Changes in eukaryotic microbial communities of vernal pools along an urban–rural land use gradient

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ABSTRACT: Urban–rural gradients are important ecological settings to investigate how human land use impacts habitats and, indirectly, inherent organisms. Seasonally flooded vernal pools are common throughout temperate forest landscapes and may represent significant sites of forest nutrient cycling. The effects of urbanization on vernal pool microbial communities, important drivers of nutrient cycling, are largely unknown; thus studies to improve our understanding of microbes and their functional roles in these habitats are needed. Eukaryotic microbial communities sampled from 30 vernal pools of the Cuyahoga River watershed (USA), located along a gradient of urban land use, were profiled with denaturing gradient gel electrophoresis, and were compared between pools using nonmetric multidimensional scaling (NMS). Microbial diversity, and, specifically, the richness and diversity of the fungal operational taxonomic units (OTUs), increased with urbanization. Vernal pool eukaryotic microorganisms formed 2 NMS clusters that differed significantly in sub-watershed urban area. However, the significance of urbanization disappeared when fungal and algal communities were analyzed separately. Water conductivity was consistently correlated with different microbial communities (e.g. eukaryotic, fungal, and algal). Fungal communities also appeared related to the carbon content of the substrate, indicating that vegetation at a local scale may be important for community structure. Almost half of the OTUs matched fungal species, which provides taxonomic evidence that the eukaryotic microbial communities of vernal pools are dominated by fungal species. Overall, our data suggest that the eukaryotic microbes of vernal pools are influenced by a variety of factors of the surrounding landscape, including urbanization, water chemistry, and vegetation type.

KEY WORDS: 18S rDNA · Denaturing gradient gel electrophoresis · Diversity · Microbial communities · Vernal pools · Urbanization · Litter quality · Richness

INTRODUCTION

In the temperate regions of North America, seasonally flooded ponds, called vernal pools, are found throughout the landscape during the spring season. Similarly, vernal pools can be found on a variety of soil types, underlying geologies, and surrounding land uses (reviewed by Colburn 2004). For example, in a study of 106 vernal pools found in Amherst, Massachusetts (USA), Stone (1992) found that these pools were distributed on soil from 7 different drainage classes and of 6 different origins. Such differences in underlying material, accompanied by the variety of other factors that can change across the landscape, can lead to spatially variable water chemistry in vernal pools. In a study of 30 vernal pools of northeastern Ohio (USA), Carrino-Kyker & Swanson (2007) found correlations between conductivity and the degree of agricultural land use and between water depth and the degree of urban area that surrounded the pools. In a smaller study of 4 vernal pools in Massachusetts, Brooks et al. (2002) also found an effect of urbanization on water

chemistry. Specifically, these authors noted higher temperature, pH, alkalinity, and conductivity in urban pools compared to non-urban pools. Such changes in the physical and chemical environment can affect members of the biological community, such as amphibians whose larval survival can be influenced by water chemistry (e.g. Freda & Dunson 1985, Laposata & Dunson 1998), insects whose development is strongly tied to the length of the hydroperiod (Brooks 2000), or microorganisms, which are perhaps the most environmentally-sensitive organisms on our planet due to their small size (McArthur 2006). This latter group of organisms has been particularly understudied in vernal pools.

Vernal pools are commonly known as sites of amphibian breeding and, as such, many studies on vernal pool ecology have focused on frogs or salamanders (reviewed by Colburn 2004). Much less attention has been given to the microbial communities of vernal pools, even though these communities are instrumental in ecosystem element cycling and energy flow in both terrestrial and aquatic habitats (Gutknecht et al. 2006, Liu et al. 2006). Vernal pools may represent 'hot spots' of nutrient cycling within the forest due to microbial processing of (1) allochthonous leaf litter inputs, which are colonized by microbes in both the dry and wet phases, and (2) the excrement and debris (e.g. exoskeletons) left behind by aquatic organisms that live in the pools (Williams 2006). The microorganisms of vernal pools comprise the base of the detrital-based food web upon which more complex organisms rely. Importantly, we do not have a clear understanding of what major taxonomic groups of microorganisms are present in these habitats, how the diversity of these communities is affected by water chemistry or urbanization, or the functional significance of these organisms in vernal pools. Carrino-Kyker & Swanson (2008) conducted a study of 5 vernal pools over 3 mo and determined that both the bacterial and microbial eukaryotic communities changed rapidly over time, possibly as a consequence of changes in water chemistry and dominant abiotic conditions. This previous study, however, was done on a small scale; thus, the effects of landscape-level influences, such as urbanization and associated water chemistry changes, on these communities are largely unknown. Further, many other previous studies of vernal pool microbial communities were completed prior to the development of environmental genomic technologies (e.g. Bärlocher et al. 1978, Laird 1988).

In the current study, we examined the eukaryotic microbial communities in 30 vernal pools within a landscape of mixed land use types and utilized an urban–rural gradient to explore effects of urbanization on microbial communities in these pools (see Carrino-Kyker & Swanson 2007). Such urban–rural gradients are interesting settings to ask basic ecological questions, test theories, and contribute to ecosystem management (McDonnell & Pickett 1990). Our study specifically addressed whether the eukaryotic microbial community was altered by changes in watershed land use, since there is conflicting evidence on whether urbanization affects the richness and diversity of eukaryotic microorganisms negatively (Jumpponen & Jones 2009), positively (Dopheide et al. 2009), or has no effect (Cousins et al. 2003, Duong et al. 2007). We also sought to determine if and how the composition of the eukaryotic microbial community was affected by urbanization. As such, the eukaryotic microbial community was profiled using denaturing gradient gel electrophoresis (DGGE) and sequence analysis of excised bands, which allowed for relative comparisons of diversity and community composition based on partial 18S rDNAs.

