Microbial Ecology

Culturability of Stream Bacteria Assessed at the Assemblage and Population Levels

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Abstract

Lotic bacterial communities can be examined at multiple levels: from the assemblage level to populations of individual species. In stream environments, as in many other systems, the percentage of bacteria that are culturable is quite low. In this study, the culturability of the overall bacterial assemblage, as well as the culturability of three common species (*Acinetobacter calcoaceticus*, *Burkholderia cepacia*, and *Pseudomonas putida*), was determined in samples collected from four streams on three dates. Colony hybridization (colonies were grown on modified nutrient agar) and fluorescent *in situ* hybridization were used to calculate the percentage of cells of a given species that were culturable. Approximately half of the overall assemblage was estimated to be viable but nonculturable cells (VBNC). The culturability of two of the species was low (0.29% for *A. calcoaceticus* and 0.46% for *P. putida*), whereas the value for *B. cepacia* (2.48%) exceeded the overall assemblage level culturability (0.90%). Overall, both bacterial assemblages and populations were dominated by VBNC. These results show quantitatively that not all members of a species that has culturable representatives are culturable when retrieved from natural populations, likely because of interspecific phenotypic and genotypic variability. Thus, the large pool of nonculturable cells includes representatives of species that are, under some circumstances, culturable.

Introduction

Our understanding of the ecology of lotic bacteria depends on using a combination of investigative approaches applied at assemblage and population levels of organization. One assemblage-level pattern that has emerged is a relative consistency in the number of planktonic bacteria in lotic systems. A literature review of over 40 studies performed in Europe, Africa, and South and North Americas between 1951 and 2003 shows that 0.01–16.5 $\times$ 10^6 bacterial cells mL^{-1} exist in stream or river water (an exception was 100 $\times$ 10^6 cells mL^{-1} in the Ogeechee River; [59]). Although a range of four orders of magnitude at first seems large, considering the diverse array of differences in the systems studied, the number is rather similar across different systems (median = 2.35 $\times$ 10^6 cells mL^{-1}; n = 51). So too is the relative consistency in heterotrophic plate counts. There is a larger range, likely a result of differences in culturing methods, of five orders of magnitude [0.001–36.3 $\times$ 10^4 colony-forming units (CFU) mL^{-1}; median = 1.15 $\times$ 10^4 CFU mL^{-1}; n = 24] enumerated as viable, culturable cells from the variety of lotic habitats.

Because of the consistent differences between total and culturable cell counts in a variety of environments, "the great plate count anomaly" [55] concept emerged as an integral part of microbial ecology. In general, the ratio of the number of culturable bacteria to the total number in any assemblage seldom exceeds 1%. This phenomenon may occur because bacterial assemblages are dominated by organisms that are not readily cultivated using conventional approaches. Cells that remain viable but cannot be cultured are thought to do so as response to natural stress, stresses that might otherwise be lethal for the cells [45]. Stress factors may include starvation, exposure to temperature outside of normal growth range, and poor nutrient conditions, osmolarity, radiation, aeration/oxidants, desiccation, or harmful compounds [13, 17, 21, 45]. If these factors damage essential cell components, the result may be either temporary (sublethal injury) or permanent (lethal injury) loss of the ability for the cell to divide [26].

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Regardless of the cause, the low culturability of bacteria from natural environments led investigators to turn to culture-independent molecular techniques to explore the prokaryotic world under the assumption that the uncultured majority represent bacterial species that have never been cultivated and are thus unknown to science [49]. On the other hand, potentially, a component of the pool of unculturable viable cells represents species that have culturable representatives or that could be cultured under different circumstances.

The plate count anomaly is generally applied to a whole assemblage. Yet, what if culturability at the species level? For bacteria, it is often assumed that if a species is cultured in the laboratory, then all viable members may be cultured, and each viable cell can yield one colony on the appropriate growth medium [37]. When taking into account physiological responses to the environment, as well as intraspecific variability, it is known that some members of a natural population of bacterial species may be cultured, whereas others may not; that is, cells may have entered into a viable, but nonculturable (VBNC) state [51]. The VBNC state is thought to exist for several reasons, including periods of starvation, responses to stress, physical or chemical injury, or an extremely low metabolism [9, 10, 41, 50]. While questions of culturability are being pursued on a species-by-species basis, we may be overestimating the number of species that are unculturable by making the assumption that all members of a laboratory-cultured species are culturable in nature. Put in another way, to what extent does the plate count anomaly apply to species that appear to be readily cultivatable?

The objectives of this study are to (1) determine if streams in the same region had similar numbers of total and culturable bacteria and (2) examine the abundance and culturability of three common, culturable gram-negative bacterial species. By combining culture-dependent and -independent methods, this study attempts to determine if members of a population show different responses to culturing compared to the assemblage as a whole.

