The effects of pH change and NO$_3^-$ pulse on microbial community structure and function: a vernal pool microcosm study

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Abstract

Forest vernal pools experience strong environmental fluctuations, such as changes in water chemistry, which are often correlated with changes in microbial community structure. However, very little is known about the extent to which these community changes influence ecosystem processes in vernal pools. This study utilized experimental vernal pool microcosms to simulate persistent pH alteration and a pulse input of nitrate (NO$_3^-$), which are common perturbations to temperate vernal pool ecosystems. pH was manipulated at the onset and microbial respiration was monitored throughout the study (122 days). On day 29, NO$_3^-$ was added and denitrification rate was measured and bacterial, fungal, and denitrifier communities were profiled on day 30 and day 31. Microbial respiration and both bacterial and fungal community structure were altered by the pH treatment, demonstrating both structural and functional microbial responses. The NO$_3^-$ pulse increased denitrification rate without associated changes in community structure, suggesting that microbial communities responded functionally without structural shifts. The functioning of natural vernal pools, which experience both persistent and short-term environmental change, may thus depend on the type and duration of the change or disturbance.

Introduction

Vernal pools are seasonally flooded habitats that, in temperate regions, are typically inundated in the spring owing to snowmelt, high precipitation, and low evapotranspiration. Although they are typically small in size compared to permanent bodies of water, they are abundant across the landscape (Colburn, 2004) and, therefore, functionally and ecologically significant. Much of the research on vernal pool organisms has focused on amphibians and macroinvertebrates, which take advantage of the fish-free habitat for breeding (reviewed in Colburn, 2004). Very little is known, however, about the microbial communities in vernal pools, even though these communities are responsible for much of the nutrient cycling and energy flow in wetland habitats (Gutknecht et al., 2006). In forests, where vernal pools receive high amounts of organic matter from allochthonous leaf litter inputs in addition to excrement and debris (e.g. exoskeletons) left behind by aquatic organisms (Williams, 2006), microbially mediated energy flow may be especially important. Consequently, further studies on the microorganisms in vernal pools are necessary to fully understand the ecological impact of microorganisms in these habitats.

Because of their small size, shallow depth, and short hydroperiod, vernal pools experience a high degree of environmental fluctuation, both spatially and temporally (Brooks, 2000; Carrino-Kyker & Swanson, 2007, 2008). Over the course of 4 months, Carrino-Kyker & Swanson (2008) noted changes to microbial community structure in vernal pools and correlated those changes with temporal fluctuations in water column pH, dissolved oxygen, and conductivity. Not only are vernal pool microbial communities temporally dynamic, but they have also been shown to vary spatially in response to water column conductivity and dissolved oxygen, to leaf litter quality, and to the degree of surrounding urbanization (Carrino-Kyker et al., 2011). However, whether the observed...
changes to microbial communities in vernal pools also affect their function is uncertain. Studies from other systems have shown that environmental conditions can simultaneously affect both microbial community structure and function (e.g. Balser & Firestone, 2005; Bell et al., 2009; Smucker & Vis, 2011); however, conflicting studies have found no association between microbial community structure and ecosystem processes when one is affected by environmental change (Rich & Myrold, 2004; Menyailo et al., 2010; Attard et al., 2011), implying functional redundancy in some communities. It has been proposed that the type and duration of the environmental change will determine whether microbial community structure is altered and if there is a corresponding change in ecosystem function (Balser et al., 2001). Despite the importance of this topic and the uncertainty concerning the effects of environmental conditions on microbial community structure and function, we are unaware of any studies that have attempted to explore these relationships in vernal pools.

Vernal pools are a good model system for studying the effects of multiple environmental conditions on microbial community structure and function because of their high degree of environmental variability and expected sensitivity to environmental change. Environmental influences on vernal pools may be persistent and associated with different soil types, underlying geologies, and surrounding plant communities and land uses (Carrino-Kyker & Swanson, 2007). However, other environmental changes may be sporadic, such as nutrient pulses associated with urban and agricultural runoff following rainfall events (Moldan & Černý, 1994). Because runoff is a significant source of water for vernal pool formation (Colburn, 2004), seasonal pulses of pollutants may have a significant effect on microbial community structure and function, but this has previously not been investigated.

Here we present the results of an experimental microcosm study that examined the extent to which altered environmental conditions influenced microbial communities and the processes they mediate. In particular, we were interested in parameters that are related to human alteration of the environment, such as pH changes that can result from acid deposition and nutrient enrichment from pollution. As such, vernal pools were simulated in the laboratory and subjected to pH manipulation and nitrate (NO$_3^-$) enrichment. The pH manipulation was established at the onset and maintained throughout the 4 months of the experiment, while the NO$_3^-$ was administered only once part way through the experiment. This simulated persistent pH changes previously observed in vernal pools across an urbanization gradient (Carrino-Kyker & Swanson, 2007) and NO$_3^-$ pulses associated with runoff (Moldan & Černý, 1994). The purpose of this study was to add to the growing literature on the responses of microbial community structure and ecosystem function to environmental changes and to specifically address this question in vernal pools, which are understudied relative to their ubiquity in temperate regions and ecological importance.