MATERIALS AND METHODS

Field sampling and environmental parameters. In March 2004, 30 vernal pools in the Cuyahoga River Basin (near Cleveland, Ohio, USA) were sampled. The pools were located in 10 sub-watersheds (n = 3 pools per sub-watershed), which ranged in size from 359 to 4899 km^2. The vernal pools were randomly located within the 10 sub-watersheds and were selected based on their existence in a deciduous forest. The percentage of each sub-watershed that represented a certain land use category was determined with digital maps (W.B. Clapham, Jr. pers. comm.) and a Geographic Information System (GIS; Fig. 1). The 10 sub-watersheds were selected to represent a gradient of urbanization (quantified as the sum of percent urban, percent suburban, and percent barren areas, which were mostly construction sites), but agricultural and natural land use classifications were also prevalent (Fig. 1). All 3 pools within a sub-watershed were located close to one another; therefore, they were similar distances from the nearest urban area (see Appendix 1). However, the urban–rural gradient reported here was quantified with a sub-watershed approach for 2 reasons: (1) sub-watersheds are natural boundaries defined by water flow direction and accumulation, allowing the inclusion of any non-point source pollutants that could run off into the pools, and (2) land use features (e.g. construction sites, golf courses, industrial development) located close to the pools were not consistent across sites, making it difficult to conduct a consistent proximity analysis. A more thorough description of how sub-watersheds were delineated and how land use was assessed can be found elsewhere (Carrino-Kyker & Swanson 2007). From each pool, 3 different sample
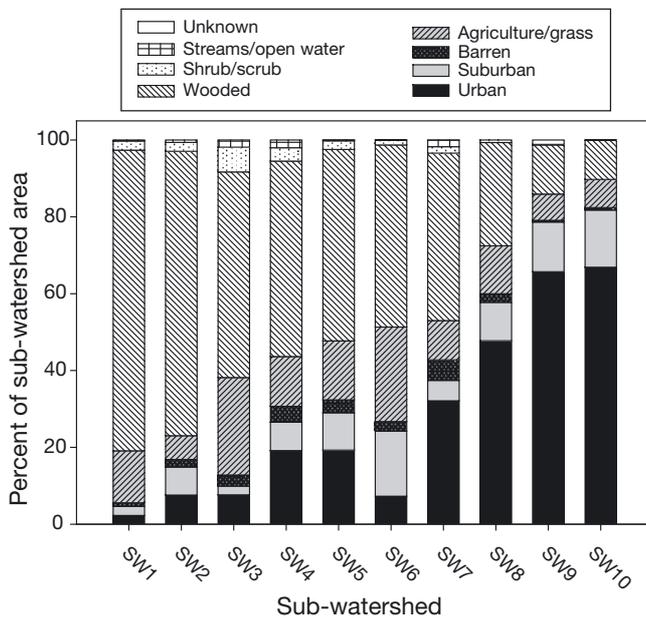
Fig. 1. GIS-characterized land use of 10 sub-watersheds of the Cuyahoga River Basin, Ohio, USA. Percent urbanization of each sub-watershed was defined as the sum of percent urban, percent suburban, and percent barren area (the lowest 3 categories on the bars). Sub-watershed (SW) numbers were assigned in order of decreasing percent natural area, which included percent wooded, percent shrub/scrub, and percent streams/open water (figure from Carrino-Kyker & Swanson 2007). GIS: Geographic information system

types were taken 1 m from the edge of the pool. Sterile pipette tips were used to sample 1 ml soil cores in triplicate, and leaf matter and water were sampled from 3 locations (ca. 3 leaves and 30 ml of water at each location). Once in the laboratory, 50 ml of the water sample were filtered through 0.2 µm polycarbonate filters; no coarse filtering of the water was conducted. The environmental parameters of pH, dissolved oxygen, temperature, and depth were measured *in situ*. Conductivity was determined on water samples that had been transported to the laboratory on ice after no longer than 7 h. Specific descriptions of these measurements were given by Carrino-Kyker & Swanson (2007). The leaves were sub-sampled 3 to 5 times and were used for percent carbon (%C), percent nitrogen (%N), and C:N with an elemental combustion system (Costech Analytical Technologies). These leaf tissue sub-samples were representative of the collected leaves, such that they included tissue from each leaf type (i.e. species) found and more tissue from leaf types that were more abundant in the sample.

DNA extraction and PCR. DNA was extracted from each sample type (500 mg of soil, 5 leaves, and half of each water filter) using the Fast DNA® Spin Kit for Soil (MP Biomedicals) and following the manufacturer's

protocol with the modifications described by Carrino-Kyker & Swanson (2008). Following the extractions, DNA from the 3 sample types was combined for each vernal pool (i.e. DNA was a composite of soil, litter, and water). Although there may be differences in the eukaryotic communities in these sample types (see Carrino-Kyker & Swanson 2008), the purpose of the present study was to examine how environmental changes affected the entire community of vernal pool organisms; therefore, the sample types were combined. Partial 18S rDNA was then PCR-amplified from these combined DNA samples using the primers and temperature cycling outlined by van Hannen et al. (1999), except that the final extension step was 30 min to eliminate DGGE double banding (Janse et al. 2004). The forward primer contained a GC clamp 5'-'CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C-3' on the 5' end (van Hannen et al. 1999). For each vernal pool sample, 4 DNA dilutions (1:5, 1:10, 1:20, and 1:40) were used in PCR reactions to limit bias (Chandler et al. 1997). The DNA concentration in each reaction ranged from 0.5 to 14.0 ng between the 1:40 and 1:5 dilutions, respectively. The PCR reactions were 50 µl in volume and contained 0.5 µM of each primer, 200 µM each dNTP, 1.5 mM $MgSO_4$, and 1 unit of Platinum® Taq DNA Polymerase High Fidelity (Invitrogen) for an automatic 'hot start' to minimize nonspecific binding of the primers to non-target DNA. Amplifications were performed on an MJ Research PTC 100 Thermal Cycler.

