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Bacterioplankton community in Chesapeake Bay: Predictable or random assemblages

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Abstract

We monitored bacterioplankton communities from Chesapeake Bay over 2 years (2002–2004) by use of denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene. Chesapeake Bay bacterioplankton exhibited a repeatable annual pattern and strong seasonal shifts. In winter, the bacterial communities were dominated by *Alphaproteobacteria* and *Actinobacteria*, whereas in summer, the predominant bacteria were members of *Alphaproteobacteria*, *Gammaproteobacteria*, *Cyanobacteria*, *Actinobacteria*, *Planctomycetes*, and *Bacteroidetes*. Phylotypes of *Alphaproteobacteria* and *Actinobacteria* present in warm seasons were different from those in cold seasons. Relatively stable communities were present in summer–fall across the sampling years, whereas winter communities were highly variable interannually. Temporal variations in bacterial communities were best explained by changes of chlorophyll *a* (Chl *a*) and water temperature, but dissolved oxygen, ammonia, nitrite and nitrate, and viral abundance also contributed significantly to the bacterial seasonal variations.

Over the past 2 decades, our view of aquatic bacterial communities has changed considerably because of the application of molecular techniques. With the advantages of cultivation independence, molecular techniques determine the structure of bacterial communities by characterization of indicative macromolecules, generally rRNA genes, directly isolated from the environments (Giovannoni et al. 1990; Ward et al. 1990). Community fingerprinting approaches, such as denaturing gradient gel electrophoresis (DGGE), provide powerful tools for comparison of bacterial communities (Muyzer et al. 1993). DGGE is a quick-fingerprint technique, and it can separate different PCR fragments, even with single base-pair difference on a denaturant gradient gel (Muyzer et al. 1993). Diversity profiles from different microbial communities can be compared according to their gel patterns and the sequences of representative bands. Simultaneous comparisons of DGGE fingerprint patterns allow rapid assessment of changes in bacterial-community structures over time and space.

Temporal variation in bacterial communities is an important and complex ecologic process. Dramatic seasonal variations of bacterial-community structures have been observed in marine, estuarine, and freshwater ecosystems (Höfle et al. 1999; Pinhassi and Hagström 2000; Selje and Simon 2003), regardless of their environmental characteristics. Physiologic predisposition and nutritional tolerance of dominant bacteria tend to maintain stable communities during certain seasons (Pinhassi and Hagström 2000). Meanwhile, bacteria are also likely influenced by abiotic characteristics and microbial food-web structures of aquatic ecosystems (Yannarell and Triplett 2005). Previous studies have reported that population structures of bacterioplankton are correlated with salinity (Crump et al. 1999; Bouvier and del Giorgio 2002), nutrients (Biddanda et al. 2001), pH and water clarity (Yannarell and Triplett 2005), substrates resource (Crump et al. 2003), phytoplankton and Chl *a* (Murray et al. 1998; Pinhassi et al. 2004), grazing (Höfle et al. 1999), and viral lysis (Fuhrman and Suttle 1993; Suttle 1994). However, given the indigenous characteristics among diverse aquatic ecosystems, environmental variables that affect the bacterial communities may also vary by site, time, and experiment.

Chesapeake Bay contains strong physical, chemical, and biological gradients and provides a representative ecosystem to study the dynamics of estuarine bacterioplankton communities. Banding patterns of 5S rRNA showed that the compositions of bacterial communities from Chesapeake Bay varied between summer and winter (Bidle and Fletcher 1995; Noble et al. 1997). By use of fluorescence in situ hybridization (FISH), Heidelberg et al. (2002) showed that Gammaproteobacteria exhibited strong seasonality in a Chesapeake Bay tributary (Choptank River). An annual DGGE fingerprint of the bacterial community at Baltimore Harbor has shown that bacterial structure was more stable

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in summer–fall than winter and spring (Kan et al. 2005). None of these studies in Chesapeake Bay examined bacterioplankon dynamics interannually, however, which leaves unclear the question of whether the bacterial-community patterns vary from year to year. Moreover, little effort has been made to understand what environmental factors contribute to annual changes in bacterial communities. The fact that bacterial communities are affected by temperature (Heidelberg et al. 2002) and salinity (Bouvier and del Giorgio 2002) suggests that bacterioplankton in eutrophic habitats are regulated by hydrologic factors in addition to nutrient availability. Bulk measurements of bacterial abundance and secondary production in Chesapeake Bay were also found strongly dependent on water temperature (Shiah and Ducklow 1994). Thus, seasonal patterns of bacterial communities should reflect the effects of aquatic environments. If the suite of environmental factors responsible for structuring the Chesapeake bacterial communities are known, then samples with similar values for these variables would be expected to contain rather similar bacterioplankton communities. Therefore, environmental variables, either stable or fluctuant on seasonal cycles, may be used to predict and interpret the occurrence of seasonality of bacterioplankton communities.

Multivariate analysis of variance is an appropriate statistical tool for defining variations of communities and relating the variations to changes of environmental variables. Nonmetric multidimensional scaling (MDS) attempts to arrange the bacterial communities in a space with certain dimensions (usually two or three dimensions), so as to identify community patterns and help to explain observed similarities or dissimilarities. MDS has been extensively applied to describe changes in bacterial communities over time or space (Crump et al. 2003; van Hannen et al. 1999). However, linking of the bacterial-community variations with environmental changes by application of MDS is not possible. Canonical discriminant analysis (CDA), another multivariate analysis, can be used to determine what environmental variables discriminate the naturally occurring patterns. CDA classifies the variables and determines the optimal combination of variables via multivariate F-tests. If the canonical discriminant functions are statistically significant, bacterial communities can be distinguished and predicted on the basis of predictor variables included in these functions.

