

Effects of large-scale poultry farms on aquatic microbial communities: A molecular investigation

Joseph A. Ringbauer Jr, Joseph B. James and Fred J. Genthner

ABSTRACT

The effects of large-scale poultry production operations on water quality and human health are largely unknown. Poultry litter is frequently applied as fertilizer to agricultural lands adjacent to large poultry farms. Run-off from the land introduces a variety of stressors into the surface waters including nutrients, antimicrobials and pathogenic bacteria. The Delaware, Maryland and Virginia (Delmarva) Peninsula has the highest concentration of broiler chickens per farm acre in the United States and provides an ideal location for studying the effects of stressors from poultry farms. We investigated potential effects by characterizing shifts in the structure of aquatic bacterial communities. DNA was isolated from microorganisms in water samples from streams and rivers at varying distances from, or having different frequencies of, litter applications. Fingerprints of 16S rDNA amplicons from bacteria in water samples collected during late summer 2001 to late spring 2002 were produced by denaturing gradient gel electrophoresis (DGGE). A statistical analysis of multiple fingerprints from each sampling location demonstrated that each site harboured a bacterial community significantly different from the communities at other sites. Similarly, the bacterial communities from each sampling time differed significantly from communities at other sampling times. Most importantly, a competitive, library-based analysis showed time of sampling (month) had a greater effect on community structure than did location.

Key words | bacterial community structure, DGGE, poultry farms

Joseph A. Ringbauer Jr
University of Missouri-Columbia,
Biochemistry Department,
117 Schweitzer Hall, Columbia,
MO 65211, USA

Joseph B. James
Fred J. Genthner (corresponding author)
National Research Council and US Environmental
Protection Agency,
National Health and Environmental Effects
Research Laboratory,
Gulf Ecology Division, Gulf Breeze,
FL 32561, USA
E-mail: genthner.fred@epa.gov

INTRODUCTION

The more than 7 billion (7×10^9) broiler chickens raised in the United States each year (USDA 2002) produce over 7 million tons (6.35 million tonnes) of litter consisting of manure, feathers, food and bedding material. Litter provides an inexpensive fertilizer; the manure it contains is a source of plant nutrients, including nitrogen, phosphorus and potassium. Litter also contains antimicrobials as millions of pounds of antiprotozoan agents and antibiotics are used in poultry production each year. Used both therapeutically and as growth promoters, antibiotics not only select for drug-resistant pathogens but may also exert selective pressure on indigenous bacteria. Run-off from litter-fertilized fields carries excess nutrients, pollutants,

antimicrobials and pathogens into nearby waterways, which may have negative effects on water quality and aquatic life. It is important to better understand these effects and develop methods to measure them.

The Delaware, Maryland and Virginia (Delmarva) Peninsula was chosen for this study because it has the highest concentration of broiler chickens per acre in the nation (Rhodes *et al.* 2000) and conditions there are favourable for studying potential effects of large-scale, concentrated animal feedlot operations (CAFOs) on surface waters.

Microbial communities in surface waters play an essential role in ecosystem function (Ford 1993). Possible

CAFO and seasonal effects were studied through analysis of changes in microbial community structure. Because less than 1% of the bacteria in aquatic systems may be readily cultured (Amann *et al.* 1995) a molecular based approach rather than traditional cultural based methods were used. Indeed, culture-independent or molecular methods are widely used in studies of microbial community structure (Amann *et al.* 1995; Head *et al.* 1998; Hugenholtz *et al.* 1998).

Denaturing gradient gel electrophoresis (DGGE), considered as a fingerprinting technique based on separations by sequence composition of PCR-amplified genes (Muyzer *et al.* 1993), was used for our study. DGGE with PCR-amplified 16S rRNA genes has been used in many laboratories to profile aquatic microbial communities (LaPara *et al.* 2000; Lane *et al.* 1985; Dumestre *et al.* 2001; Boon *et al.* 2002; Sekiguchi *et al.* 2002; Rowan *et al.* 2003). DGGE banding patterns change in accordance with changes in the predominant components of microbial populations (Theron and Cloete 2000). Thus, an analysis of Bacteria-specific PCR fragments provides a valuable tool for monitoring structure and dynamics of microbial communities (Boon *et al.* 2002).