DGGE and band profiling. Positive PCR reactions (of the 4 dilutions) for each vernal pool sample were combined equally to total 32 µl for each vernal pool. These combined PCR products were subsequently loaded onto DGGE gels such that the concentrations ranged from approximately 0.4 to 2.0 µg of PCR product for each vernal pool. Different DNA sequences present in this combined PCR sample were separated on 1 mm thick DGGE gels that were run for 16 h, as described by van Hannen et al. (1999). We included a 2 cm stacking gel containing no denaturants through which samples were electrophoresed at 150 V until they entered the gradient gel (ca. 10 min). Bands were visualized by staining with a 1:10 000 dilution of SYBR gold (Molecular Probes) in 1× Tris Acetate EDTA for 40 min and photographed on the Foto Eclipse™ and Foto Analyst® (Fotodyne) gel doc system. Approximately 100 visualized bands from each DGGE gel were excised with sterile 1 ml pipette tips. TotalLab™ gel analysis software (Nonlinear Dynamics) was used to identify distinct bands, determine band intensity (Rf value), calculate DNA quantity in each band by comparing Rf values in bands from the samples to bands of known DNA concentration, and cross compare operational taxonomic units (OTUs).

DNA sequencing. One excised band representing each OTU was re-amplified as described above ('DNA extraction and PCR' section) with GC-clamped primers. These PCR products were then subjected to DGGE to confirm that each excised band represented a single sequence. Once the bands were visually pure, they were re-amplified with non-GC-clamped primers, purified with the QIAquick® PCR Purification Kit (QIAGEN), and sent for commercial DNA sequencing (MWG Sequencing) using the non-GC-clamped forward primer. The sequences were analyzed using the BLAST tool at the National Center for Biotechnology (NCBI) GenBank database (www.ncbi. nlm.nih.gov). If DGGE bands returned the highest similarity with an uncultured environmental sample, the next highest similarity to a biologically relevant species is reported here in order to provide a taxonomically meaningful result. The DGGE band sequences were deposited into GenBank under accession numbers GU459011 to GU459054. Sequences were viewed in Chromas Lite, Version 2.01 (Technelysium) and modified prior to submission such that bases labeled with 'N' were replaced with more specific base codes following the IUPAC-IUB codes.

Data analysis and statistics. The relative abundance of each OTU in each sample was calculated using the Rf values generated with the TotalLab™ gel analysis software. Some faint bands in the DGGE gels had Rf values of 0 that were changed to 0.1 for analyses that included relative abundance. OTU richness per vernal pool was determined by counting the number of bands in each DGGE lane, and the Shannon-Weaver diversity index was calculated as described by Nübel et al. (1999). Pearson correlations between percent urban area and richness and diversity were conducted in SigmaStat, Version 3.5 (Systat Software). Changes in OTU patterns, including the presence/absence of OTUs and relative band intensity, were identified by performing the ordination technique of nonmetric multidimensional scaling (NMS) with PC-ORD, Version 5.0 (B. McCune and MJM Software). Like other ordination techniques, NMS provides a visual representation of the similarity between sites based on their community composition (i.e. on the resulting NMS graph, sites that cluster together show more overlapping taxa than sites that appear farther apart). Unlike other ordination techniques, NMS does not assume that the data are distributed normally and, as such, is appropriate for ecological data sets, which can be non-normal, arbitrary, and/or discontinuous (McCune & Grace 2002). Even though the data for the present study were proportions, the raw data were used for the analyses reported here because an arcsine, square root transformation did not change the distribution of the data nor did it considerably alter the results. NMS clusters were determined visually after examining all possible graphs (including 2- and 3-dimensional graphs) from the resulting 3-dimensional solutions. A multi-response permutation procedure (MRPP) and indicator species analysis were performed on the NMS clusters, also in PC-ORD. MRPP was used to determine whether the clusters differed significantly in their OTU composition/abundance, and indicator species analysis was used to identify specific OTUs that were possibly driving the clustering pattern. To determine whether eukaryotic community patterns corresponded with the changing vernal pool environment, the general water quality parameters we measured were compared between the NMS clusters using t-tests (also conducted in SigmaStat). If data were not normally distributed, a Mann-Whitney rank sum test was used in place of a parametric t-test. The water chemistry data were also compiled in a secondary matrix which was correlated with the NMS axes in PC-ORD. The resulting Pearson correlation coefficients allowed further investigation of the relationship between environmental parameters and microbial community structure. Richness, diversity, and NMS analyses were conducted using all microbial eukaryotic OTUs, as well as the subset of OTUs that were only non-photosynthetic protists (labeled protist), only autotrophic protists (labeled algae), or only fungi. Graphs were made in SigmaPlot, Version 10.0 (Systat Software). NMS clusters were labeled with E1 and E2 for the NMS ordination of all microbial eukaryotes. Likewise, the algal and fungal NMS clusters were labeled A1 and A2 and F1 and F2, respectively.

RESULTS

DGGE revealed 47 OTUs in total from the 30 vernal pools. Successful DNA sequences were recovered from 44 of these OTUs, which were broadly organized into 3 major taxonomic groups: fungi, algae, and protists (Table 1). Of these 44 recovered sequences, 41% matched with fungal species, specifically the groups Ascomycota, Basidiomycota, and Chytridiomycota; 27% matched with protist species, specifically the groups Alveolata, Cercozoa, and Amoebozoa; and 23% matched with algal species, specifically the groups Bacillariophyceae, Chrysophyceae, Synurophyceae, and Xanthophyceae (Table 1). Any sequences of macroscopic eukaryotes (shown in Table 1) were removed from further analyses. Three OTUs, which matched with protist sequences in the NCBI database, had low percent similarities (i.e. <80%). These OTUs were considered unknown eukaryotic microorganisms for the purpose of this study and were therefore not included in the calculations of protist richness or diversity.

