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PRIMARY RESEARCH PAPER

Urban stream denitrifier communities are linked to lower functional resistance to multiple stressors associated with urbanization

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Abstract The microbial communities in urban stream ecosystems are subject to complex combinations of stressors. These same microbial communities perform the critical ecosystem service of removing excess reactive nitrogen. We asked whether the denitrifying microbial communities in urban streams differ in their functional resistance to common urban stressors from communities from nonurban streams. We exposed inocula from a highly polluted urban stream and a nearby nonurban stream to three different stressors, added alone and in combination. Stressors represent the common urban impacts of thermal pollution (10°C), trace metal exposure (ionic silver (Ag^+)), and salinization (addition of NaCl). We used reduction in nitrite (NO_2^-) concentrations under anaerobic conditions as a proxy for denitrification potential. Nonurban stream denitrifying microbial communities were more diverse than their urban counterparts. Denitrification potential for both communities was unaffected by exposure to any individual stressor. However, denitrification rates by the less diverse urban microbial inoculum decreased in response to combined heavy metal and salt stress, while nonurban communities were unaffected. These findings support the hypothesis that higher diversity may confer greater functional resistance in response to multiple stressors and do not support the idea that stressful conditions select for communities that are functionally resilient to multiple stressors.

Keywords Denitrification · Disturbance · Diversity · Resistance · Stress · Urbanization

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Introduction

As humans continue to alter ecosystems through land use and climate change, there is a growing need to better understand and predict how biotic communities might respond to increasingly compounded and severe disturbance (Paine et al., 1998). One key element of a community's response is its degree of functional resistance, defined here as the degree of change in community function caused by a stress or disturbance. Functional resistance can be strongly affected by community structure (McCann, 2000). Given the key role microorganisms play in mediating many valuable ecosystem services, the need to better understand how microbial communities might respond to anthropogenic disturbance is particularly acute (Allison & Martiny, 2008). While there are many competing

hypotheses for how community structure might be linked to resistance for plants and animals, there have been relatively few tests of these hypotheses for naturally occurring microbial communities.

Theoretically, communities composed largely of taxa that have been repeatedly exposed to a given stress should show little change in community function when exposed to that stress. A drought, for instance, will not cause large changes in primary productivity for a drought-adapted community dominated by drought tolerant taxa. The historic disturbance regime and its selective impact on community composition play a key role in determining community resistance. We refer to this theory as the legacy hypothesis, which predicts high resistance for communities with previous exposure to stressors (White, 1979; Sousa, 1984; Paine et al., 1998). This is analogous to the pollution-induced community tolerance (PICT) concept that has been advanced in ecotoxicology (Blanck et al., 1988), which suggests that strong selection for tolerant organisms will ultimately confer a high tolerance for the whole community.

A critical assumption of both the legacy and PICT hypotheses is that selection for tolerance to certain stressors confers an ability to resist other stressors (that there is positive cotolerance among species), thus leading to "stress-induced community tolerance" (Vinebrooke et al., 2004). An alternative hypothesis suggests that if stressors select for different tolerant taxa (negative cotolerance among species), community adaptation to one set of stressors may actually increase the impact of other stressors, generating "stress-induced community sensitivity" (Vinebrooke et al., 2004). In such cases, communities with high functional diversity are more likely to include a member that can respond favorably to a particular stress, and, therefore, can maintain function through community adaptation, a concept referred to in ecology as the "insurance hypothesis" (Yachi & Loreau, 1999). That is, diverse communities are more likely to have members that can functionally replace important members lost to disturbance (Naeem & Li, 1997). A stress will not disrupt denitrification, for instance, if that function is highly redundant so that many taxa are capable of denitrification and at least one or more of the denitrifying bacteria are insensitive to or stimulated by the stress.

Urban streams tend to have higher pollutant concentrations and both higher and more rapid spikes in streamwater temperatures than nonurban streams (Paul & Meyer, 2001; Walsh et al., 2005; Somers et al., 2013). We might predict that urban denitrifiers, accustomed to these stressors, would be relatively insensitive to contaminant exposure or warming (legacy hypothesis). Alternatively, urban denitrifier communities may already be under such high degrees of stress that they have reduced functional diversity, and thus a reduced capacity to respond to new stressors (insurance hypothesis). There is some support for the latter hypothesis, since ecologists have found that urbanization and subsequent biotic homogenization can sometimes reduce functional diversity in stream macroorganisms. However, it is unclear whether ecological theory based on macroorganisms can be applied to microbial communities which are generally thought to be much more functionally redundant (Allison & Martiny, 2008).

