	9.1 ± 2.5[b]
HNF			
$\times 10^6$ g^{-1}	14.6 ± 2.9[c]	9.2 ± 3.6	3.6 ± 1.0[c]
µg C g^{-1}	17.4 ± 2.8[c]	10.9 ± 3.1	4.3 ± 1.7[c]
Naked amebas			
No. g^{-1}	555 ± 124	694 ± 130	515 ± 158
µg C g^{-1}	0.10 ±0.04	0.09 ± 0.02	0.15 ± 0.07
Myxomycete amebas[d]			
No. g^{-1}	–	–	272 ± 48
µg C g^{-1}	–	–	151 ± 81

[a] Based on densities g^{-1} and C-biomass g^{-1} (moist weight) of the thallus segment. [b]Significant differences ($p < 0.05$). [c]Significant differences ($p < 0.01$). [d] There were insufficient numbers of myxomycete amebas detected in the inner and middle segments of the lichen segments to obtain a mean value during the five sampling dates.

Table 2. Means ± S.E. for densities and C-biomass of bacteria, heterotrophic nanoflagellates (HNF), naked amebas, and myxomycete plasmodia isolated from inner, middle and outer segments of rock-dwelling foliose lichens based on dry weight of the lichen sample.[a]

Microbiota	Inner segment	Middle segment	Outer segment
Bacteria			
$\times 10^9$ g^{-1}	4.22 ± 0.79	3.27 ± 0.94	2.90 ± 0.87
µg C g^{-1}	111.0 ± 20.7	86.0 ± 24.6	75.0 ± 22.6
HNF			
$\times 10^7$ g^{-1}	6.0 ± 1.14	4.38 ± 1.21	3.1 ± 0.88
µg C g^{-1}	71.5 ± 13.9	51.7 ± 14.3	36.7 ± 10.4
Naked amebas			
No. g^{-1}	2300 ± 469	3400 ± 546	4160 ± 1247
µg C g^{-1}	0.45 ± 0.2	0.44 ± 0.1	1.24 ± 0.6
Myxomycete amebas[b]			
No. g^{-1}	–	–	1127 ± 200
µg C g^{-1}	–	–	625 ± 335

[a] Based on densities g^{-1} and C-biomass g^{-1} (dry weight) of the thallus segment. [b] There were insufficient numbers of myxomycete amebas detected in the inner and middle segments of the lichen segments to obtain a mean value during the five sampling dates.

Table 3. Proportions and frequency counts in brackets of vannellid (Van.) and non-vannellid (N. Van.) naked amebas in the inner, middle and outer segments of the rock-dwelling foliose lichens[a], including means ± S.E. for the Shannon-Weaver diversity coefficients for amebas, exclusive of the myxomycete plasmodial amebas.

	Inner	Middle	Outer
Van.[b]	0.92 (0.81) [79]	0.71 (0.44) [95]	0.70 (0.55) [128]
N. Van.	0.08 [7]	0.29 [38]	0.30 [56]
Diversity	1.31 ± 0.25	1.86 ± 0.14	2.43 ± 0.15

[a] Chi-square for the data distribution = 16.81 ($p < 0.001$, df = 2). [b] Vannellids include *Vannella* spp. and *Cochliopodium* spp. Values in parentheses are the proportion of vannellids that are *Vannella* spp.

shaped, vannellid species occurred with significantly greater frequency ($p < 0.001$, df = 2) in the inner segment than in the outer (Table 3). Moreover, the diversity of ameba taxa was greater in the outer segment than either the middle or inner segments (Table 3). Overall, a wide variety of major ameba taxonomic groups were observed in the rock lichen samples, including some representatives most commonly observed as follows: *Acanthamoeba* spp., *Cochliopodium* spp., hartmannellids, mayorellids, *Thecamoeba* spp., vahlkampfids, and *Vannella* spp. Some smaller amebas in the range of 10 to 20 µm were not identified to genus level.

Correlation of bacterial prey with HNF and ameba predators. Because HNF are major predators on bacteria, we assessed the correlation of densities of HNF with those of bacteria for data from all three segments of thalli ($r = 0.72$, $N = 15$, $p < 0.01$). As expected in a trophic hierarchy, the HNF (principal primary predators on the bacteria) are highly correlated with bacterial densities. By contrast, the correlation of the densities of naked amebas with bacteria was much less significant ($r = 0.39$, $N = 15$, $p = 0.15$). Amebas, situated at a higher trophic level than HNF, are known to prey on a much wider group of microbiota than those of HNF, including small other protists, HNF and algae as well as bacteria.