Materials and methods

Microcosm construction and treatments

Soil from four vernal pools in Northeast Ohio, USA (41° 29'53"N, 81° 25'27"W, elevation, 320 m) was randomly sampled in November 2007. All pools contained standing water at the time of sampling. The top 2–5 cm layer of soil was collected with a clean shovel, transported to the laboratory in opaque plastic bins, immediately transferred to flat plastic trays, covered with shade cloth, and left to dry at room temperature. Once dry, the soil was passed through a 2-mm sieve and stored at 4 °C prior to microcosm construction. The soil was dried and stored at 4 °C to mimic the winter temperatures and dry conditions that vernal pools experience in the field in temperate regions (Colburn, 2004).

_Fagus grandifolia_ Ehrh. leaves were sampled from a mature Beech-Maple forest located at The Holden Arboretum, Kirtland, OH (41°36'40"N, 81°17'30"W, elevation, 340 m) on the same date. Only _F. grandifolia_ leaves were used to control for variability associated with varying litter quality. Freshly fallen leaves were collected by hand, placed in plastic bins, mixed to eliminate bias by location, covered with shade cloth, and left to dry at room temperature. Once dry, 1.5-cm-diameter leaf disks were taken with a punch and stored at 4 °C.

In February of 2008, 72 microcosms were constructed in 500-mL Mason jars. Each microcosm received an equal weight (50 g) of premixed vernal pool soil and fine-grained sterile sand, 800 mg of leaf punches, and 150 mL of autoclaved deionized water. Sand was added to improve aeration and diffusion because the vernal pool soil was fine-textured. Each microcosm was assigned to one of twelve treatments (four pH treatments and three nitrogen (N) treatments in a 4 × 3 full factorial design), with six replicates per treatment (n = 72). The initial pH of the microcosms ranged from 4.00 to 4.39. The four pH treatments (pH values of 5, 6, 7, and 8) represented values found in Northeastern Ohio vernal pools (Carrino-Kyker & Swanson, 2007, 2008) and were manipulated by low-volume addition of 5 M sodium hydroxide (NaOH) based on amounts determined by titrations in a pilot study. The pH 6, 7, and 8 microcosms initially received 100, 350, and 500 µL, respectively, of NaOH. Throughout the remainder of the
study (122 days), 5 M hydrochloric acid (HCl, as in 
Chu et al., 2007) and 5 M NaOH were used to maintain 
the pH of each microcosm, which was monitored 
biweekly (see Supporting Information, Table S1). While 
HCl and NaOH may artificially raise salinity, measure-
ments of sodium ions on day 113 (range of 0.8–304 ppm) 
and chloride ions on day 114 (range of 60–320 ppm) indicated that their levels in the micro-
cosms were within the range of fresh water salinity 
(McArthur, 2006) and we expect the effects on microor-
ganisms to be minimal compared to our experimental 
treatments. On day 29, once respiration measurements 
were similar in all pH treatments (see Fig. 1), the N 
treatments were implemented such that each microcosm 
received a 20-mL solution containing 10-mg NO\textsubscript{3}– in 
the form of KNO\textsubscript{3}(+NO\textsubscript{3}), 10-mg NH\textsubscript{4}+ in the form of 
(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} (+NH\textsubscript{4}+), or sterile DI water (–N). Therefore, the +NO\textsubscript{3} and +NH\textsubscript{4}+ microcosms received 
200 μg per g of dry soil of either NO\textsubscript{3}– or NH\textsubscript{4}+. The NO\textsubscript{3}– added was similar to levels measured in 
vernal pools that received wastewater in Pennsylvania 
(Laposata & Dunson, 1998). The NH\textsubscript{4}+ addition was 
used as a comparison to determine whether any treat-
ment effects were owing to low N (nutrient) availability, 
and the DI water treatment was a control. The micro-
cosms were randomly placed in an incubator at 
16 ± 1 °C with a 12-hr light/12-hr dark cycle to simu-
late temperature and natural day/night cycle in North-
eastern Ohio in the spring, and rotated within the 
incubator to limit bias of location. The jars were 
covered with parafilm that included vents to allow air 
exchange.

**Microbial processes**

Microbial community respiration was determined on days 
1, 4, 8, 15, 22, 29, 30, 31, 94, and 122 by measuring CO\textsubscript{2} 
efllux from each microcosm with a portable open-flow 
infrared gas analyzer (IRGA; LI-6400; LI-COR® Instruments, 
Lincoln, NE). Respiration was determined by 
scrubbing CO\textsubscript{2} from the microcosm headspace and mea-
suring the change in headspace CO\textsubscript{2} concentration over 
time. We used the average of two separate scrub/flux cycles 
and a rate was calculated using the following formula:

\[
\text{Community Respiration} = (\text{dc}'/\text{dt}*\text{system volume})/\text{soil carbon}
\]

where \(\text{dc}'/\text{dt}\) was the rate of change of CO\textsubscript{2} over 10 s 
determined with the IRGA and corrected for water con-
tent, system volume included the volume of microcosm 
headspace and IRGA tubing, and soil carbon was the 
mass of carbon per gram of dried soil and leaves added to 
the microcosms. The final respiration value was 
converted to μg CO\textsubscript{2}-C (g soil C\textsuperscript{−1} day\textsuperscript{−1}).