DGGE patterns are typically analysed using digital images of the gels. Similarity coefficients are calculated using band- or curve-based analyses. Statistical tools such as principal component analysis (PCA), multidimensional scaling (MDS), two-way multiple analysis of variance (MANOVA), diversity index evaluation, regression analysis or a cluster analysis are used to evaluate similarities and differences between DGGE patterns (El Fantroussi *et al.* 1999; Dumestre *et al.* 2001; Smit *et al.* 2001; Boon *et al.* 2002; Sekiguchi *et al.* 2002; Clegg *et al.* 2003). Conclusions are then drawn giving an overview of the relationships between samples. With these analyses similarities amongst banding patterns were identified based on the effect of a predominant environmental factor (e.g. location, time, stressor). Multiple factors influencing community structure were not addressed. Our objective was to develop an analytical procedure capable of recognizing multiple, significant influences on community structure and use this procedure to investigate the relative influence of factors related to time and location.

MATERIALS AND METHODS

Study areas

The study area was in the upper Pocomoke River watershed located on the Delaware, Maryland and Virginia (Delmarva) Peninsula, northeast of Salisbury, Maryland (Figure 1a). This watershed is extremely flat and poorly drained with an extensive ditch network that extends into agricultural fields and forms the headwaters of most Pocomoke River tributaries. Poultry litter is applied to fields up to the edge of the ditches, which generally have no riparian buffers. Four surface-water sites, with varying proximity to manure applications, were chosen for study: Gum Branch (GB), a small ditch that runs through a predominantly forested area (intended to represent more pristine conditions) with some agricultural land use upstream of the sampling site; North Fork Green Run (NF), a channelized tributary of the Pocomoke River that passes through farm fields with recent manure application; South Fork Green Run (SF), a channelized tributary of the Pocomoke River that passes through farm fields in which no manure was applied for at least the past three years prior to sampling; and Pocomoke River (PR), which integrates the GB, NF and SF upstream inputs and has a wooded riparian zone near the sampling site.

A secondary study area was a single site where fresh water streams merge near Fort Pickens (FP), west of Pensacola Beach, Florida (Figure 1b). It served as a clean water control site because the Gum Branch site dried up after the first sampling and access to another control site on the Delmarva Peninsula could not be obtained.

Water sampling and DNA extraction

Water samples from all sites were collected four times over a 1-year period starting in September 2001 and repeated every three months thereafter (December 2001, March 2002 and June 2002). Water samples were collected from the upper 0.3 metres of surface water and placed on ice until processing. Water samples were first passed through a 3.0 µm nitrocellulose pre-filter (Millipore, Billerica, Massachusetts) to remove particles and large organisms. Free-living bacteria and bacteria attached to small particles

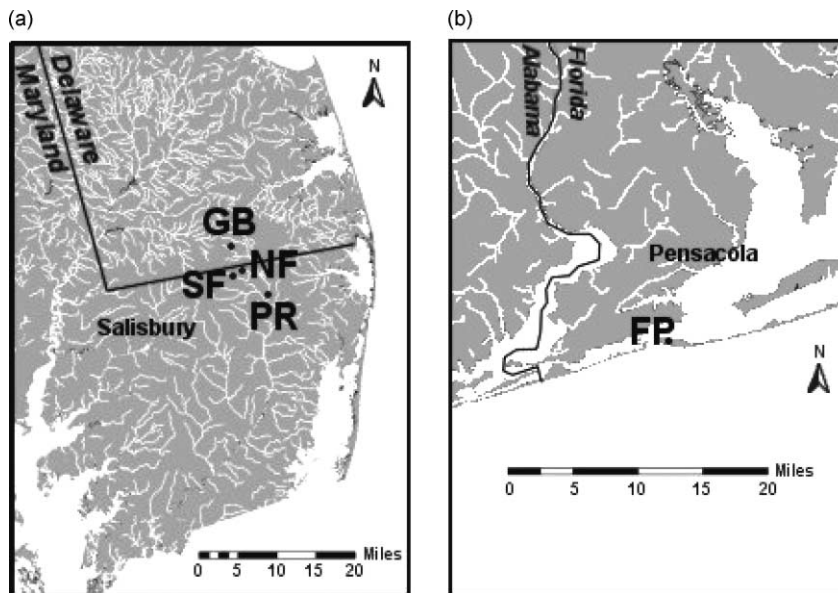


Figure 1 | Maps of the Delmarva (a) and Ft Pickens control site (b). GB, Gum Branch; NF, North Fork; SF, South Fork; PR, Pocomoke River; FP, Fort Pickens.

(<3 μm) were harvested from approximately 1 l of water by immobilizing them onto 0.22 μm Sterivex-GS filter cartridges (Millipore, Billerica, Massachusetts). Cartridges were frozen until the DNA was extracted from the microorganisms on the filter's surface. Filters were aseptically removed from the cartridges and sectioned into ~ 3 mm wide strips. DNA was extracted using the FastDNA SPIN kit for soil (Qbiogene, Inc., Carlsbad, California) according to the manufacturer's protocol except that a Mini-BeadBeater-8 homogenizer (BioSpec Products, Inc., Bartlesville, Oklahoma) was used for 2 minutes on maximum setting in place of Qbiogene's FastPrep[®] instrument in the bead beating step. Although triplicate 1 l water samples were collected at each site, a sufficient amount of DNA was extracted from a single sample to allow a minimum of three amplifications. Oligonucleotides from each amplification were separated on at least one gel. Between five and six DGGE separations were analysed from each sample.