In this study, the population structures of bacterioplankton were investigated by DGGE at three stations along the main stem of Chesapeake Bay. Sampling included three summer–fall seasons in two consecutive years (2002–2004). We reported seasonal variations of major phylotypes of bacterioplankton in Chesapeake Bay and described the annual patterns that occurred in the middle and southern bay from 2002 to 2004. We took band richness (alpha diversity) as a diversity index, and HARMONIC analysis of the diversity indicated a repeatable seasonal pattern in Chesapeake Bay. By use of a distance matrix constructed from DGGE band profiles (absence and presence of the bands), MDS defined the population structures in a multiple-dimension space, and samples with similar communities plot close to one another in two-dimensional plots. Finally, the environmental variables that may explain or predict the bacterial seasonal patterns were determined by application of CLUSTER analysis and CDA.

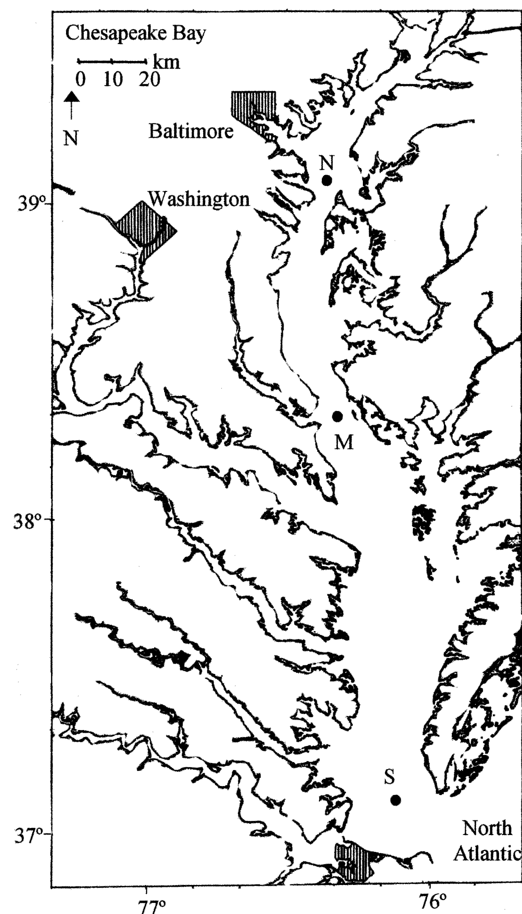

Fig. 1. Chesapeake Bay map of sampling stations. N, M, and S represent the northern, middle, and southern bay, respectively.

Materials and methods

Sample collection—Water samples were collected at three stations along the middle axis of Chesapeake Bay from September 2002 to October 2004 (Fig. 1). Stations N (39°08′N, 76°20′W), M (38°18′N, 76°17′W), and S (37°07′N, 76°07′W) represented the northern, middle, and southern bay, respectively. At each station, 500-mL surface-water samples (below 2 m) were collected from 10-L Niskin bottles mounted on a CTD rosette on board the R/V *Cape Henlopen* and filtered immediately through 0.2-μm pore polycarbonate filters (47-mm diameter) (Millipore). The filters were stored at −20°C. Water temperature, salinity, and dissolved oxygen were recorded on board. A subsample of 50 mL of water was frozen at −20°C for nutrient analysis.

Chl a and nutrients analysis—Duplicate 100-mL samples from each station were vacuum filtered (<150 mm Hg) onto 25-mm Whatman GF/C filters and Chl *a* extracted in

90% acetone for 24 hours at 4°C in the dark. Chl *a* concentration was determined fluorometrically by use of a Turner Designs 10-AU fluorometer. Nutrient data, including ammonia, nitrite and nitrate, and phosphate were determined by Technicon AutoAnalyzer II at the Horn Point Analytical Services Laboratory. The analysis followed the standard methods for chemical analysis of water and wastes proposed by USEPA (1983).

Enumeration of bacteria and viral particles—Subsamples of 50 mL of water were fixed in 0.5% glutaraldehyde for total bacterial-cell and viral-particle counting. Briefly, 200 to 400 μL of fixed sample was filtered onto a 0.02-μm pore, 25-mm Annodisc membrane filter (Whatman) under vacuum pressure less than 10 mm Hg. The cells were stained with 2.5× SYBR Gold solution for 10 minutes in the dark (Chen et al. 2001). Bacterial cells and viral particles were enumerated under blue excitation (485 nm) on a Zeiss Axioplan epifluorescence microscope (Zeiss). At least 200 bacterial cells or viral particles were counted per sample on 10 to 20 randomly chosen fields.

Extraction of nucleic acid and PCR amplification of 16S rRNA gene—Bacterial genomic DNA extraction and PCR amplification of 16S rRNA genes by 1070F and 1392R followed the protocol described previously (Kan et al. 2005). After PCR, agarose gel electrophoresis was used to detect and estimate the concentration of PCR amplicons.

DGGE and banding patterns analysis—DGGE was performed as previously described (Muyzer et al. 1993; Crump et al. 2003) with modifications. Briefly, same amounts of PCR products were separated on a 1.0-mm-thick vertical gel that contained polyacrylamide (acrylamide-bisacrylamide, 37.5 : 1) and a linear gradient of the denaturants (urea and formamide), increasing from 40% at the top of the gel to 55% at the bottom. Electrophoresis was run in a DGGE-2001 system (C.B.S Scientific) at 65°C in a 0.5 × TAE buffer and at 75 V for 22 hours. Nucleic acids were visualized by staining with SYBR Gold (Ovreas et al. 1997) and photographed with a ChemiDoc imaging system (BioRad). Defined as at least 5% of the most-intense band in the sample, bands were scored as present or absent by application of the GelcomparII software package (Applied Maths). The numbers and positions of the bands on the gel were determined on the basis of the vertical position of the bands in ladders. Banding patterns were compared with matching bands (absence and presence), and binary data were exported to Microsoft Excel for further statistical analysis.

Statistical analysis—All the statistical analyses described below were performed with HARMONIC regression, CLUSTER, MDS, and CANDISC procedures of the SAS System (SAS/STAT, 1992).