Potential denitrification rates were determined with a 
modified acetylene-block technique (Groffman et al., 
1999). On day 29, when the N treatments were adminis-
tered, the microcosms were closed with airtight lids that 
were fitted with rubber septa and cycled five times between 
vacuum and purging with 99.9% pure nitrogen gas (N\textsubscript{2}). Jars were then opened, and a syringe and needle were used to 
bubble 30 mL of acetylene through the soil. The jars were 
then reclosed and the headspace was again flushed with N\textsubscript{2} 
before adding 40 mL of acetylene with a syringe and needle to 
the head space via the rubber septa. Between day 29 and 
day 31 of the experiment, 20 mL of headspace was sampled 
with a syringe and needle at 1, 12, 16, 24, 36, and 40 h fol-
lowing the N additions, without controlling for headspace 
pressure, and stored in scintillation vials fitted with airtight 
rubber stoppers (GeoMicrobial Technologies, Ochelata, OK). Following the 16-h headspace sampling, the micro-
cosms were opened for respiration measurements (see 
above) and to collect samples for microbial community 
analysis (see below). Immediately following this sampling, 
additional acetylene was added to the soil and headspace 
as described above and the jars were again sealed with airt-
tight lids in preparation for the remaining headspace sam-
ple. The nitrous oxide (N\textsubscript{2}O) concentration of each sample 
was measured with a gas chromatograph and electron cap-
ture detector (GC-2014; Shimadzu Scientific Instruments, 
Columbia, MD). Only headspace samples were collected for 
N\textsubscript{2}O determination, which did not include any N\textsubscript{2}O that 
was dissolved in the water. Denitrification rate was calcu-
lated following Groffman et al. (1999) by first converting 
the N\textsubscript{2}O measurement in ppm to μL N\textsubscript{2}O-N/L headspace 
and then subtracting this measurement from one time
Effects of pH and NO\textsubscript{3} on vernal pool microbial communities

point by that of an earlier time point and standardizing for headspace volume and soil dry weight. The proportion of NO\textsubscript{3} that was released as N\textsubscript{2}O-N during our measurements was calculated for the 16-h and 40-h time points and summed, as an estimate of the amount of NO\textsubscript{3} that was denitrified. The 16- and 40-h time points were used because these were the final time points on day 30 and day 31, respectively, and the jars were opened between the 2 days of denitrification measurements.

**DNA extraction and molecular analysis**

On day 30 (after the 16-h headspace collection) and day 31 (after the 40-h headspace collection), microcosms were opened to randomly sample one leaf disk and two soil samples from each jar for microbial community analyses. Microbial analyses were conducted on these days to profile the communities at the same time that both respiration and denitrification measurements were made. The microbial communities in soil and leaf samples were profiled separately because they have previously been shown to differ in vernal pool habitats (Carrino-Kyker & Swanson, 2008). Samples from two microcosms in the same treatment were randomly pooled prior to DNA extraction, such that half of a leaf disk and ~125 mg of soil from each microcosm were used for the molecular work (i.e. \( n = 3 \) soil and leaf samples per treatment group for the microbial analyses). After cell lysis using a Precellys 24 Homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France), DNA was extracted separately from soil and leaf samples using the PowerSoil\textsuperscript{TM} DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA).

DNA from soil and leaf samples was used to amplify general bacteria in a PCR using 16S rRNA gene primers 338f and 926r (Muyzer \textit{et al.}, 1993, 1995) following conditions as described in Burke \textit{et al.} (2006). Fungal PCR amplified the internal transcribed spacer region of the rRNA gene using the primer set 58A2F and ITS4 (Martin & Rygiewicz, 2005) following conditions as described in Burke \textit{et al.} (2005) except the extension step was 90 s. To amplify the community of denitrifying bacteria, we targeted the \textit{nosZ} gene using the primers nosZFa and nosZRb (Rösch & Bothe, 2005) and followed conditions as described in Rösch \textit{et al.} (2002). The \textit{nosZ} gene codes for \( \text{N}_2\text{O} \) reductase, the enzyme that catalyzes the conversion of \( \text{N}_2\text{O} \) to \( \text{N}_2 \) in the denitrification pathway. The \textit{nosZ} gene is found in a number of denitrifying bacteria and is considered a good marker for this group of organisms (see Philippot & Hallin, 2006 for a review); however, the primers used here discriminate against any denitrifiers lacking the \textit{nosZ} gene, as well as gram-positive denitrifiers.

Terminal restriction fragment length polymorphism (TRFLP) profiling was used to examine the diversity and structure of the bacterial, fungal, and denitrifying communities following protocols previously described (Burke \textit{et al.}, 2005, 2006, 2008). The endonucleases MspI and HaeIII were used for bacterial TRFLP and AluI and HaeIII were used for fungal TRFLP. The denitrifier TRFLP was conducted using the restriction endonucleases MboI and TaqI (Rösch & Bothe, 2005). TRFLP profiles were generated at the Life Sciences Core Laboratories Center (Cornell University) and analyzed using the GS600 LIZ size standard and Peak Scanner\textsuperscript{TM} Software (version 1.0, Applied Biosystems 2006). For our analyses, only peaks that accounted for > 1% of the relative peak area were included (i.e. major TRFs; Burke \textit{et al.}, 2008).