In this study, we compared the functional response of two structurally distinct, naturally occurring communities of denitrifying bacteria (denitrifiers) to experimental stress treatments in laboratory microcosms. The key questions were whether the two communities differed in functional resistance to the experimental stress treatments, and, if so, whether the legacy hypothesis or the insurance hypothesis provides a better explanation for observed differences. The microcosms were assembled using sediment-extracted inoculum acquired from either a highly polluted, warm urban stream or a relatively pristine, cool forest stream (Sudduth et al., 2011; Violin et al., 2011). We focused on denitrifiers because they are a phylogenetically diverse functional group whose function (i.e., denitrification) is ecologically important and easily quantified (Peterson et al., 2001; Philippot & Hallin, 2005; Wittebolle et al., 2009). We used three different types of stressors relevant to urban streams: a trace metal known to generate oxidative stress and membrane damage (silver) (Clement & Jarrett, 1994); the salt sodium chloride (NaCl), common in urban streams and known to generate osmotic stress (Csonka, 1989); and a thermal stress treatment of sufficient magnitude (10°C) to induce transient production of heat shock proteins (Yura et al., 1993). We examined changes in the rate of nitrite reduction for both communities exposed to all combinations of these three stressors.

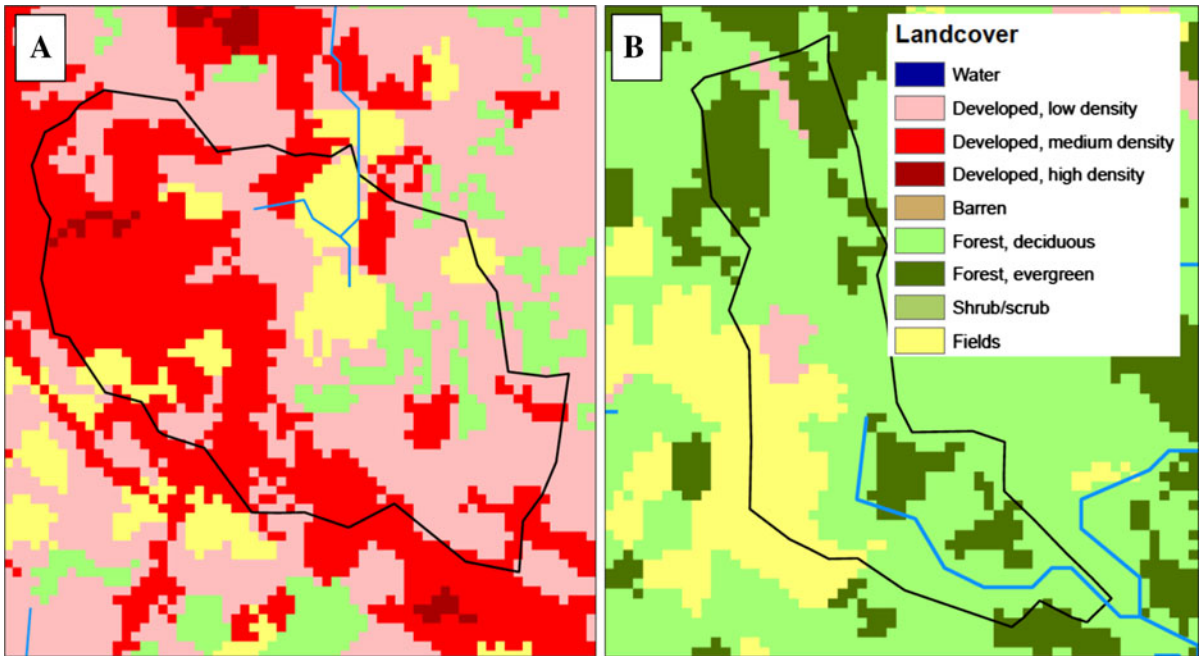

Fig. 1 Landcover maps for urban watershed (**A**) and forested watershed (**B**). Watershed delineation is in *black*

Table 1 Study stream characteristics

	Forested stream (Mud Creek)	Urban stream (Goose Creek)
Watershed area (km^2)	0.9	1.7
Impervious cover (%)[a]	0.5	39.4
Development (%)[a]	4.4	100
Flashiness[b]	0.04	0.17
EPT richness[c]	19	0

[a] Watershed land cover values calculated using 2005 land use/land cover imagery. NLCD classes 22, 23, and 24 were classified as developed areas

[b] Flashiness estimated from changes in hourly discharge between May 2007 and June 2007 (Sudduth et al., 2011)