Harmonic regression analysis: To analyze the annual pattern of DGGE band richness, we conducted harmonic regression analysis (also known as trigonometric regression or cosinor regression). In this linear regression model, the predictor variables are trigonometric functions of a single variable, usually a time-related variable. We used least-square techniques to obtain parameter estimates of the equation

$$Y_{jt} = \beta_0 + \mu_j + \sum(\beta_{1k}\sin[k\omega t] + \beta_{2k}\cos[k\omega t]) + \varepsilon, \text{ where } k = 1,2, \ldots n$$

Y_{jt} is the band richness from DGGE gel; μ_j represents the *j*th year effect; β_{1k} and β_{2k} are estimated parameters for a given k value; ω is the frequency expressed in terms of radians per unit time, that is, $2\pi/12$, where π is the constant pi = 3.1415… and 12 is the frequency of an annual cycle (12 months); the variable t is a continuous numeric value converted from time variable (e.g., starting timepoint 1 is September 2002, and 9 months later, June 2003 would be converted to 10). Significant first-order terms (i.e., $k = 1$) indicate a dome-shaped annual pattern, and second-order terms ($k = 2$) indicate a bimodal annual pattern, and so on.

Cluster analysis: To examine the relation between bacterial communities, cluster analysis (Ward's minimum-variance method) was performed. The distance matrix was calculated and constructed by Jaccard coefficient on the basis of the binary data from DGGE band patterns.

Nonmetric multidimensional scaling: Multidimensional scaling (MDS) was performed on the basis of the distance matrix. The differences between bacterial-community DGGE patterns were illustrated in two-dimension MDS plots. The band patterns with the higher similarity are plotted closer, and the band patterns with the lower similarity are located further apart. To judge the degree to which this ordination matches the distance matrix, the stress value of MDS was examined. Stress value less than 0.1 indicated a good ordination, with little risk of misinterpretation of banding patterns (Clarke 1993).

Canonical discriminant analysis: Physical, chemistry, and biological variables of Chesapeake Bay water were analyzed by CDA to identify their relative contribution in discriminating among the DGGE band patterns of bacterial communities. Nine variables included temperature, salinity, Chl *a*, dissolved oxygen, ammonia, nitrite and nitrate, phosphate, bacterial abundance, and viral-particle abundance. Because of significant pairwise correlations for some of the independent variables ($p < 0.05$), total canonical structure (TOC) was used to explain canonical discriminant functions (CDFs) (Momen et al. 1999).

Sequencing and BLAST—Representative DNA bands were excised from the gels and sequenced as previously described (Kan et al. 2005). All sequences were compared with the GenBank database by use of BLAST, and the phylogenetic trees were constructed as previously described (Kan et al. 2005).

Nucleotide sequence accession numbers—Sequences of DGGE bands obtained in this study were deposited in the GenBank database under accession numbers DQ206714 to DQ206762.

Table 1. Measurements of water-quality variables and bacterial and viral abundances for middle bay and southern bay stations during the sampling period.

Month	Water temperature (°C)	Salinity	Dissolved oxygen (mg L^{-1})	Chl *a* (μg L^{-1})	Ammonia (μmol L^{-1})	Nitrite and nitrate (μmol L^{-1})	Phosphate (μmol L^{-1})	Bacterial abundance (10^6 cells mL^{-1})	Viral abundance (10^7 cells mL^{-1})
Sep 2002 (902)*	24.4†/24.2‡	19.4/26.8	6.71/6.45	5.0/3.0	0.79/0.95	4.27/1.37	0.46/0.36	4.96/4.11	5.38/5.21
Mar 2003 (303)	1.7/4.4	15.6/22.3	12.08/11.93	22.5/14.9	0.59/3.0	17.6/2.83	0.48/0.33	0.57/0.45	0.81/0.64
Apr 2003 (403)	8.4/9.4	10.5/24.0	10.45/10.19	20.9/32.1	3.61/2.6	41.3/0.81	0.32/0.39	1.52/1.44	1.8/1.24
Jun 2003 (603)	16.9/18.2	12.7/17.2	7.79/9.85	38.5/29.7	3.41/0.65	11.5/1.2	0.42/0.31	3.8/4.64	2.74/1.73
Aug 2003 (803)	27.6/26.3	11.2/17.1	3.06/3.48	9.6/7.3	1.46/0.44	4.27/0.1	0.21/0.2	2.26/3.73	1.68/2.0
Oct 2003 (1003)	19.6/20.6	13.7/19.1	7.51/7.75	7.0/12.3	0.66/0.77	6.7/1.58	0.18/0.23	0.79/0.51	0.71/0.15
Feb 2004 (204)	1.0/3.8	10.5/15.4	11.77/7.94	5.0/4.8	0.61/3.55	15.6/12.3	0.18/0.29	0.65/0.86	0.26/0.1
Mar 2004 (304)	6.2/7.5	13.7/21.8	7.7/9.24	6.5/8.7	1.16/2.6	18/11.3	0.14/0.29	0.3/1.15	0.08/0.31
May 2004 (504)	16.5/16.5	10.5/20.7	4.75/7.15	17.1/7.5	0.46/0.69	19.4/9.81	0.17/0.19	2.78/1.83	0.99/0.71
Jun 2004 (604)	22.7/20.9	10.1/19.8	3.88/3.5	15.8/10.5	0.59/1.11	11.7/1.85	0.36/0.44	4.76/5.62	0.95/0.92
Aug 2004 (804)	26.1/25.6	13.7/24.1	6.22/6.49	6.3/8.0	0.59/1.25	4.72/0.72	0.34/0.67	3.17/3.74	3.9/3.08
Oct 2004 (1004)	16.6/16.5	12.2/16.3	4.66/5.9	18.6/20.4	0.6/0.7	5.0/1.4	0.23/0.3	3.39/3.82	3.59/3.73

* Numbers in parentheses stand for corresponding month and year (same in Figures).
† Middle bay.
‡ Southern bay.