Materials and Methods

Study Site and Sample Collection. Samples were collected from four streams—Upper Three Runs, Four Mile Creek (upstream portion), Meyers Branch, and Tinkers Creek—with minimal anthropogenic disturbance on the 768-km^2 US Department of Energy's Savannah River Site, a facility that produced weapons-grade nuclear material beginning in the early 1950s. Riparian vegetation, classified as bottomland hardwoods, was about 40% trees and 15% shrub/vine in undisturbed stream areas [61]. Samples were collected from the upper reaches of Upper Three Runs Creek (UTR), a spring-fed blackwater stream typical of the Upper Coastal Plain [61] that drains a 492-km^2 catchment [27] in Aiken Co., SC. Tinker Creek (TIN) was a tributary of UTR. To the south and adjacent to UTR and TIN was the Four Mile Branch (FOU) watershed. Because the lower reaches of this stream historically received extensive anthropogenic disturbance, samples were collected from the upper, undisturbed reaches [57], an area that had soils similar to those in the UTR watershed. Lying to the south of the other watersheds was Meyers Branch, a tributary of Steel Creek. It drains a watershed independent of the UTR and FOU watersheds. A site map of the Savannah River Site can be found in Burger and Snodgrass [8].

Five replicate water samples (about 60 mL each) were collected midstream from the four study sites on 29–30 November 1995, 23–24 February 1996, and 19–20 April 1996 and kept on ice until processed later the same day. Subsamples from each replicate were used for bacterial enumeration as described below.

Physical and chemical measurements of stream conditions taken in triplicate at time of sampling are summarized in Table 1 and included the following: pH, temperature (Oakton WD-35615 pH/mV/Temperature Meter, Singapore), turbidity (2100P Hach Turbidimeter, Loveland, CO), total dissolved solids (44600 Conductivity/TDS meter), total phosphate (Hach DR100, Loveland, CO), and flow rate (FP 101/201 Global Flow Probe, Global Water Instruments, Fair Oaks, CA). One water sample was collected at each site for chemical analysis by the University of Georgia Chemical Analysis Laboratory. Analysis included dissolved organic carbon (with an OI Model 700 total carbon analyzer, College Station, TX), nitrate–nitrite (NO_3–NO_2 with an Autoanalyzer II Continuous Flow System, Technicon/Bran-Luebbe, Buffalo Grove, IL), and a 20-element plasma emission spectrum (Jarrell-Ash 965ICP, Franklin, MA; Table 1).

Colony Enumeration and Hybridization. To enumerate culturable bacteria, a portion of each sample was used to inoculate modified nutrient agar (mNA), which consisted of one-half concentration of nutrient broth with NaCl, $MgSO_4$, $CaCl_2$, NH_4Cl, and phosphate supplements [31], by spread plating: three plates per sample at two volumes (10 and 100 μL). The total number of colony-forming units (CFU) was determined after incubation for 7 days at 23°C.

Colony hybridization followed the methods of Leff *et al.* [29] and Lemke *et al.* [32]. Briefly, about 50 CFU per water sample were transferred from the original spread plate of mNA plates to a second mNA plate on day 3 of incubation, with the remaining CFU transferred to a separate plate on day 7. Transferred CFU were blotted onto MSI MagnaLift nylon filters (Westborough, MA), lysed with 0.5 M NaOH, washed with 0.5 M Tris buffer (pH 8.0), 2× SSC [52], and 95% ethanol, and heated

Table 1. Physical and chemical variables measured for Four Mile Creek, Meyers Branch, Tinker Creek, and Upper Three Runs Creek

Variable	*Units*	*Four Mile Creek*	*Meyers Branch*	*Tinker Creek*	*Upper Three Runs Creek*
Flow	m/s	4.0 (3.1–5.3)	3.6 (3.1–4.1)	3.8 (3.2–4.4)	3.0 (5.25–6.72)
Temperature*	°C	14.3 (12.7–16.8)	14.3 (10.4–0.048)	14.7 (11.0–18.7)	17.1 (16.4–18.6)
Conductivity**	mS/cm	0.020 (0.018–0.021)	0.047 (0.043–0.048)	0.024 (0.022–0.024)	0.014 (0.014–0.015)
pH**	−log(H+)	5.63 (5.22–6.14)	6.73 (6.44–7.02)	6.23 (5.92–6.90)	5.93 (5.25–6.72)
Turbidity*	NTU	2.72 (2.08–3.54)	2.33 (1.17–3.83)	3.53 (1.65–4.81)	2.91 (1.60–3.83)
DOC	mg/L	6.35 (5.64–7.07)	4.30 (3.54–4.62)	3.46 (3.29–4.62)	8.20 (1.76–11.8)
DIC*	mg/L	1.75 (0.81–2.59)	4.92 (4.56–5.53)	1.59 (0.85–2.33)	1.75 (0.49–2.97)
NO_3–N**	mg/L	0.03 (0.02–0.04)	0.07 (0.05–0.07)	0.05 (0.01–0.09)	0.21 (0.20–0.22)
PO_4–P	mg/L	0.08 (0.06–0.10)	0.09 (0.08–0.09)	0.19 (0.13–0.25)	0.08 (0.04–0.10)
Fe**	mg/L	0.94 (0.68–1.13)	0.43 (0.30–0.60)	0.13 (0.10–0.20)	0.20 (0.20–0.20)
Na*	mg/L	3.04 (2.62–3.47)	2.76 (2.31–3.0)	2.11 (2.08–2.12)	2.50 (2.26–2.69)
Mg**	mg/L	0.38 (0.35–0.42)	0.52 (0.47–0.54)	0.39 (0.39–0.40)	0.29 (0.23–0.38)