[c] Mean number of macroinvertebrate species belonging to Ephemeroptera, Plecoptera, and Trichoptera found in summer 2006 and winter 2007 surveys (Violin et al., 2011). EPT richness is often used as an indicator of water quality; EPT species tend to occur only in clean, well oxygenated waters (Rosenberg & Resh, 1993)

Methods

Site description

The experimental microbial communities originated from Goose Creek and Mud Creek, two streams with contrasting watershed land covers in the Raleigh–Durham metropolitan area of the Piedmont region in North Carolina (USA) (Fig. 1; Table 1). We selected the streams to represent the endpoints of a gradient of urbanization intensity in the area. Goose Creek drains a watershed with 39% impervious cover. Mud Creek drains a watershed dominated by protected, mature hardwood trees and less than 1% impervious cover.

Both streams were routinely monitored for stream water quality (e.g., nitrogen, carbon, chloride, etc.) and temperature from 2006 to 2008 (Sudduth et al., 2011). Stream water samples are taken biweekly, and temperatures are recorded every 10 min with sondes (model 600XLM, YSI, Yellow Springs, OH, USA), although the sondes were malfunctioning during the period that microbial communities were collected. We measured stream water quality and temperatures, as well as sediment heavy metal concentrations (described below), because one assumption behind the legacy hypothesis is that communities from the urban stream have had previous experience in the field with the stresses tested in this study.

On June 2, 2009, we collected sediments from streams for trace metal analyses by randomly selecting five points along a permanently established study reach ($\sim$100 m) at each site. At each selected point, we demarcated a 2 m segment of the streambed (i.e.,

entire wetted width) upstream and downstream of the point and used PVC corers (6.35 cm diameter) to take multiple sediment cores until a total volume of at least 4,024 cm^3 was collected. Sediment cores were sieved (2 mm opening) and composited, resulting in a single composited sediment sample from each site. Samples were kept on ice for transport and stored at 4°C prior to analyses.

We measured the concentrations of nine heavy metals—silver (Ag), aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), and (Zn)—in sediment samples by re-sieving (1 mm opening), drying at 60°C for 48 h, and then weighing out three replicate 1 g subsamples for each site on each date. Subsamples were digested by adding 10 ml of 50% nitric acid, heating at 95°C, and then adding another 5 ml of nitric acid to each subsample. We then used inductively coupled plasma-mass spectrometry (Perkin-Elmer Elan 6000, Perkin-Elmer, Waltham, MA, USA) to measure metals concentrations. Three replicates of certified reference material STSD-3 (NRC, Institute for National Measurement Standards, Ottawa, Canada) and two method blanks were processed for every 35 samples.

Inoculum preparation and characterization

On April 30, 2009, we collected sediments from streams using the same sampling design as for the heavy metals analyses. Sediment samples were kept on ice for transport to the laboratory and stored at 4°C for less than 3 h before being used to make inocula for the microcosms. We weighed 60 g of sediment per stream into sterile tubes, added 450 ml of autoclaved spring water, vortexed at maximum power for 5 min, and then passed the resulting mixtures through sterile Whatman GF/F filters (Whatman International Ltd., Kent, UK) to create inocula from each of the two study streams. Inocula were then kept at 4°C overnight before being added to microcosms the next day.

Before adding inocula to microcosms, we first estimated the active microbial biomass in each type of inoculum to determine the approximate volumes to add in order to achieve similar microbial biomass in all microcosms. Active microbial biomass was estimated using a modified (Fierer et al., 2003) substrate induced respiration (SIR) method (West & Sparling, 1986) using autolyzed yeast extract as the substrate. Briefly, the method involved amending inocula samples (five replicates for each type) with substrate solution before measuring CO_2 production over 4.5 h. Given an essentially unlimited substrate supply and a short incubation, CO_2 production rates should be proportional to the active microbial biomass in samples (Johansson et al., 1998).

Experimental design

We assembled microcosms by adding either 50 µl of urban or 65 µl of forest community inoculum to sterile media (trypticase soy broth) spiked with 2 mM nitrite in multiwell culture plates (Corning Costar 3370; Corning Life Sciences, Lowell, MA, USA). This is a rich medium that likely favors are selected microbes, yet we intended to assay the potential of the community to perform a particular ecosystem process. We added 30% more inoculum into forest versus urban wells because SIR results suggested that the urban inoculum had approximately 30% greater active microbial biomass than the forest inoculum. Mean CO_2 production rates were 0.038 ppm CO_2/ml/h for the urban and 0.029 ppm CO_2/ml/h for the forest community inoculum. Nitrite concentrations in both inoculum types were below detection (<0.03 mM). Total volume in each well was 250 µl.