Results

Hydrologic conditions varied markedly in Chesapeake Bay (Table 1). Salinity fluctuated in the sampling years, and no annual trend was discovered. In the middle bay, the salinity varied from 10.1 to 15.6, except in September 2002, when salinity reached 19.4. In the southern bay, salinity exhibited stronger fluctuation than in the middle bay and varied from 15.4 to 26.8. Water temperature exhibited a repeatable fluctuation and reached the highest (above 25°C) in summer and lowest (1°C) in winter (Table 1). Winter was defined by the low water temperature, which sometimes extended to early spring (e.g., March 2003 and February 2004). Bacterial and viral abundance followed a similar trend as temperature: high in summer and low in winter. In contrast, dissolved oxygen, ammonia, and nitrite and nitrate peaked in the cold season and reached their lowest levels in the warm season. Concentrations of nitrite and nitrate were higher in the middle bay than in the southern bay. Chl *a* concentrations increased from early spring and peaked in summer (Table 1).

Seasonal dynamics of bacterioplankton community in 2002–2003—DGGE banding patterns showed that Chesapeake Bay bacterioplankton communities exhibited a great deal of seasonal variability (Fig. 2). Bacterial communities in early spring (March and April 2003) contain many unique populations that were not found in summer–fall (September 2002 and July, August, and October 2003). Bacterioplankton-community succession over 18 months was observed in the MDS plot (Fig. 3). Bacterioplankton populations in the northern, middle, and southern bay exhibited similar seasonal shifts. Cold-season communities (March and April 2003) shifted to a transitional community in early summer (June 2003) and after July, the community formed relatively stable summer–fall communities (July 2003, August 2003, September 2002, and October 2003).

Forty-nine phylotypes were obtained from the representative DGGE bands, and the closest phylogenetic affiliations were shown in Figs. 4 and 5. In warm seasons, phylotypes associated with *Alphaproteobacteria* (e.g., bands 1, 2, 3, 4, 13, and 40), *Gammaproteobacteria* (e.g., bands 7, 12, 14, and 21), *Cyanobacteria* (e.g., bands 15, 27, 35, and 38), *Actinobacteria* (e.g., bands 16, 34, and 48), *Planctomycetes* (e.g., bands 39 and 47), and *Bacteroidetes* (e.g., bands 37 and 45) were commonly seen. However, in cold seasons *Alphaproteobacteria* (e.g., bands 8, 9, 19, 20, 30 31, 32, and 33), *Betaproteobacteria* (e.g., bands 11, 17, and 18), and *Actinobacteria* (e.g., band 28, 43, and 44) affiliated phylotypes were found. Although *Alphaproteobacteria* and *Actinobacteria* were present in both warm and cold seasons, the composition of phylotypes shifted. Transient populations, including phylotypes related to *Alphaproteobacteria* (e.g., band 22), *Planctomycetes* (e.g., bands 26 and 49), and *Actinobacteria* (e.g., band 34) were present in June 2003 (603). Sequences of bands (3, 4, 7, 22, 40, 43, 45, 48, and 50) are identical to the bands at the same vertical positions (i.e., bands 3, 4, 7, 22, 40, 43, 45, 48, and 50). Bands 6, 24, 41, and 42 failed to be reamplified and, therefore, no sequences were obtained.

Interannual patterns for community structure and bacterial richness in 2002–2004—Pattern-forming bands were identified and highlighted in 24 samples (middle and southern bay) from September 2002 (902) to October 2004 (1004 [Fig. 6]). Bacterial communities in summer and fall were relatively stable compared with those in winter. Eighteen common bands were shared among samples from September 2002 (902), August 2003 (803), October 2003 (1003), August 2004 (804), and October 2004 (1004). In contrast, in cold seasons, 10 common bands were present, among which 5 bands appeared only in March 2003 (303), April 2003 (403), February 2004 (204), and March 2004 (304). In addition, six unique pattern-forming bands were found in cold seasons. Three of these bands were observed