Thallus morphology and water absorption. The thickness of the hyphal lamina for the inner and outer segments of the dry thallus measured with the inverted microscope were comparable (~200 µm), but after wetting, the mean thickness of the outer segment expanded to a mean of 254 µm compared to 213 µm for the inner segment ($p < 0.01$, $N = 20$). The rhizine layer of the outer segment also expanded upon wetting. The mean thickness when dry was 400 µm, but when hydrated it expanded to a mean of 450 µm ($p < 0.01$, $N = 20$). The rhizine layer on the inner segment was thin and sparsely distributed making it difficult to measure its thickness with the ocular reticule, so no data were obtained for effects of hydration. It also had a much thinner and powdery dark organic deposit in the tomentum on the lower surface. The differential increase in mean thickness of the hyphal lamina in the outer segments relative to the inner segments is also reflected in the mean ratio of the moist weights to dry weights (mw dw^{-1}) of these two segments, i.e. outer segment (6.14) and inner segment (3.71) ($p < 0.01$, $N = 20$). Based on the weight gain after hydration, the mean ± S.E. for the volume of water absorbed expressed as $cm^3\ g^{-1}$ dw was 5.13 ± 0.66 for the outer segment compared to 2.71 ± 0.22 for the inner segment ($p < 0.01$, $N = 20$). Overall, these data indicate that the outer segments of the thalli appear to be more spongiose, absorb more water per unit dry mass, and possess a more substantial rhizine lower layer (including more abundant rhizinae and dark organic matter within the tomentum) that traps more water compared to the inner segment. These differences in functional morphology may explain partially some of the differences in microbial data between the inner and outer segments of the thalli.

DISCUSSION

A substantial amount of research has been published on the biology of lichens (e.g. Nash 2008a), including ecology and physiology across widely varied geographic locales (e.g. Pitt 1927, Farrar 1976, Harrisson *et al*. 1989, Huiskes *et al*. 1997, Palmquist *et al*. 2002, Gausiaa *et al*. 2007, de Vries and Watling 2008, Armstrong and Bradwell 2011, Johansson *et al*. 2012). However, less is known about the ecology of bacterial and protist communities associated with lichens (e.g. Thompson 1958; Roberts and Zimmer 1990; Cardinale *et al*. 2006; Bates *et al*. 2011, 2012; Anderson 2014). This is one of the first studies of the microbial communities associated with rock-dwelling foliose lichens, especially focusing on likely microecological correlates of thallus functional anatomy. Collection of foliose lichens from rock surfaces offers some advantages compared to collection from bark. The lichens are more easily and entirely separated from the rock with little or no remnant of rock material;

whereas, lichens growing on bark are usually much more closely integrated with the bark surface, and collection usually involves some adhering bark material. The composition of microbial communities reported here are similar to those found for bark-dwelling lichens at the same locale (Anderson 2014), including the presence of bacteria, heterotrophic nanoflagellates, amebas and large myxomycete plasmodial amebas. However, overall the densities and biomass expressed on a dry weight basis of bacteria and heterotrophic nanoflagellates were approximately ten times larger than found with the tree bark-dwelling lichens. The density and C-biomass values, expressed per thallus moist weight, were relatively similar to the dry weight data, but lower in value due to the additional weight contributed by the bound water in the thallus sample. The boulders where the lichens were sampled were located in the same grove of trees where the tree bark lichens were collected for the northeastern locale by Anderson (2014). Some of the differences in abundances of bacteria and heterotrophic nanoflagellates between rock-dwelling and bark-dwelling lichens may be attributed partially to differences in the microclimate of the lichens, particularly orientation and disposition relative to insolation, water retention, etc. Lichens growing on the trunks of trees are oriented more vertically to the ground, and hence more subjected to effects of gravity, thus possibly incurring more rapid water loss due to drainage. This may lead to earlier water stress compared to rock-dwelling lichens, where water may be retained sufficiently to support physiological functions, without effects of water supersaturation that can restrict CO_2 diffusion and lead to suppression of algal photosynthesis (e.g. Büdel and Scheidegger 2008). Also, the bark dwelling lichens are less likely to receive favorable solar insolation than lichens attached to the surface and sloping sides of boulders where they are growing with their surfaces more perpendicular to incident illumination. The rock-dwelling lichens appeared gray-green as indicative of more highly illuminated lichens (Büdel and Scheidegger 2008). Further research is needed to determine the generality of these differences across wider environmental conditions and geographic locales, including physiological and growth measurements within the same lichen species on rock surfaces and those on tree trunks and limbs to determine if rock surface lichens are more productive and less stressed in a given environment compared to bark-dwelling lichens.

Major differences were observed in the microbial community composition among the three segments of the radial lobes of the rock-dwelling *Flavoparmelia* thalli investigated in this study (Fig. 1). Bacterial densities and C-biomass were greater in the inner, more mature segment compared to the outer segment of each lobe, while larger, myxomycete plasmodia occurred predominately in the outer segment. The densities and C-biomass of naked amebas were not so different across the three lobes, but there were substantial differences in the taxa. Flattened, fan-shaped vannellid amebas were more commonly observed in the inner segment, while non-vannellid species including more lobate amebas, occurred in the outer lobe segment. Diversity of taxa was greater in the outer lobe segment than the inner segment. It is clear that the innermost segments of a lobe are the more mature, while the outer, recently growing segments are younger in age. The outer segments also are more expanded and the margins often are less closely attached to the rock surface. Cardinale *et al*. (2012), studying different lichen species than ours, also reported that more mature segments have higher bacterial densities than younger ones. Moreover, Ellis *et al.* (2005) using ^{15}N-labeled ammonium, nitrate or glycine as nutrients reported that the labeled compounds introduced in the older portions became translocated into the younger portions of a mat-forming lichen *Cladonia portentosa*, thus possibly indicating nutrient recycling from the older part into the younger part of this lichen, perhaps mediated partially by bacterial remineralization in the older segments. The potential role of protist grazing on bacteria in mediating nutrient recycling also was suggested by Wilkinson *et al.* (2014).