Table 1. Best-matched biologically relevant DNA sequence from the GenBank database for each excised operational taxonomic unit (OTU). Organisms with <80% similarity were considered unknown microorganisms and, though they matched with protist sequences in the NCBI database, were not included in the calculations of protist richness or diversity

	Band			Closest relative	
OTU no.	Accession no.	Similarity (%)	Database match	Accession no.	Taxonomic description
1	GU459011	79	*Entamoeba chattoni*	AF149912	Unknown
3	GU459012	94	*Entamoeba chattoni*	AF149912	Protist (Amoebozoa)
5	GU459013	77	*Xiphocephalus triplogemmatus*	FJ459763	Unknown
6[a]	GU459014	80	*Cruoria pellita*	AY437664	Rhodophyta
7	GU459015	95	*Loxophyllum rostratum*	DQ190465	Protist (Alveolata; Cilophora)
8	GU459016	79	*Amoeba leningradensis*	AJ314605	Unknown
9	GU459017	98	*Navicula phyllepta*	FJ40068	Algae (Bacillariophyceae)
10	GU459018	98	*Synura uvella*	U73222	Algae (Synurophyceae)
11	GU459019	96	*Heteromita globosa*	U42447	Protist (Cercozoa)
12	GU459020	97	*Spongomonas minima*	AF411280	Protist (Cercozoa)
13	GU459021	96	*Navicula brockmannii*	AM502020	Algae (Bacillariophyceae)
14	GU459022	98	*Navicula gregaria*	AM501974	Algae (Bacillariophyceae)
15	GU459023	94	*Chilodonella uncinata*	AF300283	Protist (Alveolata; Cilophora)
17	GU459024	95	*Mallomonas tonsurata*	EF633325	Algae (Synurophyceae)
18	GU459025	93	*Spumella* sp.	EF027354	Algae (Synurophyceae)
19	GU459026	96	*Hydrurus foetidus*	FM955256	Algae (Chrysophyceaee)
20	GU459027	95	*Spumella* sp.	AJ236858	Algae (Synurophyceae)
21[a]	GU459028	97	*Phagocata sibirica*	Z99948	Platyhelminthes
22	GU459029	94	*Chilodonella uncinata*	AF300283	Protist (Alveolata; Cilophora)
23	GU459030	98	*Tribonema utriculosum*	AM490826	Algae (Xanthophyceae)
24	GU459031	98	*Gomphonema angustatum*	AM502005	Algae (Bacillariophyceae)
25	GU459032	91	*Lecythium* sp.	AJ514867	Protist (Cercozoa)
26	GU459033	98	*Hypotrichida* sp.	AF508777	Protist (Alveolata; Cilophora)
27	GU459034	95	*Oxytricha* sp.	AF508769	Protist (Alveolata; Cilophora)
28	GU459035	95	*Chalara cylindrosperma*	AF222507	Fungi (Ascomycota)
29[a]	GU459036	97	*Phagocata sibirica*	Z99948	Platyhelminthes
30	GU459037	98	*Chalara constricta*	AF222506	Fungi (Ascomycota)
31	GU459038	96	*Podosphaera tridactyla*	AB022392	Fungi (Ascomycota)
32	GU459039	93	*Blyttiomyces helicus*	DQ536491	Fungi (Chytridiomycota)
33	GU459040	88	*Chalara sessilis*	AF222514	Fungi (Ascomycota)
34	GU459041	98	*Phialocephala* sp.	EU155146	Fungi (Ascomycota)
35	GU459042	98	*Tetracladium* sp.	FJ553447	Fungi (Ascomycota)
36	GU459043	96	*Microdochum nivale*	AF548077	Fungi (Ascomycota)
37	GU459044	96	*Buellia georgei*	AJ421681	Fungi (Ascomycota)
38	GU459045	98	*Madurella* sp.	EU815932	Fungi (Ascomycota)
39	GU459046	88	*Pyrenochaeta lycopersici*	DQ898289	Fungi (Ascomycota)
40	GU459047	96	*Ophiocordyceps sinensis*	EF555097	Fungi (Ascomycota)
41	GU459048	98	*Thanatephorus cucumeris*	DQ917659	Fungi (Basidiomycota)
42[a]	GU459049	98	*Carallia brachiata*	AM235548	Viridiplantae; Magnoliophyta
43	GU459050	98	*Aureobasidium pullulans*	FJ023536	Fungi (Ascomycota)
44	GU459051	99	*Boletus rubropunctus*	FJ480426	Fungi (Basidiomycota)
45	GU459052	95	*Thecotheus holmskjoldii*	AF010589	Fungi (Ascomycota)
46	GU459053	98	*Myxotrichum deflexum*	AY541481	Fungi (Ascomycota)
47	GU459054	99	*Mrakia frigida*	DQ831017	Fungi (Basidiomycota)

[a]Considered a macroscopic organism and removed from statistical analyses

At higher urbanized sites, both richness and diversity were not eroded (Fig. 2). In fact, vernal pools in more urbanized sub-watersheds had higher eukaryotic diversity (Fig. 2b). Eukaryotic diversity had a significant positive correlation with percent urban area (Pearson correlation coefficient = 0.523; p < 0.01). Similarly, there were positive trends in richness and diversity of fungal OTUs with urbanization (Fig. 2c,d). Both fungal richness (Pearson correlation coefficient =

0.497; p < 0.01) and fungal diversity (Pearson correlation coefficient = 0.540; p < 0.01) were significantly positively correlated with percent urban area. No significant correlations were found between percent urban area and eukaryotic richness, algal richness or diversity, or protist richness or diversity.