DGGE

DGGE of PCR-amplified genes coding for 16S rRNA (Muyzer *et al.* 1993) was performed using the D-Gene system (Bio-Rad, Hercules, California). PCR amplification of

domain *Bacteria* 16S rRNA genes from community DNA for DGGE was performed using the *Bacteria*-specific forward primer GM5F (5'-CCTACGGGAGGCAGCAG-3', Muyzer *et al.* 1995) containing a GC-clamp (5'-CGC CCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCC CG-3', Muyzer *et al.* 1993) and the universal reverse primer Univ907R (5'-CCGTCAATTCCTTGGAGTTT-3', Amann *et al.* 1992). A master-mix was made containing all reagents except the template DNA. Each 50 μl reaction contained GeneAmp 10 \times PCR Gold Buffer, 25 mM MgCl_2 , 200 μM final concentration dNTPs (Muyzer *et al.* 1993), 25 pmole of each primer and 1.25 U AmpliTaq Gold (Applied Biosystems, Inc., Foster City, Arizona). Genes were amplified using a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City Arizona). Amplification using a touch-down PCR was performed as follows: initial denaturation at 94 $^\circ\text{C}$ for 5 min; 20 cycles of denaturation at 94 $^\circ\text{C}$ for 1 min, annealing for 1 min with 0.5 $^\circ\text{C}$ decrements at temperatures of 65 to 55 $^\circ\text{C}$ and extension at 72 $^\circ\text{C}$ for 3 min; 20 cycles of 94 $^\circ\text{C}$ for 1 min, 55 $^\circ\text{C}$ for 1 min, and 72 $^\circ\text{C}$ for 3 min; and final extension at 72 $^\circ\text{C}$ for 7 min. Amplicons were separated on 1-mm-thick, 35–60% denaturant (where 100% is 7M urea and 40% formamide) and 6–8% acrylamide gradient gels for 17 h in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) at 60 $^\circ\text{C}$ using a constant 70V.

After electrophoresis, the gels were stained with SYBR Gold (Molecular Probes, Eugene, Oregon) and photographed on a UV transilluminator. Gel photographs were digitized using a HP Scanjet ADF (Palo Alto, California).

Analysis of bacterial community structure

DGGE gel images were processed and the data were analysed using BioNumerics GelCompar software v.3 (Applied Maths, Sint-Martens-Latem, Belgium). PCR amplified 16S rDNA from *Ralstonia* sp., *Bacillus cereus*, *Stenotrophomonas maltophilia* and *Microbacterium* sp. provided a four-band standard for alignment and normalization of the gels. Similarities among banding patterns were calculated using Jeffrey's \times algorithm. The formula for this coefficient is $C/2 (1/T_1 + 1/T_2)$ where T_1 is the total number of bands in lane 1, T_2 is the total number bands in lane 2, and C is the number of common bands (Jeffreys et al. 1991). To show relationships among samples dendrograms were constructed using the un-weighted pair group average linkage method (UPGMA).

Three DGGE fingerprint libraries were assembled for this analysis. One library was site-specific, composed of units for each of three sites (NF, SF and PR). The second library was time-specific, possessing units for each month samples were collected (March, June, September and December). The third library was a compilation of all of the units found in the first two libraries. Data from the Gum Branch site were not included in the libraries as only one sampling month was available. Libraries including data from Ft Pickens Florida, the remote pristine site, were used when appropriate.

Library units mentioned above are, therefore, sub-sets of a main library. To further explain, separate library units were assembled from fingerprints derived from samples taken at each location and at time of sampling (month). For example, a fingerprint derived from a sample taken in March from Pocomoke River is included in both the Pocomoke River library unit (found in the site-specific library) and the March library unit (found in the time-specific library). A DGGE fingerprint is then submitted for assignment to the appropriate library units found in the chosen library. Average similarity was used to assign a selected fingerprint to the library unit yielding the largest

average similarity to all fingerprints within that unit. When using the library containing all possible units, assignment of a fingerprint to a library unit composed of fingerprints taken at the same location rather than the same month indicates that location had the dominating influence on community structure rather than month. We term this a 'competitive, library-based analysis' (Table 1). Thus, 'competitive, library-based analysis' describes the process of all library units 'competing' for the assignment of the selected fingerprint.