**Statistical analyses**

Because the microcosms were randomly placed in the incubator and rotated within the incubator throughout the study, they were treated independently for statistical tests. Differences in respiration were determined over the course of the 122-day study with a two-way repeated measures ANOVA using pH treatment and time as factors. For this ANOVA, respiration measurements made on day 1 were excluded because they were below detection in several of the pH 7 and pH 8 microcosms. Respiration differences were also determined between pH and N addition treatments individually on day 30 and 31 with two-way ANOVAS, and one-way ANOVAS were used for determining whether denitrification varied between the pH values. All ANOVAS were conducted in \textit{SIGMASTAT} version 3.5 (Systat Software, Inc., 2006). If needed, the data were natural log transformed for two-way ANOVAS or one-way ANOVAS were conducted on ranks to meet homogeneity of variance requirements. Significance was determined at \( \alpha < 0.05 \).

TRFLP profiles were compared between sample types (i.e. leaf and soil), sample date (i.e. day 30 or 31), and microcosm treatments (i.e. pH and N addition) with the nonmetric multidimensional scaling (NMS) ordination technique and with multi-response permutation procedure (MRPP) using \textit{PC-ORD} (Version 5.0; Bruce McCune and MJM Software, 1999). Because the TRFLP profiles contained relative proportions, they were arcsine-square-root transformed prior to running NMS or MRPP to improve normality (McCune & Grace, 2002). NMS ordinations were run separately for profiles generated with different TRFLP enzymes and were conducted on both sample types together, as well as for soil and leaf communities separately, for a total of 18 NMS runs. MRPP was also used to determine differences between soil and leaf communities, as well as to assess treatment differences for soil and leaf communities separately. TRFLP profiles generated with different enzymes were used in individual MRPP runs for a total of 42 MRPP runs. For our microcosms, differences between...
groups were considered statistically and ecologically significant if they were greater than by chance ($P < 0.05$) and the effect size was large ($A > 0.1$) (McCune & Grace, 2002). Pairwise comparisons following MRPP runs revealed the groups that were significantly different from each other. Prior to NMS and MRPP, outlier analysis was conducted in PC-ORD to determine any outlying samples. Samples were flagged as outliers if their distance measure was more than two standard deviations from the mean distance (McCune & Grace, 2002) and were removed from NMS and MRPP runs if they were shown to be outlying for both TRFLP enzymes. These criteria only eliminated one sample from the NMS and MRPP runs of the fungal soil samples.

Operational taxonomic unit (OTU) richness of each microbial community (bacteria, fungi, and denitrifying microorganisms) based on TRFLP profiles was determined in PC-ORD. The relationship between OTU richness for microbial community (bacteria, fungi, and denitrifiers) and microbial respiration was assessed with linear regression using SIGMAPLOT, version 10.0 (Systat Software, Inc., 2006). Richness was not regressed against denitrification rate because it was undetected in two-thirds of the microcosms (see below); therefore, reliable regressions could not be made. Regressions were conducted for the richness values of each microbial community and for each of the TRFLP enzymes. Separate regressions were made for soil and leaf samples and for day 30 and day 31 samples for a total of 24 regressions (three microbial communities × two TRFLP enzymes × two sample types × two dates). The data were tested for normality (i.e. Kolmogorov–Smirnov test) and constant variance in SIGMAPLOT when running the regressions. If assumptions were violated, regressions were re-run with CO$_2$ flux (i.e. the dependent variable) values natural log transformed and/or outliers (visualized on residual plots and prediction/confidence interval plots) removed to meet normality and equal variance assumptions. Only five regressions contained outliers that were removed. Significance for regression relationships between richness and respiration was determined at $\alpha < 0.05$. Kutner et al. (2005) was used as a guide for interpretation and diagnostic measures for the regressions.

Results

**Microbial processes**

Respiration rate differed significantly between the pH treatments ($F = 60.12$, $P < 0.001$) and over time ($F = 172.49$, $P < 0.001$), and the pH × time interaction was also significant ($F = 13.52$, $P < 0.001$; Fig. 1). The pH 7 and pH 8 microcosms showed little CO$_2$ efflux on day 1 and ended with a greater rate than the pH 5 and pH 6 microcosms on day 122 (Fig. 1). When respiration rates were compared with a two-way ANOVA between pH and N treatments individually on day 30 and day 31, significant effects of pH treatment ($F = 115.36$, $P < 0.001$ and $F = 105.91$, $P < 0.001$ for day 30 and 31, respectively), N addition ($F = 24.33$, $P < 0.001$ and $F = 10.16$, $P < 0.001$ for day 30 and 31, respectively), and pH × N treatment interactions ($F = 4.15$, $P = 0.002$ and $F = 3.15$, $P = 0.010$ for day 30 and 31, respectively) were found on both dates (Fig. 2). Pairwise multiple comparison tests (i.e. Tukey tests) within each pH treatment indicated significantly different CO$_2$ efflux between the $+$NO$_3^-$ and $+$NH$_4^+$ treatments in the pH 6, 7, and 8 microcosms on both dates and significantly higher CO$_2$ efflux in the $+$NH$_4^+$ treatment compared to the controls in the pH 6 and 8 microcosms on day 30 only (Table S2).