Community composition in the vernal pools varied between the sub-watersheds. For all eukaryotic OTUs, the vernal pools formed 2 fairly distinct clusters in the
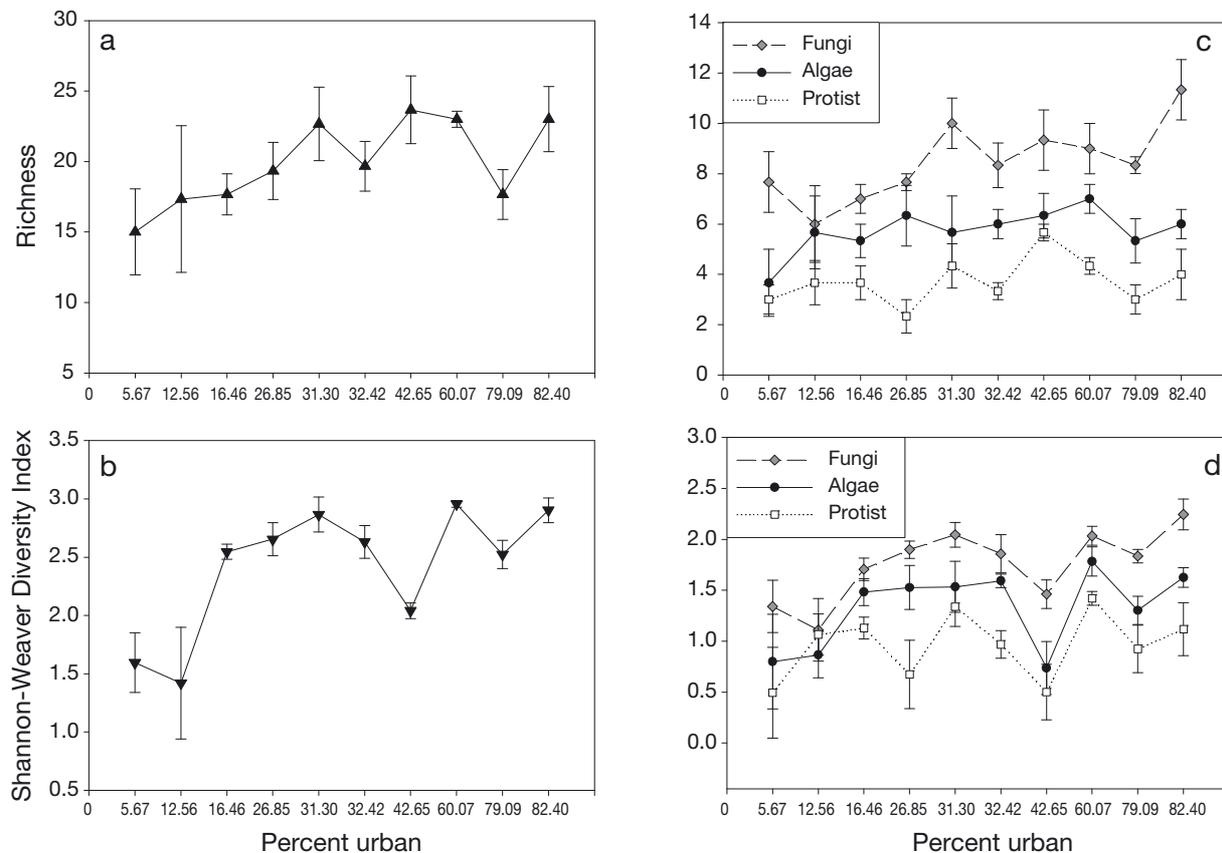
Fig. 2. Average richness and Shannon-Weaver diversity (±SE) of (a,b) the entire eukaryotic community and of (c,d) the subset of operational taxonomic units that were fungi, algae, or protists found in 30 vernal pools across a gradient of urbanization. Vernal pools were located in 10 sub-watersheds with differing degrees of percent urban area (n = 3 pools per sub-watershed). Richness and diversity were determined with denaturing gradient gel electrophoresis

NMS ordination (Fig. 3a) that were shown to be significantly different by MRPP (p < 0.001). The vernal pools in these 2 clusters differed significantly in their surrounding urbanization (p = 0.036) and dissolved oxygen (p = 0.029), with cluster E1 pools having a lower percent urban area and higher dissolved oxygen concentration (Table 2). We also found a significantly lower C:N ratio in the leaves in cluster E1 pools (p = 0.037; Table 2). The dimensions of the eukaryotic NMS were strongly correlated (i.e. Pearson correlation coefficients ≥ 0.296) with the water chemistry variables of temperature and conductivity, the %C content of the leaves, and all land use categories (Table 3). Several OTUs were found to be significant indicators of the clusters with indicator species analysis. One algal sequence (OTU 9) was a significant indicator of cluster E2 communities (Table 2). Protist sequences OTU 11 and OTU 26 were significant indicators of clusters E1 and E2, respectively (Table 2). Two fungal OTUs (numbers 34 and 38) were significant indicators for cluster E1, while cluster E2 had only 1 significant fungal indicator: OTU 35 (Table 2).