Maximum similarity was not used as each sample submitted for sorting would invariably be matched to itself. Therefore, no assignment could have been made as each sample was located in two library units (one for site the sample was taken from and another for month).

Assignment of a fingerprint to a library unit corresponding to the actual month or site was considered to be a correct assignment. The percentage of correctly assigned observations for all fingerprints was computed and reported as the estimated rate of correct classification (ERCC). Expected values for a null hypothesis of random association were also computed. Confidence limits for the percentage correctly classified were obtained from statistical tables (Sokal and Rohlf 1987) and compared with the percentages expected under the null hypothesis. When there was more than one legitimate parameter for assignment (i.e. month or site), competitive library-based analysis was used to determine which parameter, if any, correctly assigned the sample. The percentage of correct assignments attributable to each

Table 1 | Competitive library-based analysis

Library ^a	
Library units (site) ^b	Library units (month) ^c
Fort Pickens	March
North Fork	June
South Fork	September
Pocomoke River	December

^aThe library can contain units for site and/or month. Fingerprints submitted for assignment are sorted into a single library unit based on average similarity. Each fingerprint is located in all library units which correspond to its site and/or month

^bEach library unit contains all fingerprints from the site regardless of month

^cEach library unit contains all fingerprints from the month regardless of site

parameter was computed. Confidence intervals for the percentages, obtained from statistical tables (Sokal & Rohlf 1987), were used to determine whether one parameter accounted for significantly more correct assignments than the other parameter.

Quality factor, a function available in the BioNumerics software, provided an indication of the confidence of the identification. These values were obtained by comparing the average similarity between the unknown entry and the library unit's entries with the average similarity of the library unit's entries with each other. If the first value is as high or higher than the second one, the unknown entry is considered to fit well within the library unit. Thus, this quality indication takes into account the internal heterogeneity of the taxon defined in the library unit.

RESULTS

DGGE analysis

The DGGE profiles of the surface water bacterial communities from the 4 Delmarva and 1 Pensacola Beach sites sampled every 3 months from September 2001 through June 2002 are shown in Figure 2. Visual comparison of the DGGE banding patterns from duplicate samples revealed that the profiles obtained were reproducible (data not shown).

The average number of distinct bands separated on the gels from the FP site samples, 20 bands, was nearly twice that of all samples from the Delmarva Peninsula, 11 bands. The average number of bands for NF, 13 bands, was highest among the Delmarva Peninsula samples; the average number of distinct bands for SF and PR was 11 and 10, respectively. Of the September samples NF, SF and PR had approximately twice the number of bands than did the single GB sample.

Some general observations were made about bands on the gel images. Most of the bands from FP were different from those from the Delmarva sites. Several bands from the Delmarva samples occurred in only one of the replicate gels at a particular time. Most of the single missing bands occurred in the September samples. There were numerous indistinct bands between the second and third markers (Figure 2). These bands were very close together and often formed a smear on the gels. To show this variability the mean similarity values calculated from the replicate fingerprints derived from single water samples are shown in Table 3. These mean similarity values ranged from a high of 56 for the South Fork/December replicates to a low of 32 for the North Fork/March replicates.

Cluster and competitive library-based analyses

A digital image of the DGGE gel shown in Figure 2 was analysed by two different approaches: a UPGMA