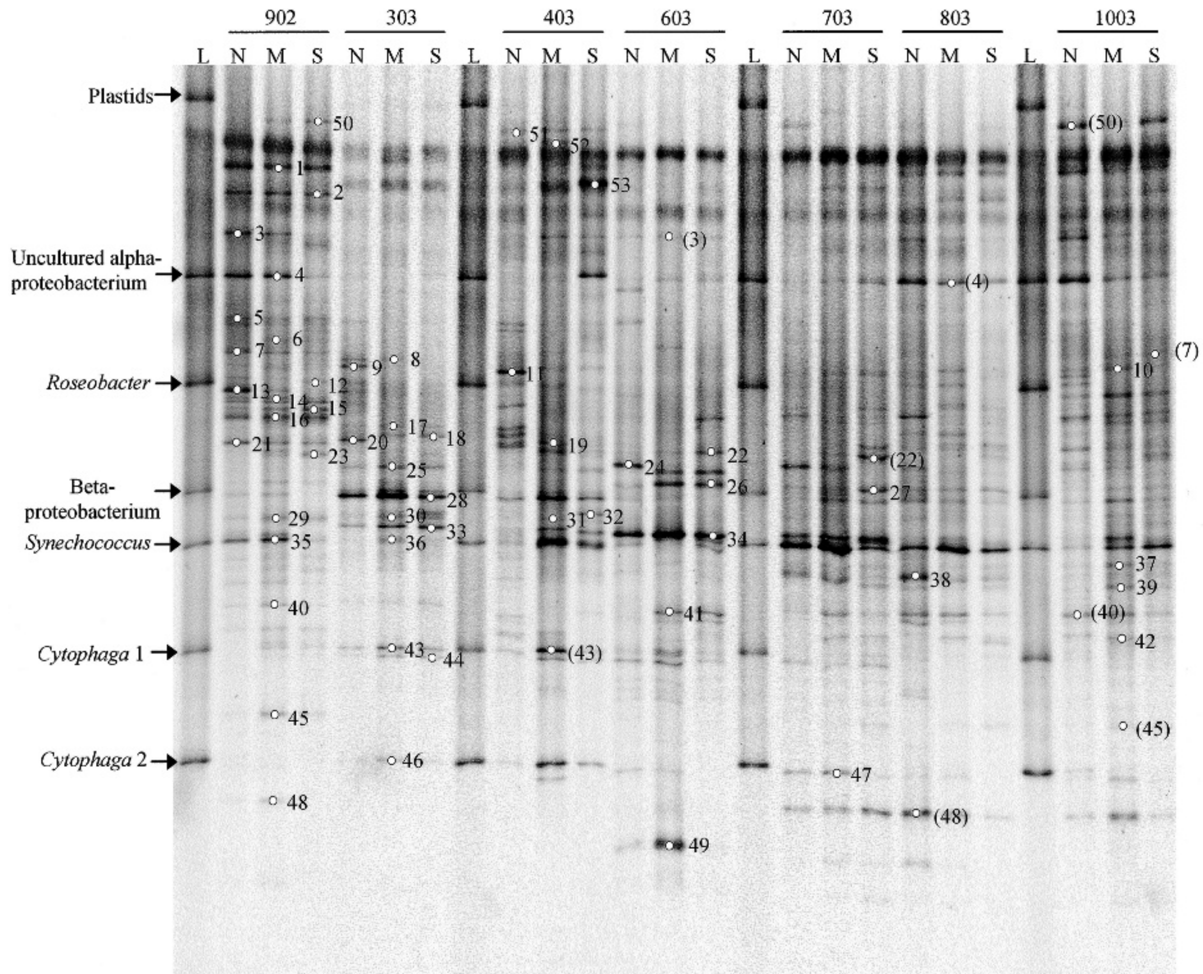

Fig. 2. Annual DGGE patterns (September 2002 to October 2003) of Chesapeake Bay bacterioplankton communities. Numbers 1 to 53 are representative bands excised and sequenced. Bands 3, 4, 7, 22, 40, 43, 45, 48, and 50 are additional bands that are sequenced to confirm that the bands at the same vertical position contain the same sequence. N, M, and S represent the northern, middle, and southern bay (Fig. 1). L is a DGGE band marker that consists of six different bacteria and one plastid as indicated.

in March 2003 (303) and April 2003 (403), and the other three were present only in February 2004 (204) and March 2004 (304).

Cluster analysis grouped the 24 bacterial communities into four classes: winter 2003, winter 2004, early summer 2003, and summer–fall 2002–2004 (data not shown). MDS analysis on these samples highlights the annual succession of the bacterial communities in Chesapeake Bay (Fig. 7). Although the samples from different years showed variability, generally the bacterial communities shift between winter and summer–fall communities. Samples from June 2003 (d and d′, Fig. 7) were different from either winter or summer–fall communities and could represent transient populations.

Although the DGGE band richness of bacterioplankton in Chesapeake Bay varied seasonally, no significant difference was observed between the middle and southern bay during the sampling period (paired *t*-test, $p = 0.18$, df $= 11$). Band richness of the middle and southern bay were used for the harmonic-regression analysis. Only the first-order cosine parameter was significant, which indicated a simple, dome-shaped repeatable annual pattern (Fig. 8; Table 2). The richness is well correlated with month, the time variable used in this study. In winter, low DGGE band richness was observed, whereas summer communities contained more diverse populations. The lowest band richness (26) was observed in February 2004, and the highest (47) appeared in August and October 2003. Increased band richness occurred in spring to early summer. Band richness remained low after winter in June 2003 but was high in June 2004 (Fig. 8).

Canonical discriminant analysis of annual patterns of bacterial communities—We included four bacterial community classes and nine independent variables in our CDA, and, hence, three canonical discriminant functions (CDFs) were computed. Only the first CDF (CDF1) and the second CDF (CDF2) were significant and accounted for 99% of the variance (Table 3). Thus, the bacterial community–environment relations were well characterized by the first two CDFs. In good accordance to MDS, bacterial communities from winter always plotted separately from summer communities, and winter communities from two

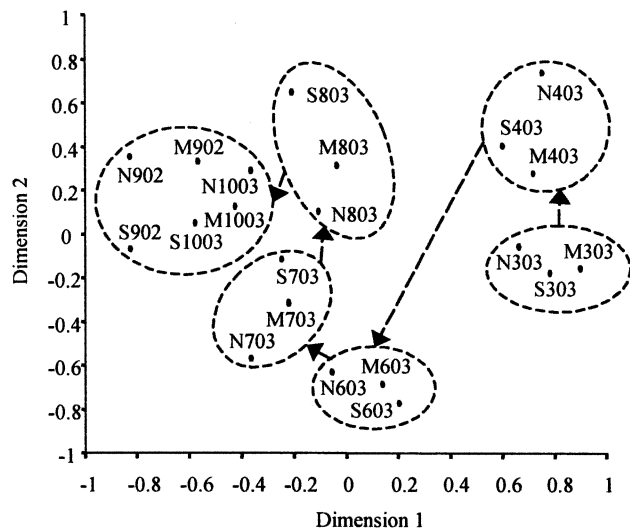

Fig. 3. MDS plots for DGGE banding patterns collected from Fig. 2. Sampling months are indicated next to each point. N, M, and S represent the northern, middle, and southern bay (Fig. 1). Stress = 0.039.

different years were also easily distinguished (Fig. 9). The samples collected in June 2003 stood out in relation to other communities.

The correlation between the original variables and the loadings of variables for a given CDF were evaluated by total canonical (TOC) structure. Among loadings on CDF1, Chl *a* was the most significant loading variable ($p < 0.0001$). Dissolved oxygen and ammonia ($p = 0.0084$) also contributed significantly to CDF1 (Table 4). Decreases in Chl *a*, dissolved oxygen, and ammonia corresponded with a transition of bacterial community from winter 2003 to winter 2004. The bacterial community of June 2003 was different from the majority of summer–fall communities and could be discriminated by CDF1 as well.

Multiple significant variables were observed in CDF2. These variables included water temperature ($p < 0.0001$), bacterial abundance ($p = 0.0001$), viral abundance ($p = 0.0012$), nitrite and nitrate ($p = 0.0024$), and dissolved oxygen ($p = 0.0093$). All these variables were associated with the transition of bacterial communities from winter to summer–fall (Table 4 and Fig. 9). However, bacterial abundance, viral abundance, nitrite and nitrate, and dissolved oxygen covaried with water temperature to some extent. Water temperature correlated positively with bacterial-cell and viral-particle abundances and negatively with nitrite and nitrate and dissolved oxygen (Table 5). So the variations between bacterial communities along CDF2 could be possibly triggered by temperature. Thus, temperature, Chl *a*, dissolved oxygen, ammonia, nitrite and nitrate, and bacterial and viral abundance generally discriminated the 24 bacterial communities into 4 distinct groups (Fig. 9).