Denitrification rates were markedly higher in the $+$NO$_3^-$ treatment, compared to the $+$NH$_4^+$ and $-$N treatments where denitrification was not detected on either day 30 or day 31 (Fig. 3). Within the $+$NO$_3^-$ treatment, there were no significant effects of pH on denitrification rate on either date ($F = 2.344$, $P = 0.104$ for day 30 and $P = 0.060$ for ANOVA on ranks conducted for day 31 data; Fig. 3). At 40 h following the NO$_3^-$ addition, 10.6% of the NO$_3^-$-N that was added to the $+$NO$_3^-$ microcosms was recovered as N$_2$O-N.

**Microbial community structure**

MRPP revealed significant differences ($A > 0.1$ and $P < 0.05$) between soil and leaf communities for bacteria, fungi, and denitrifiers (Table 1). NMS ordinations also showed differences between soil and leaf communities.
(Fig. S1). Because of these differences between soil and leaf communities, they were analyzed separately to determine the effects of microcosm treatments (pH and N addition) and date (day 30 or 31). The soil and leaf ordination diagrams shown here used the TRFLP enzymes HaeIII for bacteria, HaeIII for Fungi, and TaqI for denitrifiers. The other TRFLP enzymes produced similar results (Fig. S2). For the NMS graphs shown in Fig. 4, the leaf bacterial and soil denitrifier NMS ordinations resulted in two-dimensional solutions, while the soil bacterial, leaf denitrifier, and fungal ordinations resulted in three-dimensional solutions. For NMS ordinations that resulted in three-dimensional solutions, the graphs shown here include the two dimensions that explained the most variation (i.e. higher $R^2$ values). NMS and MRPP showed community differences between pH treatments for both bacteria and fungi (Table 1; Fig. 4a–d). For bacteria, this was apparent in both soil and leaf samples (Table 1; Fig. 4a and b), but fungi were only affected in leaf samples and the effect size (i.e. A statistic) was lower than for bacteria (Table 1; Fig. 4c and d). TRFLP profiles using the nosZ gene showed no effect of pH on soil denitrifier communities (Table 1; Fig. 4e). For the leaf denitrifier communities, the TaqI enzyme showed no significant effect of pH on denitrifier community structure (Table 1; Fig. 4f), but this result was not consistent across enzymes as the MboI enzyme indicated a significant pH effect (Table 1); however, this difference was only between the pH 8 microcosms and the remaining three pH treatments (Fig. S2). Thus, these results are inconclusive as to whether pH influenced the denitrifier community structure in leaf samples. Contrary to the pH treatment, the addition of NO$_3^-$ or NH$_4^+$ had little effect on microbial community structure, even for denitrifiers, which use NO$_3^-$ as an electron acceptor (Table 1; Fig. 4). Similarly, there was no effect of sampling date on microbial community structure (Table 1; Fig. 4). The differences in community structure between pH treatments and substrate type were owing to both the presence/absence of and changes in the relative abundance of individual terminal restriction fragments (TRFs; Tables S3–S5). In general, TRFs were consistent between the N treatments (Tables S6–S8).

**Table 1.** MRPP results for each microbial community TRFLP profile

<table>
<thead>
<tr>
<th>Grouping factor</th>
<th>Bacteria</th>
<th>Fungi</th>
<th>Denitrifiers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mspl</td>
<td>HaeII</td>
<td>MboI</td>
</tr>
<tr>
<td>Leaf vs. soil</td>
<td>A 0.55</td>
<td>P &lt; 0.001</td>
<td>A 0.14</td>
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<tr>
<td>Leaf samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH treatment</td>
<td>0.26</td>
<td>&lt; 0.001</td>
<td>0.36</td>
</tr>
<tr>
<td>N addition</td>
<td>0.014</td>
<td>0.041</td>
<td>0.013</td>
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<tr>
<td>Date</td>
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<td>0.00075</td>
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<tr>
<td>pH treatment</td>
<td>0.23</td>
<td>&lt; 0.001</td>
<td>0.15</td>
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<tr>
<td>N addition</td>
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<td>0.054</td>
<td>0.061</td>
</tr>
<tr>
<td>Date</td>
<td>0.017</td>
<td>0.013</td>
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</tbody>
</table>

Community differences between pH treatment (5, 6, 7, and 8), N addition (+NO$_3^-$, +NH$_4^+$, and --N), and sample date (day 30 or day 31) were determined independently on soil and leaf samples because the communities were significantly different between these two sample types. TRFLP enzymes were also analyzed separately. Differences between groups were considered significant if they were greater than by chance ($P < 0.05$) and had a large effect size ($A > 0.1$) (McCune & Grace, 2002). Significant differences are shown in bold and were confirmed by visualizing non-metric multidimensional scaling (NMS) graphs (see Fig. 4, Figs S1 and S2).
Regressions of richness vs. respiration

CO₂ flux was positively correlated to bacterial richness, but negatively related to the richness of nosZ-containing denitrifiers (Table 2; Fig. 5). Although there were some discrepancies between TRFLP enzymes, these relationships were significant for the bacterial community in three of the four soil samples and were unanimously significant.