When the individual groups were analyzed with NMS, both the fungi and the algae showed a cluster pattern (Fig. 3b,c; a reliable NMS diagram was not generated with protist data). The fungal clustering was similar to the overall eukaryotic clustering, suggesting that the fungi may be strongly affecting the overall eukaryotic NMS. MRPP also showed these clusters to be significantly different (p < 0.001). The vernal pools in the fungal clusters differed in their conductivity (p = 0.001) and %C content of the leaves (p = 0.002), both which were lower in cluster F1 (Table 2). Further, the %C of leaves and water conductivity, along with water temperature and dissolved oxygen, were strongly correlated with fungal NMS dimensions (Table 3). There were several significant indicator OTUs for the fungal clusters, all of which matched with sequences in the group Ascomycota (Tables 1 & 2). The clustering of the algal NMS diagram showed that 6 pools differed significantly from the rest in their composition of algae (p < 0.001 with MRPP analysis). Only conductivity was significantly different between the algal clusters (p = 0.022) and was lower in cluster A2 pools (Table 2).
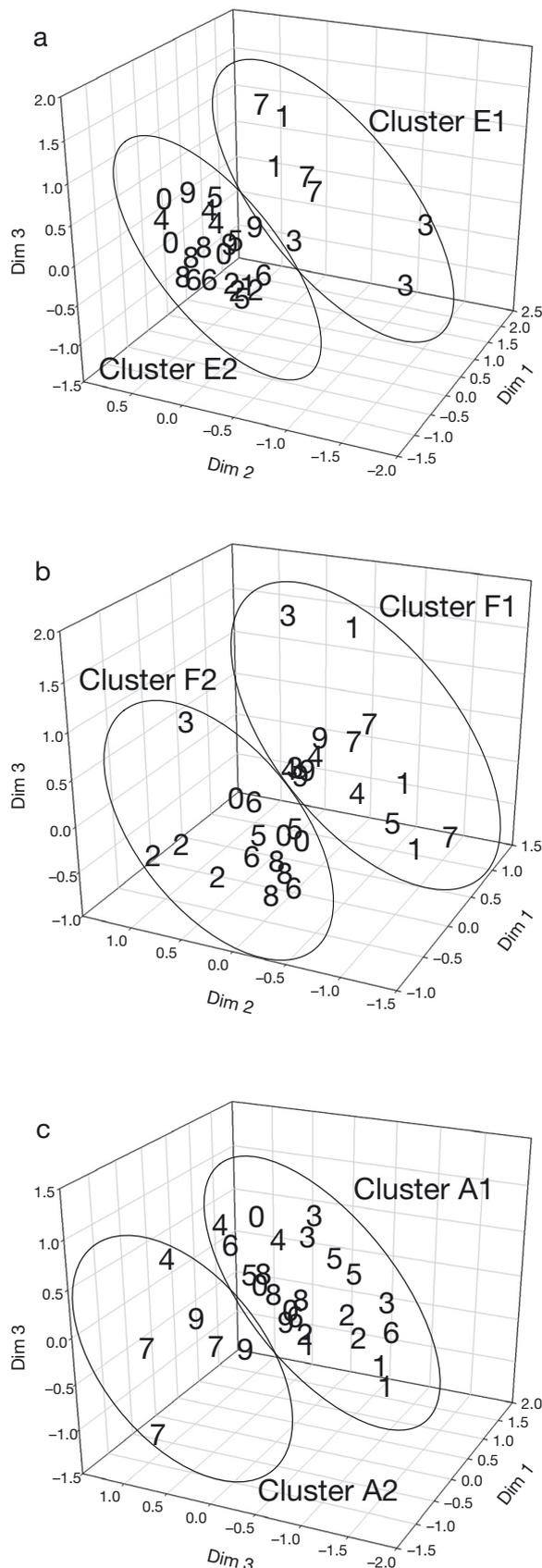
When the algal NMS dimensions were correlated with the secondary matrix, water temperature and dissolved oxygen, leaf %C and C:N, and all land use categories were strongly correlated (Table 3). For the algal NMS, significant indicators were OTUs 18 and 20 for cluster A1, and OTUs 19 and 23 for cluster A2 (Table 2).

DISCUSSION

The primary aim of this study was to determine whether relative differences in eukaryotic microbial community structure in vernal pools varied with urbanization at a landscape scale. We observed an increase in microbial diversity and, specifically, in fungal richness and diversity with increasing urbanization. The lack of eroded richness and diversity with human impact suggests that vernal pool microorganisms, which are tolerant of the naturally varying environmental conditions of an ephemeral habitat (Carrino-Kyker & Swanson 2007, 2008), may not experience decreased diversity with urbanization. In some studies where macroscopic organisms were surveyed, a positive relationship between human population density and species richness was found (Balmford et al. 2001, reviewed by Arújo 2007). It has been argued, at least for macroscopic organisms, that this positive relationship is due to the fact that humans, along with many other species, benefit by living in regions of high environmental heterogeneity (Luck 2007, Hugo & van Rensburg 2008). Urbanization may be increasing habitat heterogeneity in vernal pools, in both space and time, resulting in higher levels of diversity. Studies of stream bacterial (Cody et al. 2000) and ciliate (Dopheide et al. 2009) communities also found higher richness and diversity at sites more impacted by human activity. It is plausible that in urbanized watersheds, increased nutrient runoff and changes in water chemistry following storm events create greater temporal heterogeneity in aquatic habitats, leading to increases in overall microbial diversity. Forest fragmen-

Fig. 3. Three-dimensional nonmetric multidimensional scaling (NMS) ordination diagrams showing the similarity between the vernal pool (a) eukaryotic community, (b) fungal community, and (c) algal community. Stress values for each NMS were 13.70% for the eukaryotic ordination (a), 13.78% for the fungal ordination (b), and 11.34% for the algal ordination (c). Each pool is represented by a number indicating the sub-watershed within which that pool was located. On each graph are 2 clusters which are identified by circles and labeled with an E, F, or A for the eukaryotic, fungal, and algal NMS ordinations, respectively. Clusters were visually determined and found to be significantly different in their community structure with a multi-response permutation procedure. Dim: NMS dimension

Table 2. Descriptive statistics for vernal pool environmental parameters and significant indicator species of each nonmetric multidimensional scaling (NMS) cluster. Values for the environmental parameters are means ± SE; p-values for the environmental parameters were determined with t-tests, while the p-values for the indicator species were determined with indicator species analysis. *p < 0.05. OTU: operational taxonomic unit