A total of 715 experimental microcosms were assembled. 360 were inoculated with forest inoculum and 355 were inoculated with urban inoculum. To test community responses to disturbance, microcosms were incubated anaerobically for 20 h with or without pollutants (2 ppb Ag^+ added as $AgNO_3$ (silver) alone, 250 ppm Cl added as NaCl (salt) alone, or a mixture of 2 ppb Ag^+ and 250 ppm Cl at either 28 or 38°C (see Table S2 in supporting information for levels of replication). While the addition of $AgNO_3$ also adds nitrate, an important denitrification substrate, the concentrations added (7.3×10^{-4} ppm nitrate) are orders of magnitude less than nitrate concentrations in the media (51×10^4 ppm nitrate) and are unlikely to lead to additional stimulation of denitrification rates.

We chose to test salt as a stressor because Cl^- concentrations are typically elevated in urban streams, particularly in areas where road salts are applied (Kaushal et al., 2005; Gobel et al., 2007). We used [Ag] as our trace metal addition because Ag is known to have antimicrobial effects, has historically been an important wastewater pollutant, and is expected to become a more common environmental pollutant as a

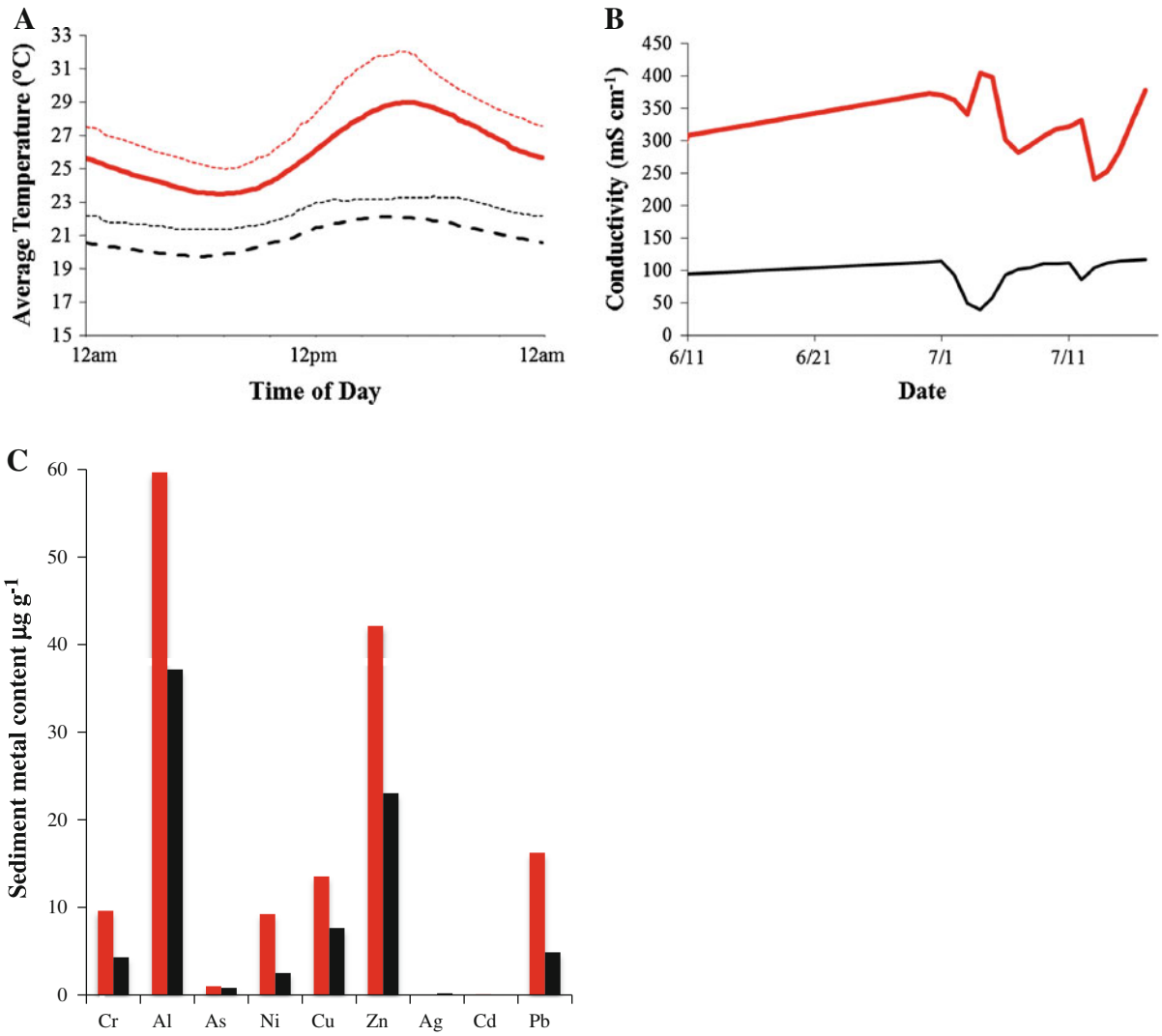

Fig. 2 Comparison of conditions in the urban (*red*) and forested stream (*black*). For temperature (**A**), the *bold line* indicates average temperature over this period, while the *lighter line* indicates the maximum temperature observed over this time period. Conductivity (**B**) shows raw measurements using a YSI Sonde for the period June 11–July 17th, 2009. Metals data (**C**) are the results from samples collected in June 2009 and analyzed by an ICP-MS following persulfate digestion

result of the increasing use of Ag nanoparticles in consumer products (Fabrega et al., 2011). Both silver and salt have been shown previously to affect denitrification rates (Throback et al., 2007; Seo et al., 2008), but represent two fundamentally different types of stress. Salt exposure leads to osmotic stress, and to survive nonlethal exposures microbes must invest significant resources in accumulating compatible solutes or altering membrane composition (Poolman & Glaasker, 1998; Tsuzuki et al., 2011). Silver generates significant oxidative stress and membrane damage (Clement & Jarrett, 1994; Lok et al., 2006).

We chose to test a 10°C temperature increase because temperature differences between the two streams are often this large during summer months and urban streams frequently exhibit large increases in temperature following rain events (Fig. 2) (Sudduth et al., 2011; Somers et al., 2013). Furthermore, microbial responses to heat stress typically involve the investment in heat shock proteins and the inhibition of cell growth (Yura et al., 1993; Ye et al., 2012). We have reason to expect that because the mechanisms by which microbes respond to each of these physiological stressors differ, that there are likely to be important