Fig. 4. Nonmetric multidimensional scaling ordination graphs showing the bacterial (a, b), fungal (c, d), and denitrifier (e, f) community structures as determined by TRFLP. TRFLP enzymes shown here are HaeIII for the bacteria (a, b), HaeIII for the fungi (c, d), and TaqI for the denitrifiers (e, f). The pH treatments are indicated in the legend, while N treatments are represented by squares (+NO₃⁻), diamonds (+NH₄⁺), and circles (-N). Ellipses show the general change in community structure between pH treatments (where significant, see Table 1). The two sample dates (day 30 and day 31) are not shown on the graphs, but no differences in community structure were seen between dates (see Table 1).
for the denitrifier community in leaf samples (Table 2). No significant regressions were found using fungal richness as the independent variable (Table 2).

Discussion

The effect of pH

In our microcosms, pH was found to influence the community structure of soil and leaf bacteria and leaf fungi within 30 days following the pH manipulation. Such community changes with pH have also been shown in vernal pools (Carrino-Kyker & Swanson, 2008; Carrino-Kyker et al., 2011) and upland soils (Fierer & Jackson, 2006; Roux et al., 2010). These compositional changes could be owing to different microbial taxa having unique physiological constraints, such as the maintenance of proton motive force, which allows survival within narrow pH ranges (i.e. pH has a direct effect on the community structure; Booth, 1985). Another possibility is that pH influenced microbial community structure indirectly because pH influences a number of other environmental characteristics in aquatic systems, such as nutrient availability and redox potential (Stumm & Morgan, 1996). See Lauber et al. (2009) for further discussion on why environmental pH change could affect microbial community structure directly or indirectly in soil. The bacterial community was affected in both soil and leaf samples, but changes to the fungal community were only observed in leaf samples, suggesting that fungi were more abundant and more active in the leaf samples than in soil. This is typical for wetlands where fungi often have a substantial role in decomposing leaf litter, but can be in low abundance or absent from soils owing to anoxic conditions (van der Valk, 2006). Further, in the leaf samples, the effect of pH on fungal community structure was weaker than for bacterial community structure. Roux et al. (2010) also found a weaker influence of pH change on fungal communities than on bacterial communities in upland soil and suggested that this result may be owing to the wide pH optimum for fungal growth (often 5–9 pH units for many fungal species). Improved competitive abilities of bacteria in response to pH increases could also limit fungal response to pH changes through direct or indirect competitive interactions (Roux et al., 2010). Regardless of the reason for the community pH response, it was accompanied by significant differences in microbial respiration.

The microcosms with more basic pH had significantly higher CO₂ efflux throughout most of the study. We believe the initial low CO₂ efflux values in the pH 7 and pH 8 microcosms resulted from the microcosm having the greatest departure from the initial pH of 4.00–4.39 and any CO₂ released from microbial respiration and dissolved in the water would be used for buffering via the carbonate system (Wetzel, 2001). Between day 1 and day 29, CO₂ efflux in each pH treatment increased and decreased in a pattern similar to that of microbial growth in culture (Madigan et al., 2003). We believe the populations in each microcosm initially experienced exponential growth, and by day 29, had declined to a size that could

<table>
<thead>
<tr>
<th>Sample type and day</th>
<th>Regression equation</th>
<th>$R^2$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial richness</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MboI</td>
<td>Soil day 30</td>
<td>$y = -0.447x + 43.686$</td>
<td>0.0168</td>
</tr>
<tr>
<td></td>
<td>Soil day 31*</td>
<td>$y = 0.00517x + 3.439$</td>
<td>0.00185</td>
</tr>
<tr>
<td></td>
<td>Leaf day 30</td>
<td>$y = 0.348x + 30.777$</td>
<td>0.0422</td>
</tr>
<tr>
<td></td>
<td>Leaf day 31*</td>
<td>$y = 0.0110x + 3.398$</td>
<td>0.0456</td>
</tr>
<tr>
<td>HaeII</td>
<td>Soil day 30</td>
<td>$y = 0.462x + 25.701$</td>
<td>0.0180</td>
</tr>
<tr>
<td></td>
<td>Soil day 31*</td>
<td>$y = -0.00268x + 3.588$</td>
<td>0.000858</td>
</tr>
<tr>
<td></td>
<td>Leaf day 30*</td>
<td>$y = 0.00952x + 3.390$</td>
<td>0.0375</td>
</tr>
<tr>
<td></td>
<td>Leaf day 31</td>
<td>$y = 0.485x + 29.075$</td>
<td>0.0859</td>
</tr>
<tr>
<td><strong>Fungal richness</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alul</td>
<td>Soil day 30</td>
<td>$y = -0.496x + 48.513$</td>
<td>0.132</td>
</tr>
<tr>
<td></td>
<td>Soil day 31*</td>
<td>$y = -0.00955x + 3.669$</td>
<td>0.0254</td>
</tr>
<tr>
<td></td>
<td>Leaf day 30</td>
<td>$y = -1.712x + 51.866$</td>
<td>0.423</td>
</tr>
<tr>
<td></td>
<td>Leaf day 31*</td>
<td>$y = -0.0393x + 3.870$</td>
<td>0.326</td>
</tr>
<tr>
<td>TaqI</td>
<td>Soil day 30</td>
<td>$y = -0.726x + 41.256$</td>
<td>0.302</td>
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<tr>
<td></td>
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<td>$y = -1.869x + 53.895$</td>
<td>0.270</td>
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<tr>
<td></td>
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<td>0.702</td>
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<tr>
<td></td>
<td>Leaf day 31</td>
<td>$y = -1.564x + 46.890$</td>
<td>0.489</td>
</tr>
</tbody>
</table>

Richness was determined from TRFLP profiles and was used as the independent ($x$) variable. Microbial respiration was measured as CO₂ flux and was used as the dependent ($y$) variable. The richness determined from different sample types (leaf or soil) and on different dates (day 30 or day 31) was regressed independently against microbial respiration values. Different TRFLP enzymes were also analyzed separately. Significant regressions ($P = 0.05$) are shown in bold.