Parameter	Eukaryote NMS Cluster E1 (n = 8)	Cluster E2 (n = 22)	p	Fungi NMS Cluster F1 (n = 15)	Cluster F2 (n = 15)	p	Algae NMS Cluster A1 (n = 24)	Cluster A2 (n = 6)	p
pH	7.54 ± 0.38	7.62 ± 0.12	0.944	7.51 ± 0.21	7.68 ± 0.15	0.309	7.67 ± 0.12	7.32 ± 0.47	0.378
Dissolved oxygen (mg l⁻¹)	11.72 ± 1.08	8.08 ± 0.87	0.029*	9.85 ± 1.00	8.25 ± 1.12	0.292	8.47 ± 0.84	11.35 ± 1.39	0.127
Temperature (°C)	7.26 ± 0.54	6.07 ± 0.85	0.467	5.35 ± 0.89	7.43 ± 0.88	0.106	6.85 ± 0.69	4.52 ± 1.51	0.148
Conductivity (µmho cm⁻¹)	134.13 ± 35.59	288.68 ± 46.46	0.064	128.13 ± 22.25	366.80 ± 56.79	0.001*	289.38 ± 42.10	79.83 ± 25.55	0.022*
Depth (cm)	7.41 ± 1.29	8.07 ± 0.82	0.677	7.50 ± 1.17	8.29 ± 0.73	0.569	8.04 ± 0.69	7.32 ± 2.12	0.678
%N	1.48 ± 0.13	1.31 ± 0.068	0.218	1.29 ± 0.11	1.41 ± 0.062	0.272	1.34 ± 0.063	1.42 ± 0.18	0.581
%C	33.15 ± 1.43	37.19 ± 8.84	0.227	31.78 ± 1.99	40.45 ± 1.47	0.002*	36.60 ± 1.74	34.15 ± 2.18	0.510
C:N	23.11 ± 1.36	29.41 ± 1.65	0.037*	25.96 ± 2.05	29.51 ± 1.71	0.193	27.98 ± 1.27	26.76 ± 4.81	0.724
Percent urban area	22.12 ± 6.09	45.07 ± 5.58	0.036*	35.58 ± 6.78	42.32 ± 6.75	0.487	35.46 ± 5.37	52.91 ± 8.47	0.091
Percent agricultural area	16.84 ± 2.58	12.40 ± 1.27	0.094	13.23 ± 1.47	13.93 ± 1.93	0.771	14.58 ± 1.40	9.60 ± 0.97	0.091
Percent natural area	61.04 ± 4.90	42.38 ± 5.12	0.051	50.96 ± 5.91	43.75 ± 6.19	0.407	49.92 ± 4.89	37.12 ± 7.78	0.124
Indicator species[a]	—	OTU 8	0.008*	OTU 34	—	0.031*	OTU 18	—	0.016*
	—	OTU 9	0.035*	—	OTU 35	0.026*	—	OTU 19	0.0002*
	OTU 11	—	0.0006*	OTU 38	—	0.0004*	OTU 20	—	0.0016*
	—	OTU 26	0.028*	—	OTU 39	0.0002*	—	OTU 23	0.0052*
	OTU 34	—	0.0008*	—	OTU 45	0.019*			
	—	OTU 35	0.0002*						
	OTU 38	—	0.003*						

[a]Only significant indicator species are shown. The identity of each of these operational taxonomic units is listed in Table 1

tation and plant community changes in urban watersheds could also create spatial variability across a vernal pool in terms of light availability and water temperature, leading to greater within-pool heterogeneity and microbial diversity. For microorganisms, however, it may be that the effect of urbanization on richness and diversity is specific to different habitats or regions. Indeed, others have noted a decrease (e.g. Jumpponen & Jones 2009) while a greater number of other studies have found no change in microbial richness and diversity with anthropogenic pollutants (e.g. Dorigo et al. 2002, Cousins et al. 2003, Duarte et al. 2004, Duong et al. 2007, Borin et al. 2009). Although metrics of richness and diversity can be useful, when applied to microbial communities, they are often insensitive to environmental changes (Morris & Blackwood 2007); thus the effects of urbanization reported here may be more strongly impacting microbial community composition.

It is more common that microbial communities change in their taxonomic composition with human impacts, which has been consistently documented in scientific literature (e.g. Cody et al. 2000, Dorigo et al. 2002, Cousins et al. 2003, Duarte et al. 2004, Duong et al. 2007, Borin et al. 2009, Dopheide et al. 2009). We also found that the community composition of eukaryotic microbes in vernal pools was related to the percent of each subwatershed that was urban area. This observation is likely due to the fact that some species of protists, algae, and fungi are better adapted to the environmental conditions created by urbanization than others. That each eukaryotic cluster resulting from the NMS ordination had significant indicator OTUs associated with them lends support to the conclusion that some species are better adapted to the urban pools than others. This is further supported by the observations that the eukaryotic community structure was significantly affected by dissolved oxygen concentration, while the fungal and algal community structures were significantly affected by conductivity. These general water quality characteristics are known to be influenced by urbanization, with more impacted aquatic habitats often experiencing lower dissolved oxygen concentration and higher conductivity due to polluted runoff from developed areas (Daniel et al. 2002, Miserendino et al. 2008).

Table 3. Pearson correlation coefficients showing the relationship between vernal pool characteristics (e.g. water chemistry, substrate litter quality, and sub-watershed land use) and nonmetric multidimensional scaling (NMS) dimensions (labeled Dim). NMS was performed for the entire eukaryotic microbial community (Eukaryote) as well as the portion of the community identified as fungi (Fungi) or autotrophic protists (Algae). Because our study had n = 30, strong correlations were identified as a Pearson correlation coefficient ≥ 0.296 (Zar 1999)

	Eukaryote			Fungi			Algae		
	Dim 1	Dim 2	Dim 3	Dim 1	Dim 2	Dim 3	Dim 1	Dim 2	Dim 3
pH	−0.094	0.100	0.132	−0.247	−0.075	−0.115	0.045	−0.035	−0.153
Dissolved oxygen (mg l^{-1})	0.066	−0.262	0.226	0.348	0.045	−0.055	−0.089	−0.071	0.324
Temperature (°C)	−0.349	−0.334	−0.048	0.064	0.158	−0.492	0.379	−0.259	−0.259
Conductivity (μmho cm^{-1})	−0.332	0.081	−0.404	−0.201	0.247	−0.412	0.244	0.220	−0.181
Depth (cm)	0.029	−0.291	−0.201	−0.100	0.166	0.049	0.170	0.187	−0.205
%N	−0.120	−0.227	−0.066	0.022	0.155	−0.180	0.111	−0.115	0.135
%C	−0.246	−0.016	−0.302	−0.329	0.277	−0.213	0.096	−0.244	−0.329
C:N	−0.087	0.211	−0.165	−0.272	0.047	−0.004	−0.136	−0.068	−0.310
Percent urban area	−0.237	0.469	0.098	−0.244	−0.145	−0.078	−0.335	0.109	0.397
Percent agricultural area	0.184	−0.431	−0.224	0.283	0.232	0.054	0.333	0.268	−0.234
Percent natural area	0.210	−0.404	−0.047	0.193	0.098	0.067	0.286	−0.196	−0.377