Mean (and range) are presented for November, February, and April sampling periods (symbols denote significant difference in parameter between streams for $^*p \leq 0.05$–0.01; $^{**}p < 0.01$).

(80°C, 2 h) to anneal DNA to the filter [54]. Hybridization followed the Boerhinger-Mannheim Genius system protocol (Indianapolis, IN).

Three species-specific probes were used at the appropriate hybridization temperatures: 5′-AGC ATC CTA TCG CTA GGT A-3′ for 16S rRNA gene of *Acinetobacter calcoaceticus* [6] at 49°C, 5′-CCC ATC GCA TCT AAC AAT-3′ for 23S rRNA gene of *Burkholderia cepacia* [53] at 47°C, and 5′-GCT GGC CTA ACC TTC-3′ for 16S rRNA gene of *Pseudomonas putida* [53] at 50°C. Oligonucleotide probes were labeled with digoxigenin and purified through a NucTrap probe purification column (#400701, Stratagene Cloning Systems, La Jolla, CA). Probe specificity was verified by hybridization to target American Type Culture Collection (ATCC; Manassas, VA) type strain controls and lack of hybridization to closely related species. Control strains were as follows: *B. cepacia* (25416), *P. putida* (12633), *A. calcoaceticus* (23055), *Burkholderia solanacearum* (11696), *Pseudomonas fluorescens* (13525), *P. aeruginosa* (10145), *P. chlororaphis* (9446), *Aeromonas hydrophila* (7966), *Comamonas testosteroni* (11996), *C. acidovorans* (15668), and *Hydrogenophaga pseudoflava* (33668). Filters with control DNA were included in all hybridizations.

Following hybridization, filters were processed by the recommended Boerhinger-Mannheim procedure, and autoradiographs were produced by exposing filters saturated with Lumiphos or CSPD (Boerhinger-Mannheim) to X-ray film for 1–2 h. Only hybridized CFU that were as dark or darker than controls were scored as positives (i.e., weak positives were not included, yielding conservative estimates of abundance).

Fluorescent in Situ Hybridization. To enumerate populations without culturing, a portion of each sample was mixed with buffered paraformaldehyde (50 mL water: 150 mL preservative) and stored at 4°C until processed for fluorescent *in situ* hybridization (FISH). Preservative was 4% paraformaldehyde in 0.5× phosphate-buffered saline [PBS; 1× PBS = (7.6 g NaCl, 1.9 g $Na_2HPO_4{\cdot}7H_2O$, 0.7 g $NaH_2PO_4{\cdot}2H_2O$) (liter 0.2 μm, filtered $dH_2O)^{-1}$, pH 7.2].

FISH was performed quantitatively by direct concentration and hybridization on a filter disk following the method of Lemke *et al.* [34]. Particles in the preserved sample were concentrated with 0.2-μm pore-size Anodisc 25 filter (Whatman, Maidstone, UK), which was rinsed with 1 mL filtered dH_2O and 1 mL 0.1% Nonidet P-40, transferred, sample-side up, to a petri dish with the lid containing filter paper saturated with wash buffer [0.9 M NaCl, 0.02 M Tris (pH 7.2), 0.1% sodium dodecyl sulfate (SDS)]. Texas-red-labeled probe [40 μL; 5 ng μL^{-1} in 6× SSC, 0.02 M Tris (pH 7), 0.1% SDS, 0.01% PolyA] was placed on each filter and incubated in the dark for 4 h.

Oligonucleotide probes and hybridization temperatures were 5′-CCT CTG TTC CGA CCA-3′ at 47°C for 16S rRNA for *B. cepacia* and 5′-GCT GCC TCC CGT

AGG AGT-3′ at 48°C for the domain Bacteria (EUB338) [2]. Probes for 16S rRNA for *A. calcoaceticus* (49°C) and *P. putida* (48°C) were also used, and the sequences are given above. Controls for these hybridizations were ATCC-type strain cultures and included *A. calcoaceticus* (ATCC 23055), *B. cepacia* (ATCC 25416), *P. fluorescens* (ATCC 13525), *P. putida* (ATCC 12633), and *Halobacterium salinarium* (negative EUB338; Ward's Biology, Rochester, NY). Excess probe was removed by filtration, and filters were rinsed twice with wash buffer (400 μL) at the respective hybridization temperature. Filters were washed by placing the filter in a clean petri dish, adding warm wash buffer (200 μL), and incubating for 10 min at the hybridization temperature. This sequence was repeated and followed by two dH_2O (400 μL) rinses. Filters were mounted on glass slides, and cells in 10 (EUB338) or 50 (*A. calcoaceticus*, *B. cepacia*, and *P. putida*) random fields were counted using epifluorescence microscopy (1000×; filter set 41004 for Texas Red, Chroma Tech. Corp., Brattleboro, VT; exciter HQ560/55, dichroic Q595LP, emitter HQ645/75).