*Indicates a regression where the dependent variable (CO₂ flux) was natural log transformed to meet normality and homogeneity of variances.

†Indicates a regression where an outlier(s) was removed.
be maintained throughout the remainder of the experiment. By day 29, the CO₂ efflux was similar for all the pH treatments, suggesting that each microcosm had reached CO₂ equilibrium and the microorganisms had adapted to the new pH. Following the N additions on day 29, when the soil in the microcosms was disturbed by our acetylene additions, CO₂ efflux was higher in the pH 7 and pH 8 treatments across N addition treatments. This result was consistent throughout the remainder of the study and, we believe, reflects the respiration rate of the adapted microbial community in a buffered aquatic system, which is more reflective of a natural environment. In natural aquatic habitats, a number of studies have noted higher microbial respiration at higher pH, perhaps because of inhibition of microbial activity accompanying the high levels of aluminum common under acidic conditions (Rao et al., 1984; Dangles et al., 2004; Dorea & Clarke, 2008). Others have suggested that, in soils, fungal respiratory activity is highest at low pH, while that of bacteria is highest under more basic conditions (Blagodatskaya & Anderson, 1998). Our measurement of CO₂ efflux in the current study encompasses respiration from a wide range of microorganisms, both prokaryotic and eukaryotic, thus limiting conclusions about the activities of specific microbial groups. However, significant regressions were found between CO₂ efflux and the richness of certain microbial groups; specifically, both leaf and soil bacterial richness had a positive relationship with respiration. Similar to the current study, McGrady-Steed et al. (1997) and Bell et al. (2005) demonstrated that increased bacterial richness is correlated with higher CO₂ flux in aquatic microcosms, suggesting that increases in bacterial species traits can have an impact on carbon cycling in aquatic systems. However, a review by Nielsen et al. (2011) found that positive relationships between richness and respiration were more likely to be found in microcosm studies where 10 species or less were manipulated, but this relationship is less clear in studies including more diverse communities. The relationship between richness and respiration observed in the current study might not accurately reflect natural settings, where bacterial richness is typically higher than in a microcosm where dominant, fast-growing organisms might be selected for (Nielsen et al., 2011). Interestingly, we found a negative relationship between denitrifier richness (in both leaf and soil samples) and CO₂ efflux, which, to our knowledge, is the first documentation of such a relationship. It may be that our observed increases in denitrifier richness signify increasingly anoxic conditions in our microcosms and, therefore, a general reduction in CO₂ production, which could explain the negative relationship between denitrifier richness and CO₂ efflux. Examining the mechanism behind this relationship will require additional study, but the significant regressions between bacterial richness and CO₂ efflux in our study, coupled with our finding that the bacterial and fungal community composition was different between microcosms with different CO₂ flux rates, suggest that the difference in microbial respiration between treatments could be the result of microbial community structural changes.

Unlike the bacterial and fungal communities, the structure of the nosZ-containing denitrifiers, at least in soil, was unchanged between pH treatments; and unlike respiration rate, denitrification rate was unchanged between pH treatments. There is evidence to suggest that denitrifiers perform optimally at the pH of their native environment (Parkin et al., 1985; Cavigelli & Robertson, 2000; Šimek et al., 2002). Specifically, Šimek et al. (2002)
showed that, in soils where the pH was manipulated, the activity of denitrifiers was likely to be highest at the natural pH. However, we saw no significant change in denitrification rate between the pH treatments of our microcosms. We suggest that vernal pool denitrifiers are metabolically flexible because under natural conditions they commonly experience seasonal pH fluctuations between 5 and 8 (Colburn, 2004; Carrino-Kyker & Swanson, 2007). Therefore, denitrifiers in vernal pools are likely adapted to this fluctuating pH and may maintain high denitrification rates throughout this range. Consequently, pH fluctuations may not influence denitrification rate in vernal pools in ways expected for upland soil denitrifiers.