The importance of dissolved oxygen and conductivity for eukaryotic microbes was also shown by the indicator species analysis. Of the 7 indicator OTUs between the eukaryotic NMS groups, 3 were protists and 3 were fungi. In aquatic habitats, dissolved oxygen is of paramount importance for the distribution of microbial eukaryotes; for example, aquatic fungi are reliant on dissolved oxygen to carry out decomposition with different species thriving in high versus low oxygen conditions (Medeiros et al. 2009). In vernal pools, the conductivity likely includes measures of essential nutrients, namely phosphorus and nitrogen ions, which are often high in urban runoff (Carpenter et al. 1998). These nutrients are important determinants for the survival of different algal and fungal species. Phosphorus availability is particularly important for the distribution of golden-brown algae (Wetzel 2001), which comprise 3 of the 4 significant OTUs for the algal NMS groups. Nutrient availability is also important for the decomposition activity of aquatic fungi (Gulis et al. 2006), and all significant OTUs for the fungal NMS groups were in the Ascomycota, a group that contains over 90% of all aquatic hyphomycetes (Webster 1992). Vernal pool conductivity can also be related to increased salt content in runoff from road deicing agents (Karraker et al. 2008), and certain algal and fungal species may be more tolerant of these high salt levels.

It is worthwhile to note that urbanization is not the only factor known to affect dissolved oxygen or conductivity, and other characteristics of the landscape may be important for vernal pools, as urbanization was no longer a significant factor in structuring the fungal and algal communities when they were examined alone. For example, conductivity can be affected by soil type that varies across the landscape (Wierenga et al. 1969). In the same 30 pools used in the current study, agricultural land use was found to influence conductivity (Carrino-Kyker & Swanson 2007). Although percent agriculture was not significantly different between any of the NMS clusters in the current study, it was strongly correlated with both eukaryotic and algal NMS dimensions. Water temperature, which we found consistently correlated with NMS dimensions, affects the dissolved oxygen content of water (Wetzel 2001) and can vary in shallow water bodies at multiple scales (e.g. spatially across a landscape as wetland area changes, Li et al. 2009; or temporally as air temperatures rise and fall, over the course of a day, Podrabsky et al. 1997, or season, Bonner et al. 1997). Regardless of the factors responsible for the change, our data indicate that the composition of eukaryotic microorganisms in vernal pools varied with urbanization at a landscape scale, and such a change in microbial community composition may have important consequences for ecosystem function (Kinzig et al. 2001, Loreau et al. 2002, Morin & McGrady-Steed 2004), which should be explored in future studies of these habitats.

Our results also suggest that local site-specific characteristics may be just as important as urbanization in structuring the eukaryotic community of vernal pools. We found the OTU composition of eukaryotic and fungal communities to be significantly influenced by litter quality, and at least 1 dimension resulting from the eukaryotic, fungal, and algal NMS ordinations was strongly correlated with at least 1 aspect of litter quality. Similarly, Cousins et al. (2003) found that the structure of arbuscular mycorrhizal fungi was influenced by vegetation type just as much as by land use. Further, Ochimaru & Fukuda (2007) found that the richness of fungi involved in decomposition was higher in urban forests compared to rural forests and suggested that

this was due to differential litter distribution, not the effects of land use. Contrary to our findings, Verb et al. (2001) saw little change in algal community structure with different vegetative litter substrates in a meso-cosm study after 56 d. However, in their study, Verb et al. (2001) used fairly coarse groups of vegetation with mixed deciduous forest substrate comprising 1 group. We argue that changes in litter quality on a finer scale (e.g. between different species of trees that comprise the deciduous forest) may be important, as qualitative data we collected using canopy pictures over the pools suggest that the main sources of leaf litter into cluster E1 and F1 pools were *Acer* trees, which are relatively easily decomposed, while the canopy trees over cluster E2 and F2 pools contained higher numbers of *Fraxinus*, *Quercus*, *Platanus*, and *Populus* leaves (data not shown). Thus, in landscape-scale studies, the vegetation at a more local scale cannot be ignored, as litter quality can affect both community structure as well as ecosystem function (Alarcón-Gutiérrez et al. 2009, Kotilainen et al. 2009, Ndaw et al. 2009). Our observation that the majority of OTU sequences were saprotrophic fungi, which are involved in the decomposition of allochthonous leaf litter into vernal pools, provides further support that litter quality and local environmental factors are important for vernal pool microbial community structure. Future studies should explore the influence of leaf litter composition and quality on vernal pool microbial communities, as they appear to play a role in structuring these communities that form the basis of the aquatic food web, but were not quantitatively measured in the current study.

Overall, we found that urbanization was associated with an increase in microbial richness and diversity and an altered microbial community composition in vernal pools. This suggests that there is a suite of eukaryotic microbial species which are able to tolerate the fluctuations in water chemistry associated with urbanization and are likely aided by their adaptations to the ephemeral environment of vernal pools. These changes in microbial community composition may also influence ecosystem function, and future studies should investigate this. Urbanization was not the only factor found to affect microbial community composition. Litter quality and other local water chemistry changes may be just as important as larger landscape-scale influences on vernal pool microbial communities. Therefore, when conducting landscape-scale studies, local effects on the microbial community cannot be ignored, as they may be equally important for structuring microbial communities to landscape variability.

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Appendix 1. Distance of each pool to the nearest urban area, as determined with ArcGIS®, version 9.3 (ESRI) and 1992 United States Geological Survey land use maps

Sub-watershed	Distance to nearest urban area (m)		
	Pool 1	Pool 2	Pool 3
SW1	283.46	333.84	513.73
SW2	560.32	697.77	776.65
SW3	377.63	355.65	176.40
SW4	108.88	173.80	247.32
SW5	234.14	245.39	268.78
SW6	57.75	52.82	65.38
SW7	42.74	22.74	38.79
SW8	11.89	16.04	0[a]
SW9	42.09	55.58	104.75
SW10	9.90	28.07	61.16

[a]Pool located within the 30×30 m area that was classified as urban, even though it was found in a forested area