synergistic effects between them. For example, silver exposure is known to cause membrane damage, and heat stress is known to cause depletion in osmoprotectants (Ye et al., 2012) changes which would each significantly reduce the capacity of individual microbes to effectively protect themselves from salt exposure. Each of our treatments was intended to induce a high level of stress compared to field conditions (the highest Cl concentrations measured in our urban stream are $\sim$60 mg Cl L^{-1}, the highest Ag concentrations in urban stream sediments are at $\sim$1.2 μg g sediment, and the highest temperature reached in our highly urban stream during the late spring is 33°C. These experimental treatment levels were guided by field data, but are more extreme than recorded field conditions in each case.

Microcosms were made anaerobic by placing them in airtight chambers with gas generating sachets that create an anaerobic atmosphere (BD GasPak EZ Container System, BD Diagnostics, Franklin Lakes, NJ, USA). We used indicator strips to check that all chambers reached and maintained anaerobic conditions.

As with Wittebolle et al. (2009), we estimated net ecosystem denitrification of the microbial communities by calculating the difference between the mean nitrite concentration of the negative controls (i.e., no inocula added) and the final nitrite concentration in each inoculated microcosm at the end of the incubation. Final nitrite concentrations were quantified spectrophotometrically on a plate reader (BMG Labtech, Cary, NC, USA).

Characterizing denitrifier communities

To explore potential links between initial community diversity and resistance, we used terminal restriction fragment length polymorphism (TRFLP) profiles to estimate operational taxonomic unit (OTU) richness and evenness. While communities may have changed in structure during the course of the experiment, our objective was to test the functional response of the initial community to treatments, not to examine community shifts. We focused, therefore, on characterizing the initial denitrifier communities in samples collected from the study streams.

We extracted DNA with PowerSoil kits (MoBio Laboratories, Carlsbad, CA, USA) from stream sediments collected on April 30, 2009 for making inocula. Extractions were done in triplicate for each stream. Following extraction, PCRs with *nirK* primers (FAM labeled nirK1F and nirK5R) (Braker et al., 1998) and Taq Master Mix (Sigma-Aldrich, St. Louis, MO, USA) were used to amplify denitrifier DNA. *nirK* is a functional gene that encodes the copper containing form of nitrite reductase, a key enzyme in the denitrification pathway. Each of 33 *nirK* PCR cycles consisted of 30 s at 95°C, 30 s at 46°C, and 45 s at 72°C. Three separate *nirK* PCRs were done for each DNA extraction. There is another form of nitrite reductase encoded by *nirS*, but we were unable to amplify *nirS* genes (using primer set nirS1F and nirS6R) (Braker et al., 1998) from these samples.