Total Number of Bacteria. To determine the total number of bacteria, a portion of each sample was preserved with formaldehyde (1.5% final concentration). Bacteria were concentrated by vacuum onto 0.2-μm pore black polycarbonate filters (Poretics, Livermore, CA) and rinsed with 1 mL dH_2O. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg mL^{-1} final concentration) for 5 min [22, 47]. Fluorescent cells in 10 random fields were counted using filter set 31000 for DAPI (1000×; Chroma Tech. Corp.; exciter D360/40, dichroic 400DCLP, emitter D460/50645/75).

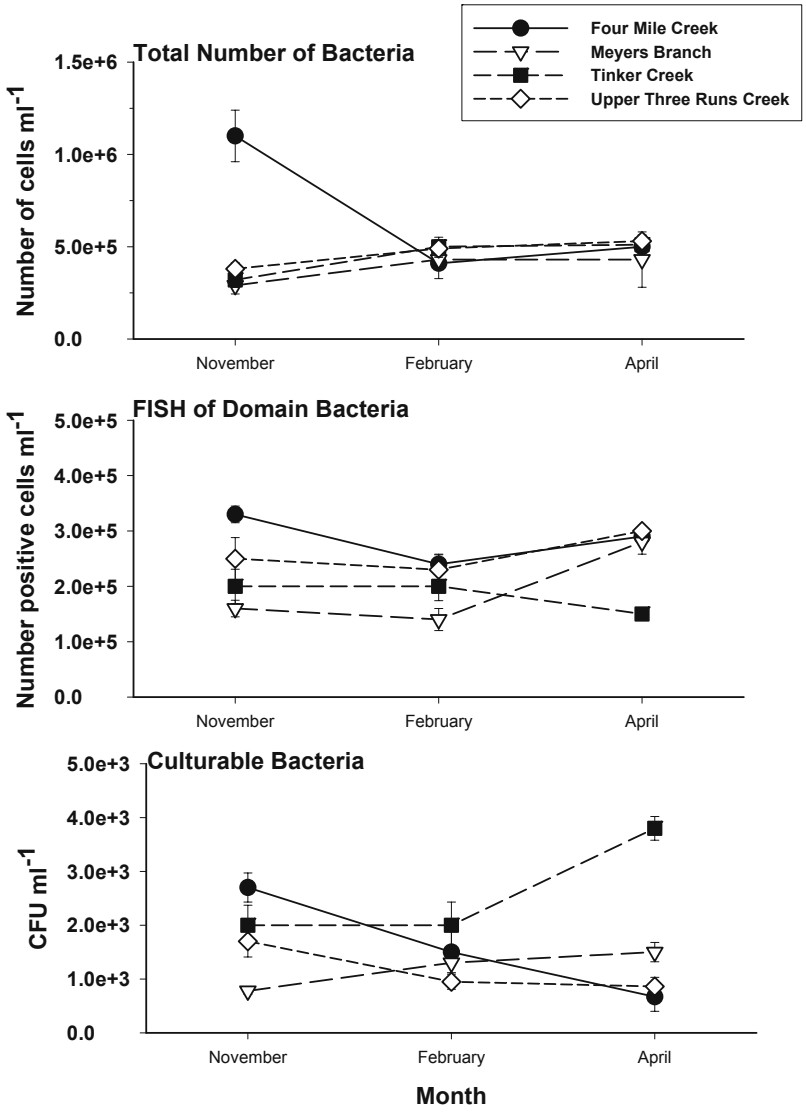

Figure 1. The total number of bacteria, number hybridized with domain Bacteria-specific probe, and total number of culturable bacteria found in four streams in similar drainages in South Carolina sampled at three seasonally different times of the year. *Symbols* represent averages ($n = 3$) ± standard error (SE).

Data Analysis. The number of CFU per milliliter hybridizing a given probe was determined by multiplying the proportion of CFU that hybridized the species-specific probe by the number of CFU per milliliter. The percent of cells that were culturable for each species was calculated based on the ratio of species CFU and species-level probe FISH. The overall percentage of cells that were culturable was calculated as the ratio between the total CFU and cells hybridizing the domain Bacteria probe. Percentages were arcsine-transformed before statistical analysis. Arcsine transformation was performed to stabilize variance in a proportion (i.e., to bring very high and low values toward the center of the normal distribution).