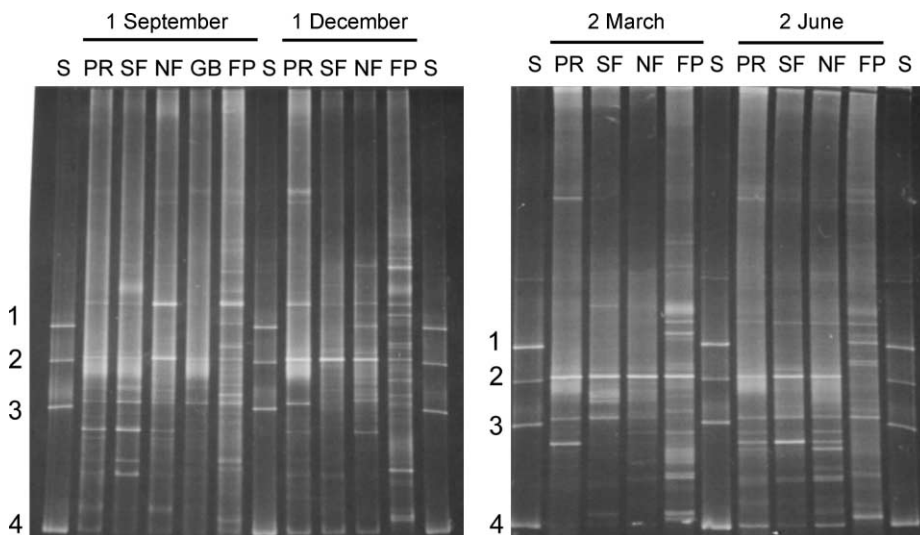


Figure 2 | DGGE profile of bacterial communities from surface waters in the Delmarva Peninsula and Pensacola Beach, Florida (Fort Pickens). Lanes: S, standards; FP, Fort Pickens; NF, North Fork; SF, South Fork; PR, Pocomoke River; GB, Gum Branch. Standards: 1. *Ralstonia* sp. 2. *Bacillus cereus* 3. *Stenotrophomonas maltophilia* 4. *Microbacterium*. The dates above the lanes indicate when the samples were taken.

dendrogram and a competitive library-based analysis. The UPGMA dendrogram shown in Figure 3 was created from the digitized banding pattern data of a gel which possessed a single fingerprint from all the samples taken. This dendrogram, selected as being representative of all separations, shows that the FP site samples formed a single cluster with 26% similarity to all the Delmarva samples. Most of the Delmarva samples, however, grouped by month of sampling rather than by location, more so for the first half of the year when both Delmarva samples from March and June formed separate clusters. The March cluster was 37% similar to the rest of the Delmarva samples, whereas the June cluster was 52% similar to the rest of the Delmarva samples. Samples taken during the second half of the year (September and December) did not completely cluster by month. Two (SF and PR) of the December samples clustered separately while the remaining December sample (NF) grouped with the September samples from SF and PR. The GB and NF September samples grouped together.

Two libraries, containing units composed of either month or site data, created from all data from the replicate

amplifications and multiple separations, were then probed (average similarity) with all gel fingerprints. For the time-specific library containing units for each month, the ERCC was 90%. The 95% confidence interval was 79–97% (Sokal & Rohlf 1987). Random chance alone would allow for a 25% correct identification since there were four sampling months. For the site-specific library containing units for each Delmarva site, NF, SF and PR, the ERCC was 80% with a 95% confidence interval of 66 to 90% (Sokal & Rohlf 1987). Since there were three sites, random chance alone allows for a 33% correct identification.

One large library was assembled by combining the month and site libraries mentioned above (Table 1). All sample data were submitted to this library for sorting (Table 2). Samples were allowed to sort to one of the eight library units (March, June, September, December, NF, SF, PR and FP) by average similarity. All 23 FP fingerprints sorted to the FP library unit and not to their respective month library unit. Seventy Delmarva samples were submitted to the library for sorting. The majority, 43/70 (61%), of the Delmarva fingerprints sorted to their respective

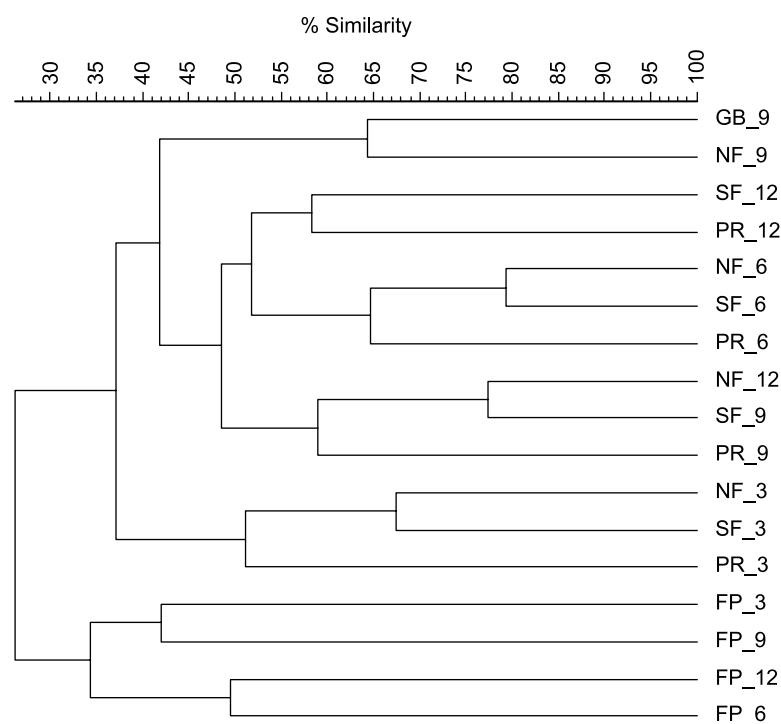


Figure 3 | Dendrogram of microbial populations from samples analysed by DGGE. The DGGE gels were analysed using BioNumerics software (Applied Maths, Austin, Texas) with the Jeffrey's X similarity algorithm, average similarity, and UPGMA dendrogram algorithms. The first two letters are the site and the last number is the month. Fort Pickens, FP; North Fork, NF; South Fork, SF; and Pocomoke River, PR. March, 3; June, 6; September, 9; December, 12.