Products from the three PCRs per extraction were combined to yield one product pool per extraction. Since three extractions were done per stream, this ultimately yielded three PCR product pools per stream. PCR product pools were then cleaned with Qiaquick PCR purification kits (Qiagen, Germantown, MD, USA), checked for appropriate sizes by agarose gel electrophoresis, and then used to generate TRFLP products with endonuclease Hae III (New England Biolabs, Ipswich, MA, USA). Subsequent electrophoresis runs were done with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

To process TRFLP data, we used T-REX software (Culman et al., 2009) to determine a baseline threshold for filtering true peaks from background noise. Each peak corresponds to a unique restriction fragment length and represents an OTU. After filtering, we aligned peaks using a clustering threshold of one base pair. Samples with less than five peaks were eliminated. We transformed TRFLP data to relativized abundances by standardizing each peak area by the within-sample total peak area for all further analyses.

Data analyses

To determine whether denitrifier community OTU richness and evenness were lower for the urban versus forest denitrifier communities, we conducted one-tailed Student's *t*-tests using data from *nirK* TRFLP profiles. OTU richness is simply the total number of restriction fragment lengths identified in a TRFLP sample, while OTU evenness was quantified by calculating the Gini coefficient (*G*), which ranges from zero to one. A value of zero represents a perfectly

even community, while values close to one indicate a highly uneven community dominated by few taxa (Wittebolle et al., 2009). We used the ineq function (in package ineq) to calculate G values.

Besides diversity, urban and forest denitrifier communities may have also differed in composition (i.e., species identity). To explore this possibility, we tested the null hypothesis of no difference in composition between streams by using the adonis function in vegan (Oskanen et al., 2010) to conduct a permutational multivariate analysis of variance (perMANOVA) (Anderson, 2001). perMANOVA is similar to redundancy analysis and calculates a pseudo F-statistic by comparing the total variance explained by sample identity (in this case, urban vs. forest) to that explained by random permutations ($n = 9{,}999$) of identities. We used relativized abundances and Bray–Curtis distance measures.

To assess the effect of treatments on denitrification, we conducted a four-way analysis of variance (ANOVA) with fixed factors, including community type (urban or forest), the presence of oxidative ($AgNO_3$), osmotic (NaCl), and thermal (28 vs. 38°C) treatment. The model including a term for a four-way interaction had a higher AIC value than the model that only included all possible three-way interactions, so for ease of interpretability, we re-ran the model only considering three way interactions. Following ANOVA, we compared mean denitrification between reference (i.e., no pollution, 28°C) and treated microcosms *within* the same community type. To adjust for multiple comparisons, we used the Tukey honest significant differences method with a family wise confidence level of 0.95.

Results

In the month following our sediment sampling (June 9–July 17, 2009), we recorded stream water temperatures that were typically ~5°C higher in the urban stream throughout the day. Temperature differences between the two streams regularly reached 8–9°C in the late afternoon of sunny days (Fig. 2A). The highest temperature measured in Goose Creek during this early summer monitoring period was 32°C, while the highest temperature observed in Mud Creek tributary was 23°C. Stream water chloride concentrations were higher in the urban (mean = 31.0 mg/l) versus forest (mean = 13.0 mg/l) stream (Student's t test, one-tailed: $P = 0.002$) with conductivity values always higher in the urban Goose Creek (Fig. 2B). Sediment heavy metal content was significantly higher in the urban stream (Student's t test, one-tailed: $P < 0.05$) (Fig. 2C).

OTU richness tended to be higher for forest (mean = 44 OTUs) versus urban (mean = 16 OTUs) denitrifier communities, although the difference was only marginally significant (Student's t test, one-tailed: $P = 0.053$). Communities from the forested stream (mean G value = 0.63) were significantly more even than those from the urban stream (mean G value = 0.87) (Student's t test, one-tailed: $P = 0.021$). In contrast to the differences in diversity between streams, denitrifier community composition was not significantly different between the urban and forest streams (perMANOVA: $F_{1,3} = 7.457$, $P = 0.099$).