Statistical comparisons of results were accomplished by two-way analysis of variance to detect differences among streams at the three different times; a Tukey's test for multiple mean comparisons was used to confirm differences. Statistical analyses were completed using the SigmaStat computer program (ver. 2 for Windows, Jandel Corp., 1992–1995). If assumptions of normality (Kolmogorov–Smirnov test) and/or equal variance (Levene median test) were not met, nonparametric analysis was performed using the Kruskal–Wallis (K-W) test followed by the Student–Newman–Keul (SNK) test for mean differences.

Results

Bacterial Assemblage-Level Responses. Few differences were observed in overall bacterial numbers, based on DAPI staining, among the four streams (Fig. 1). Except for an unusually high number of bacteria in November at FOU (1.1×10^6 cell mL^{-1}; $p < 0.001$), the total number of bacteria in stream water was about $0.3–0.5 \times 10^6$ cells mL^{-1}. Even though the number of cells hybridizing the domain Bacteria probe differed significantly among streams (FOU = UTR > TIN = MB; $p < 0.001$) and seasons (April > February = November; $p = 0.028$), all measurements fell between 0.1 and 0.3×10^6 cells mL^{-1}. CFU mL^{-1} were typically $0.7–2.7 \times 10^3$ with an unusually high number detected in TIN in April (3.8×10^3; $p < 0.001$).

There were significant differences in percent of culturable bacteria (CFU $DAPI^{-1} \times 100$) among streams ($p < 0.001$) because of the high CFU mL^{-1} at TIN in April (Fig. 1); yet, when all samples from each site were considered together, about 0.38% of bacteria were culturable with similar values among the sampling times [$p = 0.35$; November, 0.45 ± 0.07% (avg. ± SE, $n = 20$); February, 0.33 ± 0.04% ($n = 20$); April, 0.36 ± 0.7% ($n = 19$)]. Although only 0.3–0.4% of the bacterial assemblage was culturable, just over one half of the bacteria were EUB+ (Table 2). Because very few of the EUB+ cells were culturable (avg. = 0.90%), the VBNC portion of the assemblage (EUB+ − CFU) was estimated to be about 48.8–59.8% of the total. No difference was found among streams or seasons for this value (data not shown).

Bacterial Population-Level Responses. There were few differences among the four streams in the abundance of the three bacterial species examined (Fig. 2). The number of *A. calcoaceticus* detected by FISH was similar among sites but varied seasonally; abundance was nearly twice as high in November (avg. = 1.74×10^4 cells mL^{-1}) than in the other months ($0.88–1.02 \times 10^4$ cells mL^{-1}; $p < 0.001$). Few *A. calcoaceticus* colonies were detected, with the greatest number found in February and April TIN samples (81 and 123 CFU mL^{-1}, respectively; $p = 0.013$).

The number of *B. cepacia* cells, based on FISH, ranged from 0.26 to 0.76×10^4 cells mL^{-1} ($p > 0.25$; Fig. 2) and did not exhibit the higher November peak as was seen with *A. calcoaceticus*. However, colony hybridization results revealed more variability. There were more culturable *B. cepacia* in November ($p < 0.001$), and at that time, the highest number was found in FOU stream water (453 CFU mL^{-1}; $p < 0.001$).

Unlike the other two species, significantly fewer *P. putida* were detected by FISH in November (0.31×10^4 cells mL^{-1}) than the other sampling dates ($p < 0.001$; Fig. 2). Differences among streams were small, yet statistically significant (UTR = FOU > MB = TIN; $p < 0.001$). All streams had a low number of culturable individuals (range 5–26 CFU mL^{-1}) throughout the study.

An estimate of the culturability of the different species was obtained by dividing the CFU per milliliter by the number of cells from FISH hybridizing a given species-specific probe. To have a point of reference to compare species culturability to assemblage culturability, the same

Table 2. The number of active and culturable bacteria in stream water from four stream sites in South Carolina

Month	*% EUB+*[a]	*% Culturable of EUB+*[b]
November	60.25	0.77
	(8.38; 20)	(0.07; 20)
February	49.16	0.83
	(6.22; 20)	(0.14; 20)
April	53.46	1.15
	(7.53; 15)	(0.31; 15)
Average	54.36	0.90[c]
p value	0.548	0.316

Values represent averages with standard errors; *n* in parentheses; *p* values are for comparison of months.
[a]% EUB+ = (number of bacterial cells positive to domain Bacteria probe mL^{-1})/(total number of bacteria mL^{-1}) × 100.
[b]% Culturable of EUB+ = (CFU mL^{-1})/(number of cells positive to domain Bacteria probe mL^{-1}) × 100.
[c]Average % culturable of viable greater than % culturable of total ($p < 0.001$).