Table 2 | Competitive, library-based analysis

Fingerprints submitted for sorting site_month ^a	Assigned library unit site or month/quality factor ^b					
	Analyses from replicate DGGE gels ^c					
FP_3	FP/B	FP/B	FP/B	FP/B	FB/B	ND ^d
FP_6	FP/B	FP/B	FP/B	FP/B	FP/B	FP/B
FP_9	FP/B	FP/B	FP/B	FP/B	FP/B	FP/B
FP_12	FP/B	FP/B	FP/B	FP/B	FP/B	FP/B
NF_3	3/B	3/B	3/B	3/B	NF/B	12/C
NF_6	6/B	6/B	6/B	6/B	6/B	NF/B
NF_9	9/B	9/B	9/B	9/B	NF/B	NF/B
NF_12	NF/B	NF/B	NF/B	FP/C	9/B	12/B
PR_3	3/B	3/B	3/B	3/B	PR/B	9/B
PR_6	6/B	6/B	6/B	PR/B	PR/B	PR/B
PR_9	9/B	9/B	9/B	9/B	PR/B	ND
PR_12	PR/B	PR/B	PR/B	PR/B	12/B	12/B
SF_3	3/B	3/B	3/B	3/B	SF/B	ND
SF_6	6/B	6/B	SF/B	SF/B	PR/B	3/C
SF_9	9/B	9/B	9/B	9/B	SF/B	ND
SF_12	12/B	12/B	12/B	12/B	12/B	SF/B

^aFort Pickens, FP; North Fork, NF; South Fork, SF; and Pocomoke River, PR. March, 3; June, 6; September, 9; December, 12

^bQuality factor, a function available in BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium), provided an indication of the confidence of the identification. A–E, highest confidence to lowest confidence

^cA minimum of three amplifications from a single water sample were separated on replicate DGGE gels

^dND, not done

month library unit. Thirty-one per cent of the Delmarva samples (22/70) sorted to their respective site library unit. Taken together these data provide an ERCC for the Delmarva samples of 92% with a 95% confidence interval of 79 to 97%. The remaining 8% sorted to neither their corresponding month or site unit. All but three of the assignments scored a quality factor of 'B', suggesting a fairly high degree of confidence in the assignments. The three that scored the lower factor 'C' were incorrect assignments.

DISCUSSION

Similarities and differences in the DGGE banding patterns correspond to similarities and differences between the sequences and composition of amplified 16S rRNA genes for each sample. These patterns allow for comparisons of the bacterial community from one sample site or time to that of another. The number of bands is also an indicator of differences in community diversity. The average number of

distinct bands separated on the gels from the FP site samples was twice that of all samples from the Delmarva Peninsula (20 vs. 11) and most of the bands from FP were different from those from the Delmarva sites. This indicated that the microbial diversity of 16S rRNA genes was higher in this pristine subtropical stream than in the surface waters of the Delmarva Peninsula.

As shown in [Table 3](#) there was variability in the banding patterns among PCR amplifications and DGGE gel separations from a single water sample; thus, a representative dendrogram, created using data from a single digitized DGGE gel, was presented ([Figure 3](#)). This dendrogram showed that the FP site samples formed a single cluster apart from the Delmarva Peninsula samples ([Figure 3](#)), indicating that the microbial community of the FP site was quite different from the Delmarva population. This result was expected since FP is in a subtropical climate and over 1,500 kilometres away from the Delmarva Peninsula. Microbial communities separated from each other by a great distance and in a different climate are bound to be distinct from each other. Thus, this site, which completely lacked a poultry influence, was so different in its bacterial community structure that it did not provide a useful, unimpacted, control environment.

It is interesting to note that, in addition to the separation between the FP and Delmarva samples ([Figure 3](#)), most of the Delmarva samples grouped by month not by site. The March samples formed a group separate from all the remaining Delmarva samples. The remaining samples tended to group by month although not all the months separated in such distinct groups as March samples. Based on this dendrogram, it appeared that month was a more

important factor than site in influencing the microbial community.