ANOVA results indicated that the communities from the two streams differed significantly in their responses to multiple stressors (Table 2). There was a significant three-way interaction between community type (i.e., urban or forest stream) and the presence of both trace metal and salt stress ($F_{1,700} = 6.09$, $P < 0.014$). Individual stressors had no effect (Ag or heat) or a stimulatory effect (NaCl) on nitrite reduction rates for both microbial communities. In combined salt and trace metal stressor treatments, however, there

Table 2 Results of three-way ANOVA with fixed factors for net denitrification

	df	Sum of squares	Mean square	F-ratio	P-value
Stream	1	1.106	1.106	9.342	0.002
Pollution	3	12.065	4.022	33.961	<0.001
Temperature	1	3.589	3.589	30.306	<0.001
Stream:pollution	3	3.179	1.060	8.948	<0.001
Stream:temperature	1	0.015	0.015	0.128	0.721
Pollution:temperature	3	1.274	0.425	3.585	0.014
Stream:pollution:temperature	3	0.099	0.033	0.278	0.841
Within groups (residual)	699	82.772	0.118		

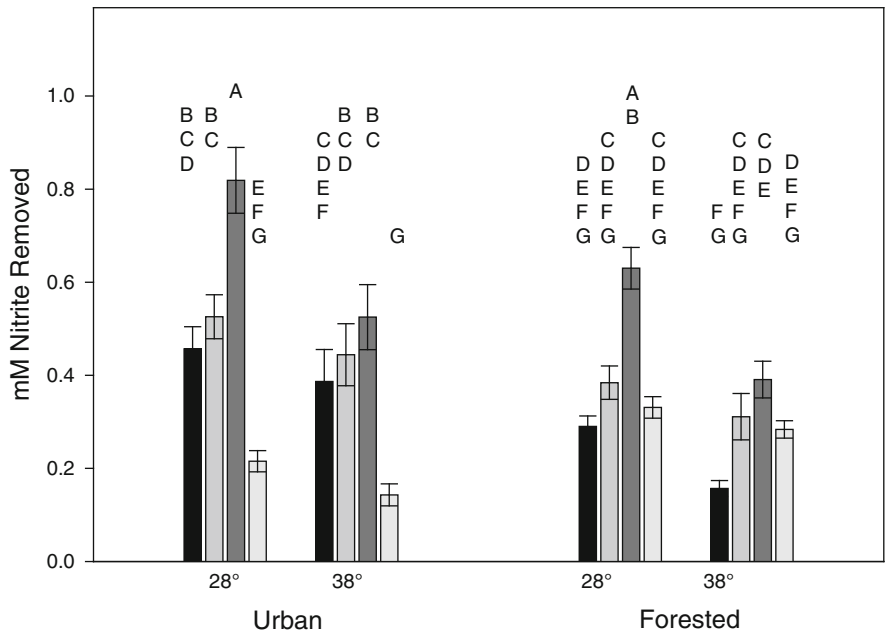

Fig. 3 Net denitrification (as estimated by removal of nitrite) under different experimental conditions (incubated at 28 or 38°C, without pollutants (*black*), with silver (*medium gray*), with salt (*dark gray*), or with both silver and salt (*light gray*) for urban and forested stream communities. Error bars represent ±1 standard error. *Letters* designate treatments that were significantly different across the entire experiment using Tukey's least significant difference

were significantly reductions in nitrite reduction rates by the urban stream microbes. In contrast, the forest stream microbial community was functionally resistant to the combination of these two stressors, with no observed change in nitrite reduction rates relative to control treatments. Temperature did not significantly interact with any of the other experimental factors, although lower rates of nitrite reduction were generally measured under the higher temperatures within each treatment (Fig. 3).

Discussion

Our results are consistent with the hypothesis that greater diversity leads to greater functional resistance (the insurance hypothesis). Prior exposure to high pollutant loads and higher temperatures apparently did not select for greater functional resistance as would be predicted by the legacy hypothesis or PICT model. Despite limited exposure to these stresses in the past, forest communities were able to maintain denitrification rates equivalent to those in control treatments when dosed with high levels of osmotic and oxidative stress, while urban communities exhibited a significant loss in function. This higher degree of functional resistance in the community with higher diversity only manifested itself when the communities were exposed to multiple stressors.

Microbes collected from our urban stream had been exposed to significantly higher chloride concentrations, stream water temperatures, and trace metal concentrations than their counterparts collected from a nearby forested stream (Sudduth et al., 2011; Violin et al., 2011; Somers et al., 2013). The legacy hypothesis, therefore, predicts that the urban stream microbial community would possess greater functional resistance to the three urban stressors than the "naïve" community collected from the nonurban stream. This expectation was not met. Instead, net denitrification for both community types was stimulated (at 28°C) or unaffected (at 38°C) by the salt treatment, and rates were unresponsive to either the temperature or silver treatment. These results suggest that, individually, no single chemical alteration was sufficiently stressful to slow the metabolic process of denitrification possibly because both communities had multiple members that could continue to denitrify in the face of the moderate disturbance.