Discussion

Seasonal succession of Chesapeake Bay bacterial communities—Chesapeake Bay bacterioplankton communities experienced strong seasonal succession from 2002 to 2004. The temporal differences in community structure were greater than the spatial differences during any sampling month. This result was consistent with previous studies in Chesapeake Bay and other estuaries (Noble et al. 1997; Selje and Simon 2003; Kan et al. 2005). DGGE fingerprints of bacterial communities and MDS plots indicated that the composition of bacterioplankton differed from winter to summer and supported our results of LH-PCR (length heterogeneity–PCR) and clone library (Kan et al. unpubl. data). Changes in community composition between winter/early spring and summer were rapid rather than gradual, which suggests that few phylogenetic groups were able to overcome the environmental stresses over seasons. Although how community replacement occurs is not clear, seasonality of bacterial succession may link to the environmental variables and intrinsic activity of the major phylotypes in the communities. Bacterial counts and bacterial growth followed the same trend (Wikner and Hagström 1991), which indicates that this pattern was also reflected in the population size and activity.

Recurrent annual patterns of bacterioplankton—Our DGGE fingerprints demonstrated reoccurring annual patterns in Chesapeake bacterioplankton. During annual succession, summer–fall communities appeared to be more stable than winter communities. Significant pattern-forming bands in summer–fall communities recurred in 3 years, which suggests that they represent an indigenous estuarine community. This stability is likely the result of high bacterial-growth rates and a relatively long residence time that allows estuarine bacterioplankton to overwhelm allochthonous populations of marine and freshwater populations (Crump et al. 2004). However, considerable interannual variations were observed in winters. Recurrent, stable summer–fall bacterioplankton communities and variable winter communities appear to be regular features of this annual pattern. Seasonally variable but annually reassembling bacterioplankton communities have been reported in a high mountain lake (Pernthaler et al. 1998), California coastal waters (Fuhrman et al. 2004), and two temperate rivers (Crump et al. unpubl. data). However, one study conducted over 3 consecutive years on a humic lake in the Northern Highland State Forest in Wisconsin indicated little similarity of bacterial community composition from year to year (Kent et al. 2004), which suggests that population dynamics may vary by site because of indigenous characteristics of the aquatic system.

Factors driving the phylogenetic succession in Chesapeake Bay—Our results provide plausible explanations for seasonal variations of bacterial communities in Chesapeake Bay. The annual shift in bacterial compositions appeared to be associated with the environmental variables. Successful classification of bacterioplankton by use of environmental variables (Fig. 9) suggested that Chesapeake Bay undergoes predictable seasonal changes from year to year. Four classes of bacterioplankton that resulted from cluster analysis were reconstructed along linear functions (CDF1 and CDF2) that were computed by CDA. Among nine