Library-based analyses, based on genotypic fingerprints of fecal indicator bacteria, are commonly used in source-tracking (for a review see [Scott *et al.* 2002](#)). These library-based, source-tracking methods involve the construction of library units each possessing fingerprints from indicator organisms obtained from several known animal sources (e.g. human, wild animal, domestic animal). Fingerprints generated from these same indicators (isolated from water contaminated with fecal matter) are submitted for assignment to a library unit for source identification. Source-tracking through the use of libraries is based on the premise that only one correct assignment exists.

Our competitive library-based analysis is, however, not based on a single correct assignment nor the submission of unknown fingerprints to the library. Rather, multiple correct assignments are possible. It was therefore necessary to determine if any or both of the factors investigated (month as a proxy for season and site as a proxy for CAFO impact) could indeed be employed successfully for partitioning samples. In the case that libraries specific to both factors correctly identified samples (an ERCC significantly above random chance), the samples were submitted to a library with all significant factors available as library units. If one factor was more important to the microbial composition than another it was expected that the sample would partition a significantly greater percentage of submitted samples to the most important factor. In this study, while both site and month libraries were individually successful at identifying samples, when both options were present, samples were correctly identified with month significantly more often than with site. Thus, the analysis used in this work can be applied to other studies of community structure by examining not only time and location but other parameters such as salinity, nutrients, stressors and temperature, thereby, providing greater insight between changes in DGGE banding patterns and ecology.

Results of our competitive library-based analysis suggest that, for the FP site, location rather than month had the largest influence on microbial community structure ([Table 2](#)). This result is not surprising since, as was discussed with cluster analysis, the average temperature throughout the year at the subtropical FP site does not vary

Table 3 | Mean of similarity values among DGGE banding patterns from multiple analyses of single water samples

Site	March	June	September	December
Ft. Pickens	42	42	48	54
North Fork	32	38	37	52
South Fork	44	37	42	56
Pocomoke River	33	36	47	40

as widely as it does in the temperate climate of the Delmarva. In addition since the FP profiles were far different from the Delmarva profiles the average similarities would be greater with location. The Delmarva profiles, however, sorted primarily with month (61%) rather than site (31%) indicating that the time of year had a greater influence on microbial community structure than location (Table 2). Although these data may suggest otherwise, a CAFO impact cannot be completely disregarded, as none of the Delmarva sites was totally pristine. The site judged the least exposed (SF) did not have chicken manure applied to the surrounding fields for at least 3 years prior to our sampling. The microbial community of SF was, however, similar to NF where manure was applied yearly. At least three possible explanations can be offered. It may be that CAFOs have a minimal effect on the surface water microbial populations. Conversely, it may be that the impact is long lasting (at least 3 years) on surface water microbial populations. CAFOs may have a more substantial effect on the microbial communities found in the sediment and those attached to particulate matter in the streams. Finally, the hydrology of the sampling areas on the Delmarva is such that, unless there is a very intense storm, there is essentially no runoff from the litter-amended fields (S. Jordan, personal communication). As our samples were taken at base flow, it is likely the streams were predominantly groundwater.

Since *Muyzer et al.* (1993) introduced the genetic fingerprinting technique of DGGE to the study of microbial community ecology, many other researchers have exploited DGGE to examine geographical, temporal or stressor effects on microbial community structure (*Ferris et al.* 1996; *El Fantroussi et al.* 1999; *Iwamoto et al.* 2000; *Riemann et al.* 2000; *Ogino et al.* 2001; *Smit et al.* 2001; *Boon et al.* 2002; *Sekiguchi et al.* 2002; *Rowan et al.* 2003). Several approaches have been used to analyse DGGE fingerprints. Cluster analysis (*Dumestre et al.* 2001), two-way analysis of variance (*Clegg et al.* 2003), principle component analysis (*Sekiguchi et al.* 2002), probability-based similarity index (*Rowan et al.* 2003) and multidimensional scaling (*Boon et al.* 2002) are among some of the statistical approaches used to analyse these fingerprints of microbial communities. We believe this to be the first time a competitive library-based approach was used for interpreting the DGGE fingerprint data derived from environmental samples.

Blending this approach with cluster analysis enhanced the ability of DGGE community profiles to evaluate effects of stressors on the aquatic microbial communities.