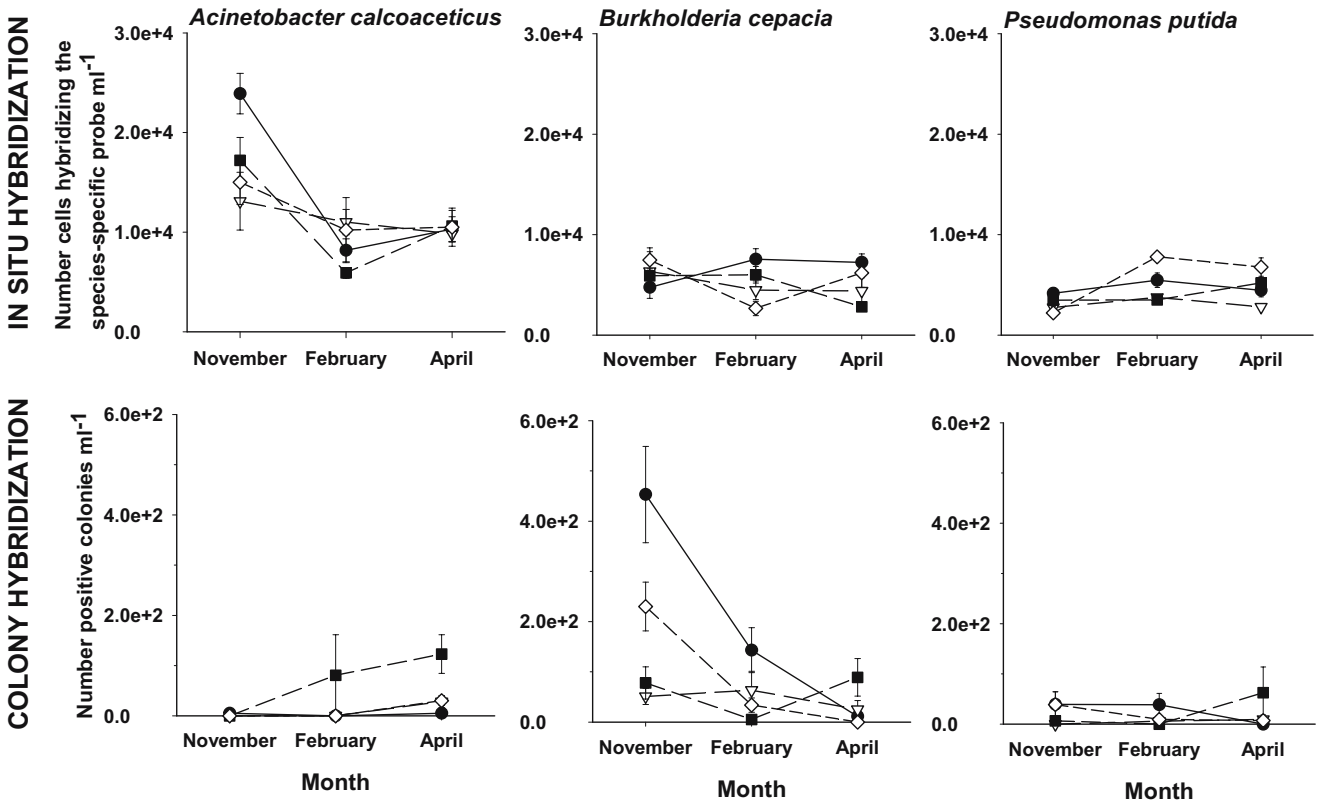

Figure 2. Fluorescence in situ hybridization (FISH) and colony hybridization measurements for three different gram-negative bacterial species. Samples taken from four different streams at three seasonally different times of the year; *symbol* = average (n = 3) ± SE. *Solid circle* = FOU, *open triangle* = MB, *solid square* =TIN, *open diamond* = UTR.

calculation was performed for the assemblage using the number of CFU and the number of cells hybridizing the domain Bacteria probe (Table 2). With the exception of *B. cepacia* in November, the number of culturable cells in a given species did not exceed 1.5% on average (Fig. 3). Percent culturable was generally similar among streams with the only significant differences ($p < 0.001$) by species being lower *A. calcoaceticus* in TIN during February and higher during April (1.2–1.5%) and very high *B. cepacia* at FOU in November (13.4%). *P. putida* showed no differences among streams or seasons.

Discussion

Overall, bacterial abundance (based on the number of total, active, and culturable bacteria) was similar among all stream sites in spite of differences in physical and chemical features. In this study, the total number of bacteria was between 0.3 and 0.5 $\times$ 10^6 mL^{-1} (except in November) and falls within the range of measurements reported in other studies in the Northern Hemisphere (e.g., [19, 30, 33, 34, 40, 46]). The total number of bacteria did not vary more than one order of magnitude within any given stream.