The effect of NO$_3^-$ pulse

Denitrification rate was high in microcosms that received the NO$_3^-$ pulse, but not detected in the absence of NO$_3^-$, as expected, as denitrification is a facultative respiratory process. Respiration was also significantly affected by the N treatment and was higher in the +NH$_4^+$ microcosms, compared to the +NO$_3^-$ microcosms in the higher pH treatments. This suggests a possible nutrient limitation that was overcome by adding (NH$_4$)$_2$SO$_4$. Unlike microbial processes, the addition of NO$_3^-$ or NH$_4^+$ did not lead to immediate microbial community structural changes for any of the groups examined (bacteria, fungi, and denitrifiers). It is likely that the lack of community responses to N is the result of the short time that they were exposed to NO$_3^-$ or NH$_4^+$, because evidence from studies on chronic N inputs has shown effects on both soil bacterial and fungal communities (Demoling et al., 2008; Hassett et al., 2009; Edwards et al., 2011). However, studies on denitrifier communities have shown no community composition changes in response to NO$_3^-$, both after 14 days of incubation in the laboratory (Deiglmayr et al., 2006) and after over 50 years of field fertilization (Enwall et al., 2005). It is possible we saw no changes in denitrifier community structure because a significant portion of denitrifiers lacks the nosZ gene (Jones et al., 2008) and the PCR primers used to amplify nosZ are known to discriminate against gram-positive bacteria (Throbäck et al., 2004). In addition, acetylene, which was added to measure denitrification of our microcosms, actively inhibits the reduction of N$_2$O to N$_2$ (Yoshinari & Knowles, 1976) and we profiled the denitrifier community with the gene that codes for N$_2$O reductase, which could limit our conclusions. However, in the studies by Deiglmayr et al. (2006) and Enwall et al. (2005) described above, the narG gene (codes for NO$_3^-$ reductase) was targeted, suggesting that the lack of response to NO$_3^-$ might be more biological than methodological; however, additional studies are necessary to fully understand how NO$_3^-$ addition impacts vernal pool denitrifier communities.

Although we profiled the community shortly after NO$_3^-$ addition, our data suggest that substantial increases in denitrification rate were not necessarily preceded by changes to the community structure of nosZ-containing denitrifiers in our microcosms. There is conflicting evidence in the literature on whether changes in denitrification are correlated with changes to denitrifier community structure or abundance. A number of studies have found no link between function (including measurements of potential denitrification rate and denitrifying enzyme activity) and denitrifier community structure or the copy number of denitrification genes (Rich & Myrøld, 2004; Deiglmayr et al., 2006; Kandeler et al., 2006, 2009; Ma et al., 2008; Hallin et al., 2009; Attard et al., 2011; Dandie et al., 2011), while others have documented changes to activity in response to changes in denitrifier community composition or gene abundance (Cavigelli & Robertson, 2000; Rich et al., 2003; Dong et al., 2009; Morales et al., 2010). It has been suggested that the amount of conflicting evidence indicates that denitrifier activity may in some cases, such as under ideal levels of oxygen and carbon, be influenced by the denitrifying community, while at other times is predominantly affected by environmental factors (Wallenstein et al., 2006). Further, the conflicting reports may be because absolute denitrification rates are more influenced by the availability of NO$_3^-$ in the environment, while overall denitrification efficiency is controlled by the abundance of denitrification genes (e.g. Hallin et al., 2009; Graham et al., 2010). Therefore, further experimentation is needed to fully understand the relationship between denitrification and the community structure and population size of the organisms performing this process. However, it is well known that denitrifiers comprise a taxonomically diverse group of organisms that include more than 50 genera and are known to use other respiratory pathways, including aerobic respiration and sulfate reduction (reviewed in Knowles, 1982; Philippot & Hallin, 2006). It is plausible that denitrifiers in our microcosms simply used other electron acceptors for respiration in the absence of NO$_3^-$.

Conclusion

Overall, our results indicate that pH could have an effect on both microbial community structure and ecosystem function in vernal pools, especially in leaf samples where fungi might be more active. Pulses of NO$_3^-$, however, may have a substantial and immediate impact on function that is not dependent on a prior change in microbial community structure. Thus, short-term increases in NO$_3^-$...
availability, such as after a rainfall event, might have a different effect on microbial communities in vernal pools than longer-term environmental changes, such as pH. Our results lend some support for the idea that different microbial groups respond differently (in terms of their structure and function) to different types of environmental change (Balser et al., 2001).

Acknowledgements

We thank Dr Wendy Mahaney, Dr Juan C. López-Gutiérrez, and Charlotte Hewins for their help with the design and implementation of this experiment and Dr Darren Bade at Kent State University for GC access.

References


Effects of pH and NO₃ on vernal pool microbial communities


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Nonmetric multidimensional scaling (NMS) ordination graphs showing the bacterial (a,b), fungal (c,d), and denitrifier (e,f) community structures as determined by terminal restriction fragment length polymorphism (TRFLP).

**Fig. S2.** Nonmetric multidimensional scaling ordination graphs showing the bacterial (a,b), fungal (c,d), and denitrifier (e,f) community structures as determined by terminal restriction fragment length polymorphism (TRFLP).

**Table S1.** Measured pH values (mean ± standard deviation) of microcosms at various time points throughout the study.

**Table S2.** Multiple comparison tests of CO₂ efflux measured on day 30 and day 31 within each pH and N treatment.

**Table S3.** Average relative abundance in each pH treatment (*n* = 18 microcosms) for the bacterial terminal restriction fragments.

**Table S4.** Average relative abundance in each pH treatment (*n* = 18 microcosms) for the fungal terminal restriction fragments.

**Table S5.** Average relative abundance in each pH treatment (*n* = 18 microcosms) for the denitrifier terminal restriction fragments (determined by profiling the *nosZ* gene).

**Table S6.** Average relative abundance in each N treatment (*n* = 24 microcosms) for the bacterial terminal restriction fragments.

**Table S7.** Average relative abundance in each N treatment (*n* = 24 microcosms) for the fungal terminal restriction fragments.

**Table S8.** Average relative abundance in each N treatment (*n* = 24 microcosms) for the denitrifier terminal restriction fragments (determined by profiling the *nosZ* gene).

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