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REFERENCES

- Amann, R. I., Stromley, J., Devereux, R., Key, R. & Stahl, D. A. 1992 Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Appl. Environ. Microbiol.* **58**, 614–623.
- Amann, R., Ludwig, W. & Schleifer, K. 1995 Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**, 143–169.
- Boon, N., De Windt, W., Verstraete, W. & Top, E. M. 2002 Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. *FEMS Microbiol. Ecol.* **39**, 101–112.
- Clegg, C. D., Lovell, R. D. & Hobbs, P. J. 2003 The impact of grassland management regime on the community structure of selected bacterial groups in soils. *FEMS Microbiol. Ecol.* **43**, 263–270.
- Dumestre, J-F., Casamayor, E. O., Massana, R. & Pedros-Alio, C. 2001 Changes in bacterial and archaeal assemblages in an equatorial river induced by the water eutrophication of Petit Saut dam reservoir (French Guiana). *Aquatic Microbial Ecol.* **26**, 209–221.
- El Fantroussi, S., Verschuere, L., Verstraete, W. & Top, E. M. 1999 Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. *Appl. Environ. Microbiol.* **65**, 982–988.
- Ferris, M. J., Muyzer, G. & Ward, D. M. 1996 Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations

- inhabiting a hot spring microbial mat community. *Appl. Environ. Microbiol.* **62**, 340–346.
- Ford, T. E. (ed.) 1993 *Aquatic Microbiology: An Ecological Approach*. Blackwell, Oxford.
- Head, I. M., Saunders, J. R. & Pickup, R. W. 1998 Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microb. Ecol.* **35**, 1–21.
- Hugenholtz, P., Goebel, B. M. & Pace, N. R. 1998 Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* **180**, 4765–4774.
- Iwamoto, T., Tani, K., Nakamura, K., Suzuki, Y., Kitagawa, M., Eguchi, M. & Nasu, M. 2000 Monitoring impact of *in situ* biostimulation treatment on groundwater bacterial community by DGGE. *FEMS Microbiol. Ecol.* **32**, 129–141.
- Jeffreys, A. J., Turner, M. & Debenham, P. 1991 The efficiency of multi-locus DNA fingerprint probes for individualization and establishment of family relationships, determined from extensive casework. *Am. J. Hum. Genet.* **48**, 824–840.
- Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. 1985 Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. *Proc. Natl Acad. Sci.* **82**, 6955–6959.
- LaPara, T. M., Nakatsu, C. H., Pantea, L. & Alleman, J. E. 2000 Phylogenetic analysis of bacterial communities in mesophilic and thermophilic bioreactors treating pharmaceutical wastewater. *Appl. Environ. Microbiol.* **66**, 3951–3959.
- Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. 1993 Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**, 695–700.
- Muyzer, G., Teske, A., Wirsén, C. O. & Jannasch, H. W. 1995 Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch. Microbiol.* **164**, 165–172.
- Ogino, A., Koshikawa, H., Nakahara, T. & Uchiyama, H. 2001 Succession of microbial communities during a biostimulation process as evaluated by DGGE and clone library analyses. *J. Appl. Microbiol.* **91**, 625–635.
- Rhodes, K., Hueston, W., Mashima, T. & Vicari, A. 2000 Environmental Consequences of the Use of Veterinary Antimicrobials in Concentrated Animal Feeding Operations. In: *Part One: Compilation of the Use and Characteristics of Selected Antimicrobials*. Virginia-Maryland Regional College of Veterinary Medicine, College Park, Maryland.
- Riemann, L., Steward, G. F. & Azam, F. 2000 Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Appl. Environ. Microbiol.* **66**, 578–587.
- Rowan, A. K., Sanpe, J. R., Fernside, D., Barer, M. R., Curtis, T. P. & Head, I. M. 2003 Composition and diversity of ammonia-oxidizing bacterial communities in wastewater treatment reactors of different design treating identical wastewater. *FEMS Microbiol. Ecol.* **43**, 195–206.
- Scott, T. M., Rose, J. B., Jenkins, T. M., Farrah, S. R. & Lukasik, J. 2002 Microbial source tracking: Current methodology and future directions. *Appl. Environ. Microbiol.* **68**, 5796–5803.
- Sekiguchi, H., Watanabe, M., Nakahara, T., Xu, B. & Uchiyama, H. 2002 Succession of bacterial community structure along the Changjiang River determined by denaturing gradient gel electrophoresis and clone library analysis. *Appl. Environ. Microbiol.* **68**, 5142–5150.
- Smit, E., Leeftang, P., Gommans, S., van den Broek, J., van Mil, S. & Wernars, K. 2001 Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. *Appl. Environ. Microbiol.* **67**, 2284–2291.
- Sokal, R. R. & Rohlf, F. J. 1987 *Introduction to Biostatistics*, 2nd edn. W.H. Freeman and Co., New York.
- Theron, J. & Cloete, T. E. 2000 Molecular techniques for determining microbial diversity and community structure in natural environments. *Critical Rev. Microbiol.* **26**, 37–57.
- USDA (United States Department of Agriculture) National Agricultural Statistics Service 2002 Poultry Production and Value, 2001 Summary, <http://usda.mannlib.cornell.edu/reports/nassr/poultry/pbh-bbp/plva0402.pdf>

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