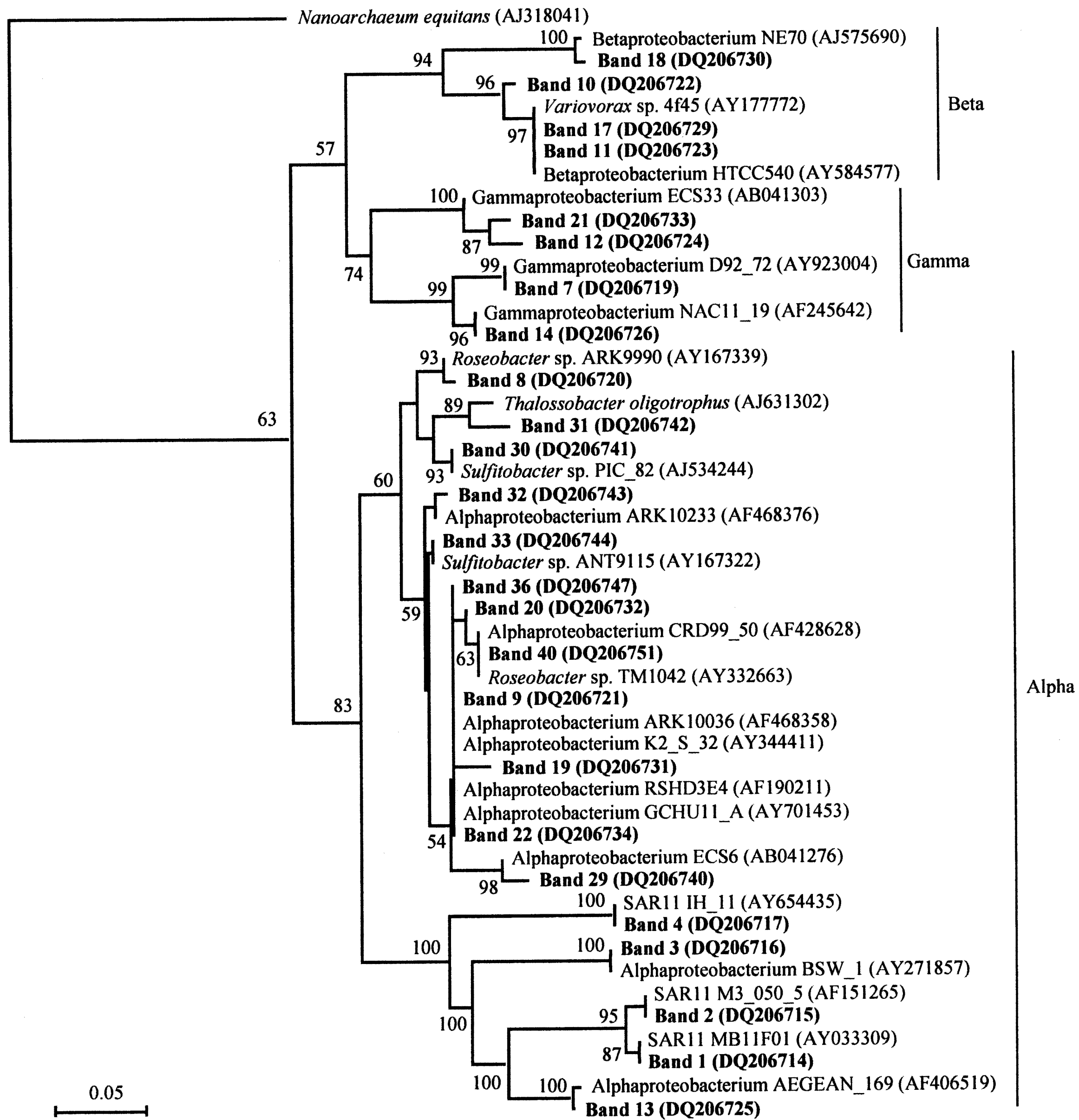

Fig. 4. Phylogenetic affiliations of DGGE band sequences related to *Proteobacteria*. Bands were excised from DGGE gel shown in Fig. 2. Sequences from this study are in boldface type. Bootstrap values were based on 1,000 replicated trees. *Nanoarchaeum equitans* is used as an outgroup. Scale bar represents 0.05 substitutions per site.

hydrologic and biological factors used for CDA, Chl *a*, temperature, dissolved oxygen, ammonia, nitrite and nitrate, bacterial abundance, and viral abundance corresponded significantly to changes in the bacterial communities.

Chl *a* was the most important variable in CDF1. Chl *a* and phytoplankton are important forces in structuring bacterial communities and archaeal communities (Murray et al. 1998; Kan and Chen 2004). During phytoplankton-bloom senescence, bacterial abundance, cell activity in hydrolytic enzyme, and growth rates increase substantially, which are potentially associated with significant shifts in bacterioplankton species composition (Riemann et al. 2000). Recent studies indicated that not only the phytoplankton biomass but also the differences in phytoplankton species composition also lead to pronounced shifts in bacterioplankton composition (Pinhassi et al. 2004). In Chesapeake Bay, surface Chl *a* concentration increases in early spring and remains high during summer, with moderate fluctuations from July to September (Malone et al. 1991). Significant difference of Chl *a* concentration was observed between winter/early spring 2003 and 2004. The appearance and disappearance of unique phylotypes of bacterial communities and changes in the relative abundance (i.e., band intensity) demonstrated that the population structure in winter 2003 was different from that in winter 2004, and, thus, the variation is likely associated with phytoplankton (diatom) blooms. Furthermore, high

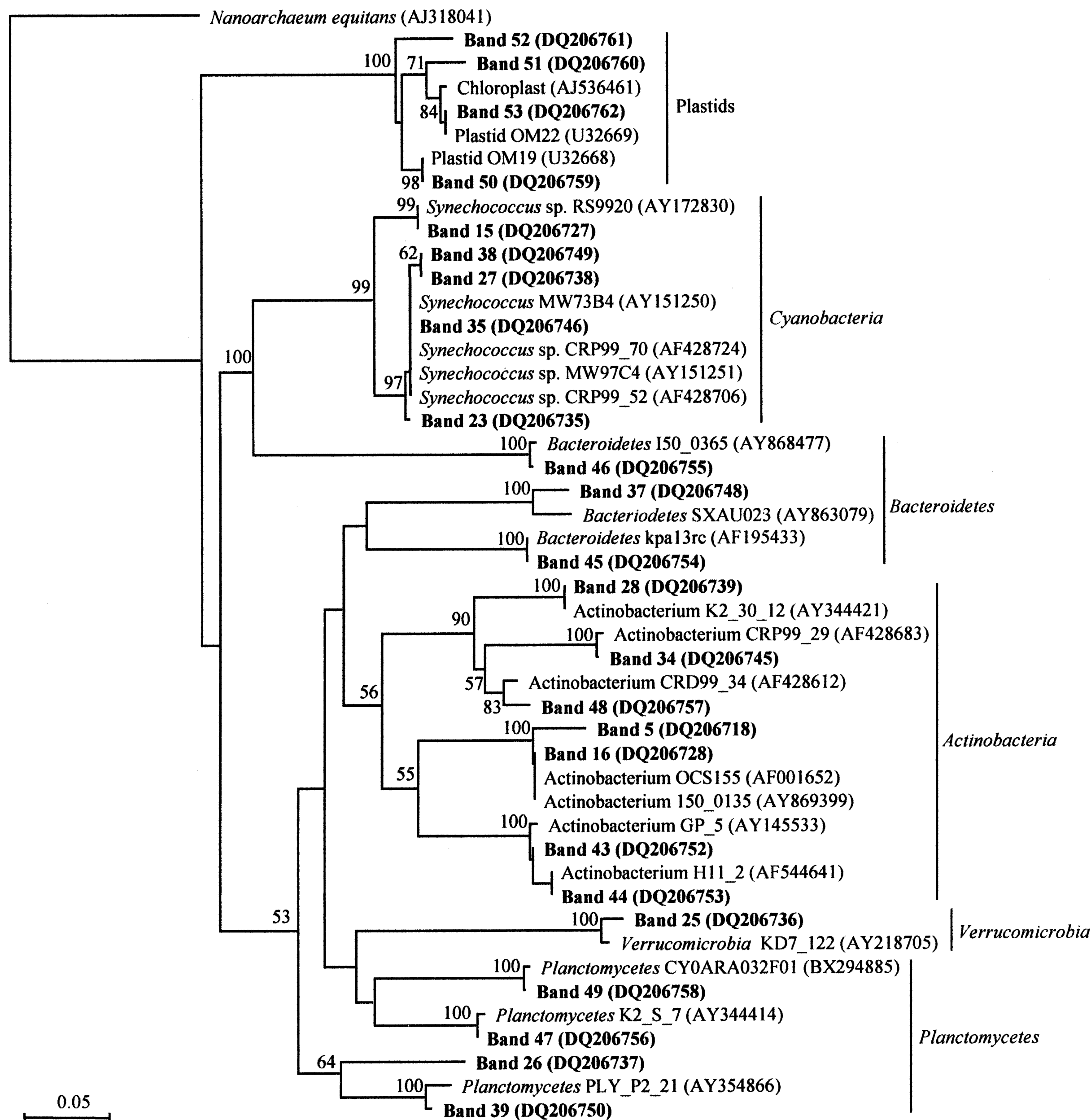

Fig. 5. Phylogenetic affiliations of DGGE band sequences related to Plastids, *Cyanaobacteria*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, and *Planctomycetes*. Bands were excised from DGGE gel shown in Fig. 2. Sequences from this study are in boldface type. Bootstrap values were based on 1,000 replicated trees. *Nanoarchaeum equitans* is used as an outgroup. Scale bar represents 0.05 substitutions per site.