Except for TIN in April, the number of culturable bacteria fell between 0.7 and 2.7 $\times$ 10^3 CFU mL^{-1} in all seasons in the streams examined and represented a portion of the aerobic and facultative heterotrophs. Our results fall well within the range of values found by other studies (e.g., [3, 7, 20, 30, 33], 0.01–363 $\times$ 10^3 CFU mL^{-1}). Some of the variation in the number of CFU may be accounted for by a wide range of culturing factors (i.e., variation in media type, incubation temperature, and length) used by different investigators, but differences among streams also likely play a part. CFU abundance has been found to vary temporally and spatially in association with changes in water chemistry [34, 35].

Across different studies, a strong linear relationship exists between total bacterial number in stream water and the number of CFU, in spite of differences in methods employed (Fig. 4). This demonstrates that the percentage of cells that are culturable is remarkably consistent across different lotic ecosystems. From the regression results, one would predict that the average percent of culturable bacteria from a stream would be 0.61%.

FISH provides a culture-independent method of examining the abundance of targeted taxa. In addition, FISH permits detection of cells with high number of ribosomes, whereas nongrowing cells give either no or a diminished signal [15, 38, 48, 58]. About 55% of the total number of bacteria hybridized with the domain Bacteria probe. In more nutritionally rich environments, such

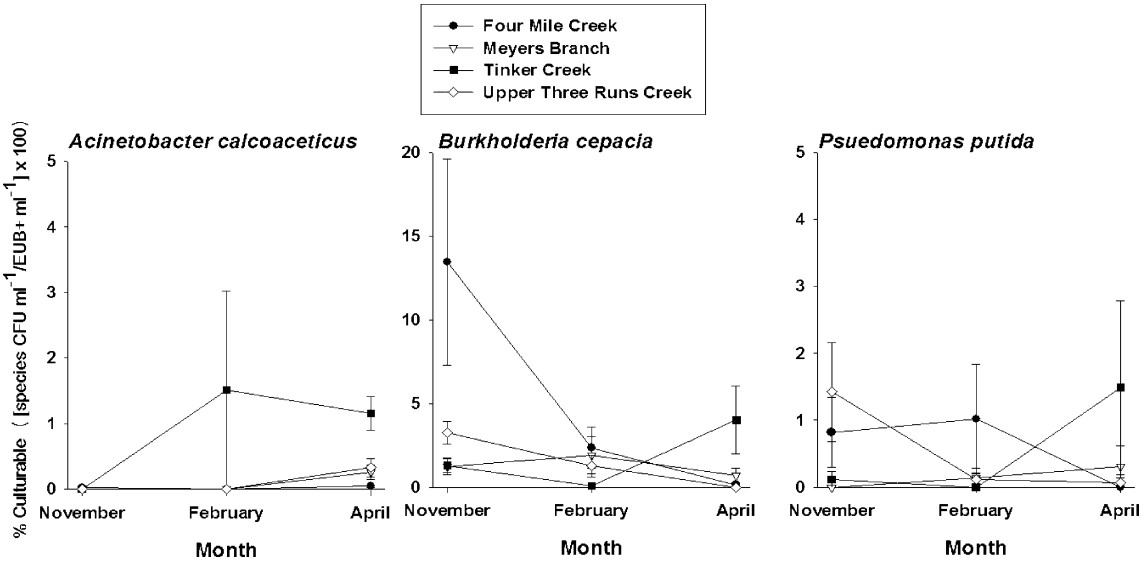

Figure 3. The percent culturable [(species CFU mL^{-1}/EUB+ mL^{-1}) × 100] by species taken from four different streams at three seasonally different times of the year; *symbol* = average (n = 3) ± SE. *Solid circle* = FOU, *open triangle* = MB, *solid square* =TIN, *open diamond* = UTR.

as activated sludge, up to 80% of cells hybridized with the domain Bacteria probe [39]. Members of the domain Bacteria detected by FISH represented on average 31.8% (range 3.11–74.42%) in the Cuyahoga River [35], whereas just over 50% (range 20–79%) were positive for EUB338 probe in 19 different freshwater aquatic ecosystems [5].

The abundance of each of the three species examined was similar among the study streams, and the differences among sampling dates were limited. On average, the number of FISH+ *A. calcoaceticus*, *B. cepacia*, and *P. putida* cells were 3.1, 1.5, and 1.0% of the total number of cells (DAPI), respectively. In spite of the fact that these species have known cultivatable representatives, the percent culturable did not exceed 0.4% for *A. calcoaceticus* and 0.6% for *P. putida*. Only *B. cepacia* had much higher culturability (range 1.22–4.81%). To our knowledge, only one other study reports the percent culturable for the species studied here, and those results are for stream sediments along Four Mile Creek, not water [42]. Results from McNamara *et al.* [42] showed 0% (August) to about 5% (February) culturability for *A. calcoaceticus*, 0% to about 3% for *B. cepacia*, and 0% to about 19% for *P. putida*. Thus, in both sediment and water, culturability