It is not immediately clear how differences in maturity, including differences in longevity of the three lobe segments, may affect the observed microbial patterns of distribution. However, the comparative morphology of these three segments of the thallus may be more significant, especially in relation to water holding capacity. Currently, water relation in lichens is not fully understood, but there is good evidence that much of the absorbed water is accumulated in the hyphae, leaving gaseous, perhaps more hydrophobic spaces among the hyphal strands (e.g. Jahns 1984, Honneger *et al*. 1996). Water translocation within the thallus is driven largely by water potential gradients, especially during wetting and drying (Honneger 1991, 2008). In our preparations, the thallus inner segment was green and capable of absorbing water. It became noticeably greener upon hydration as is typical of healthy hydrated thalli. The inner segment is generally more crustose and has a thinner and more powdery tomentum on the lower cortex and rhizine layer than the middle and outer segments. The middle segment

exhibits a morphology intermediate between inner and outer segments. Our data indicate that the outer lobe segments absorb more water per unit dry weight than the inner segment, and are typically more spongiose and pliable after hydration compared to the hydrated innermost segment. Part of this difference can be explained by the greater expansion of the hyphal and rhizine layers during hydration of the outer segment, thus accumulating more water per unit weight than the inner segment. This larger water holding capacity, coupled with a much more robust and open porous rhizine layer on the lower surface of the outer segment, may provide a more favorable environment to support communities of larger amebas, including plasmodial myxomycetes. The inner layer with a less robust rhizine layer appears to be more closely attached to the rock surface, and among other factors may explain the greater presence of amebas with a flattened or fan-shaped morphology that can invade and flourish in the thinner available space.

The greater abundances of bacteria and heterotrophic nanoflagellates in the inner segment may be due to less competition by amebas, especially larger ones, for bacterial prey. A significant, positive correlation (combining all data from the three segments) between bacterial prey and HNF, one of the primary bacterial predators, is consistent with previous findings for foliose lichen communities on bark (Anderson 2014). The correlation of ameba densities with bacteria was substantially less than the correlation of HNF with bacterial densities, probably due to the amebas being situated at a higher level of the trophic hierarchy, and consuming a broader range of prey beyond bacteria. The presence of more abundant myxomycete plasmodia in the outer segments of each thallus, coincident with a lower density of bacteria, may be attributed to the high predation pressure of the combined suite of bacterial predators, especially the contribution of the very large myxomycete plasmodia.

Previous research in various geographic locations has reported the occurrence of myxomycetes on rocks, where they are associated with algae, bryophytes and lichens (e.g. Kukwa 2005, Schnittler *et al*. 2010, Edinger 2013). However, this study appears to be the first report of the association of myxomycetes within microbial communities associated with rock-dwelling lichens, and particularly in relation to the functional morphology of the lichen thallus. Further studies are needed to clarify the taxonomic status of the myxomycetes and their possible life cycle while associated with the lichen and its microbial communities. The amount of C-biomass of myxomycetes contributed to the total biomass of the microbial community is substantial, averaging ca. 150 μg g^{-1} moist weight and 1,400 μg g^{-1} dry weight of lichen thallus. The latter is substantially higher than the largest value reported for amebas and myxomycetes associated with bark lichens (ca. 500 μg g^{-1} dry weight) reported by Anderson (2014). While myxomycetes are known to prey on a wide range of microbiota (including bacteria, fungi, small algae, and smaller heterotrophic protists) and perhaps detrital particles (Anderson 2014), further research is needed to more fully establish their place in the food webs of microbial communities associated with lichens in general, and particularly with rock-dwelling lichens. Overall, this research adds to a growing body of literature on the microecology of lichens, and provides additional evidence of the role or eukaryotic microbes as co-inhabitants of the lichen complex micro-communities. Additional research is needed to decipher the complexities of sources of nutrients supporting photosynthesis at the base of the heterotrophic food webs within this complex trophic system. This includes the significance of cyanobacteria, when present, as a source of fixed nitrogen to support the lichen microecosystem (e.g. Nash 2008c, Palmquist *et al*. 2008), either as symbiotic photobionts or epibionts in bacterial biofilms growing on the thallus (Honegger 2008). Further investigations are needed on the fate of organic compounds derived from the lichen symbiosis that are consumed by the bacterial and eukaryotic microbes, and how these C-sources vary in relation to different environments and climatic conditions where the lichens are growing.

Acknowledgements. This work was partially supported by funds from the Department of Mathematics, Science and Technology at Columbia University Teachers College, where N. Bock is a graduate student. This is Lamont-Doherty Earth Observatory Contribution Number 7876.

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Received on 29th November, 2014; revised on 3rd January, 2015; accepted on 12th February, 2015