In contrast, urban stream denitrifier function was significantly reduced when exposed to combined stressors. The maintenance of high denitrification rates in these more severe and selective disturbance, such as the *combined* silver and salt treatment, likely requires multiple tolerance mechanisms, such as efflux pumps, the accumulation of compatible solutes, and the alteration of cell membranes, which are each independently energy intensive and costly (Gadd &

Griffiths, 1977). Denitrifying organisms that possess the capacity to resist all three stressors are likely to be rare. Because the physiological responses to each of these stressors are likely to differ, we might also expect negative cotolerance among species (Vinebrooke et al., 2004). Perhaps greater functional diversity is associated with greater resistance only when very different types of selective stressors are involved (Wittebolle et al., 2009).

Since laboratory microcosms differed primarily in the type of inoculum given, initial community structure was likely an important factor in driving observed differences in functional resistance. Multiple aspects, including richness, evenness, and composition, define community structure. Based on the *nirK* TRFLP data, we can conclude with reasonable confidence that at least one subset of the urban and forest denitrifier communities had comparable compositions. While we cannot reliably estimate denitrifier diversity at the species level with TRFLP data, we can use TRFLP data to estimate diversity at a taxonomic resolution above the species level (i.e., the OTU level) (Blackwood et al., 2007; Fierer et al., 2007). These results suggest that *nirK* denitrifier OTU diversity, in terms of both richness and evenness, was higher in the forest versus urban community. However, it is important to note that taxa carrying other genes associated with the denitrification pathway (*nirS* and *nosZ*) can show differential responses to environmental stresses (Enwall et al., 2010).

According to the insurance hypothesis, greater diversity increases the odds of having community members that can respond favorably to a disturbance and/or functionally replacing important (but sensitive) members and, thereby, maintaining high functioning. Consistent with this theory, forest denitrifier communities were more diverse *and* more resistant than urban denitrifier communities. These findings are consistent with several other recent studies. Clements et al. (2008) demonstrated that metal polluted stream biofilm communities were more sensitive to UV-B exposure than their counterparts from a reference stream. Wittebolle et al. (2009) documented a positive relationship between denitrifier community evenness and resistance to salt stress.

Our experiment was designed to minimize all differences in laboratory microcosm conditions other than the source of microbial inoculum, so we conclude that initial community structure was the only important factor in driving observed differences in functional resistance.

Uncontrolled abiotic differences (e.g., salinity, organic matter, nutrients, pH, etc.) between urban and forest stream sediments used to make the inocula are potentially important confounding factors in this experimental design. To address this issue, we filtered and twice diluted inocula with spring water and trypticase soy broth, actions that should have reduced concentrations of chemicals that may have carried over into the inocula from sediments. Moreover, since the media used in all microcosms had high nutrient concentrations, any potential differences in nutrient concentrations between urban and forest inoculum should have been overwhelmed by being supplied well in excess of biological demand in the media. We should note that trypticase soy broth is a relatively nutrient rich medium and may have provided an advantage to fast-growing, opportunistic taxa. Although our experiment was a short-term incubation, if the communities from the more frequently disturbed urban stream contained a larger abundance of opportunistic taxa, differential growth of these groups may explain the higher rates of nitrite consumption observed in the control treatments exposed to urban inoculum.

Since estimates of functional resistance were based on differences between reference and treated microcosms within the same community type, differences between urban and forest stream inocula would not have affected our interpretations of whether a particular community type was resistant or sensitive to a treatment. For example, as long as all urban microcosms had similar pH, we can use differences in net denitrification between reference and treated microcosms to determine functional resistance for urban communities.

In summary, our results suggest that anthropogenic impacts, such as urbanization, may change both the composition and the functional diversity of naturally occurring microbial communities. Microbes in urban streams are subject to multiple stressors, including not only pollution and high temperatures, but also flashy hydrology, altered channel stability, and increased nutrient concentrations (Walsh et al., 2005). Such changes can decrease functional diversity which may, in turn, lead to decreased functional resistance. For urban stream denitrifiers we should consider the extent to which contaminant exposure and thermal stress may

contribute to reported declines in denitrification efficiency in urban streams (Mulholland et al., 2008). Although we urge caution in extrapolating results from this highly simplified laboratory study to the field, our results raise a number of intriguing questions about the potential feedbacks between the contamination and eutrophication of surface waters. While the added complexity may make our experimental results more difficult to interpret relative to more traditional, culture-based experiments that only include known denitrifier species, we argue that our approach is a step in the right direction toward a more realistic assessment of how highly complex microbial communities might respond to additional stressors in environments with differing degrees of pre-existing disturbance.

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