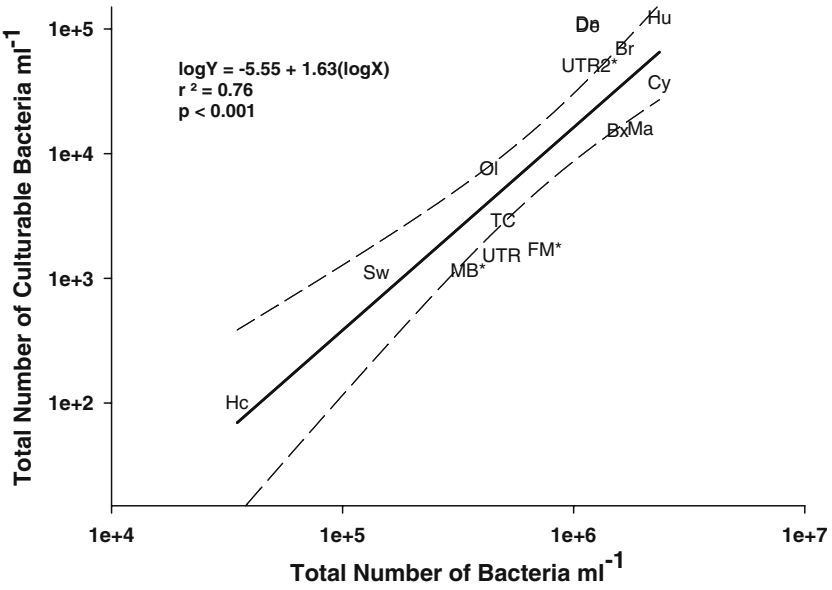

Figure 4. Relationship between total and culturable bacteria in lotic habitats from 13 locations (*dotted lines* indicate 95% confidence intervals). Values were log 10 transformations of median values obtained from literature or average measurements found in the present study. Key: Br (Breitenbach, Germany; [40]), Bx (Bixon Creek, OH; [30]), Cy (Cuyahoga River, OH; [28]), De (Dee, England; [44]), Dn (Danube River, Bratislava, Slovakia; [14]), FM (Four Mile Creek, SC; this study), Hc (Honeycut; [7]), Hu (Hull, England; [19]), Ma (Mahoning, OH; [30]), MB (Meyers Branch, SC; this study), Ol (Ogilvie, Yukon Territory, Canada; [24]), Sw (Swift River, Yukon Territory, Canada; [1]), TC (Tinker Creek, SC; this study), UTR (Upper Three Runs Creek, this study), UTR2 ([32]). Notes: superimposition of Dn and De; an * has been placed by data generated from this study.

of described species with culturable representatives is typically under 10%, suggesting that there is intraspecific variation in response to culture conditions.

There are two possible explanations for the low culturability of these species. First, it is possible that within a species, there are strains that differ in their culturability and cultivated strains are not representative of the culturability of a species as a whole. Second, the populations of these three species could be dominated by VBNC. The fact that FISH detection is dependent on the presence of sufficient ribosomes in a cell strongly suggests that cells detected in the study were viable. Overall, bacterial assemblages and populations both appear numerically dominated by VBNC. This suggests that the pool of nonculturable cells includes representatives of species that are, under some circumstances, culturable, as well as types of bacteria that have thus far defied cultivation.

As molecular techniques begin to describe assemblages of bacteria in nature, a renewed interest in culturing bacteria emerges (e.g., [25, 56]). Even though the number of culturable cells increases proportionally as cell number increases (Fig. 4), only about 1% of a bacterial assemblage can be accounted for by culturing, suggesting that the remaining 99% of bacterial cells represents a potentially astounding number of unculturable bacterial species. Yet, through application of FISH, DGGE, and compilation of 16S rRNA clone libraries, microbial ecologists typically estimate that less than 75 bacterioplankton operational taxonomic units exist in a given freshwater environment (e.g., [4, 12, 16, 36]). The subset of species exhibiting the trait of culturability in a freshwater habitat has not been described as well on the molecular level. We do know that while the majority of freshwater 16S rRNA cloned sequences are beta-proteobacteria [18, 23, 43, 60], our bias in culturing has led to an overrepresentation of copitrophs, such as those from the gamma-proteobacteria [58, 62], a trend that begins to change with increased culturing under oligotrophic conditions. Table 2 shows that on average, only about 0.9% of the viable bacteria produced CFU on media. Thus our results suggest that it may be easy to overlook species, even if they are thought to be "culturable." Prokaryotic life cycles have been described for a few species, but further study is required to better understand how a wider variety of species divide, grow, reproduce, mature, senesce, and die. Few free-living populations of any species possess the energy and resources to reproduce constantly, and therefore, in the case of the bacteria, will not always be culturable. Because a substantial number of "nonculturable" cells retain demonstrable metabolic activities [11, 26], it seems likely that a reproductive (i.e., highly culturable) phase is a brief segment of the life cycle being carried out by a small portion (<1%) of the population.

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