concentration of Chl *a* associated with samples in June 2003 explained why that bacterial community stood out from other communities. However, one study showed no relation between bacterial metabolism or composition and the distribution of Chl *a* along two transects of Chesapeake Bay rivers (Bouvier and del Giorgio 2002). These results suggest that changes in Chl *a* alone are not enough to drive the bacterial community successions.

Bacterioplankton are also affected by nutrients (Biddanda et al. 2001). Ammonia and nitrate are important nitrogen sources for heterotrophic bacteria (Kirchman et al. 2003). For example, elevated ammonia concentrations favor the growth of ammonia-oxidizing bacteria (AOB), which were found to be more abundant in Chesapeake Bay than in other marine environments (Ward 1982). Subsequent observations of the depth distribution of ammonia-oxidation rates indicated that most nitrification occurs in the surface waters (Ward and O'Mullan 2002). Our results showed that shifts in surface-water bacterial communities were significantly related to changes of ammonia ($p = 0.0084$) in CDF1 and to changes in nitrite and nitrate ($p < 0.05$) in CDF2. Another significant factor for both CDF1 and CDF2 was dissolved oxygen. Although hypoxia is generally restricted to the bottom waters of Chesapeake Bay,

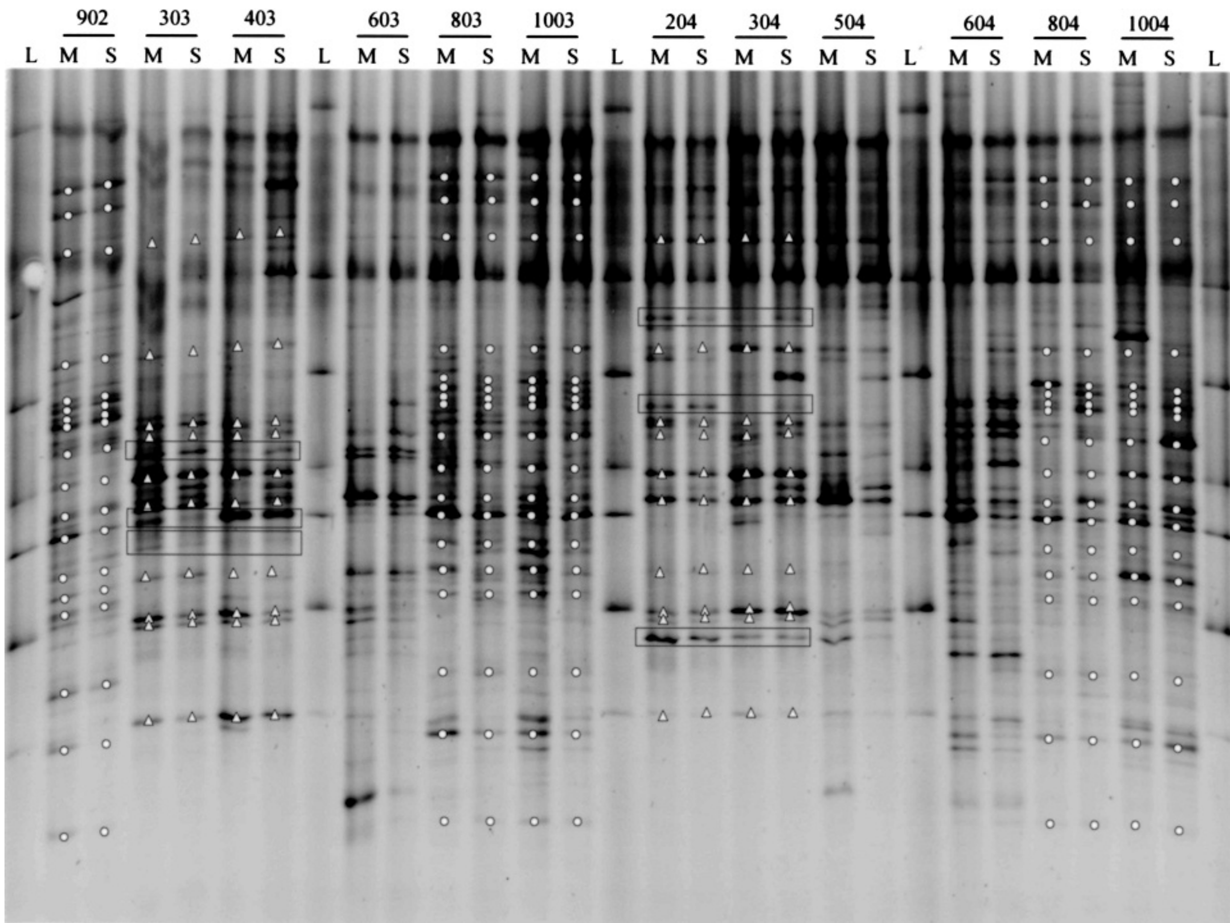

Fig. 6. Interannual variations (September 2002 to October 2004) of Chesapeake Bay bacterioplankton communities revealed by DGGE. Only samples from the middle (M) and south (S) bay were analyzed. Symbols categorize the bands as important pattern-forming bands in summer–fall (open circle) and winter (open triangle). Unique bands appeared in winter 2003 or 2004 are shown in the rectangular box. L is DGGE band marker (same as Fig. 2).

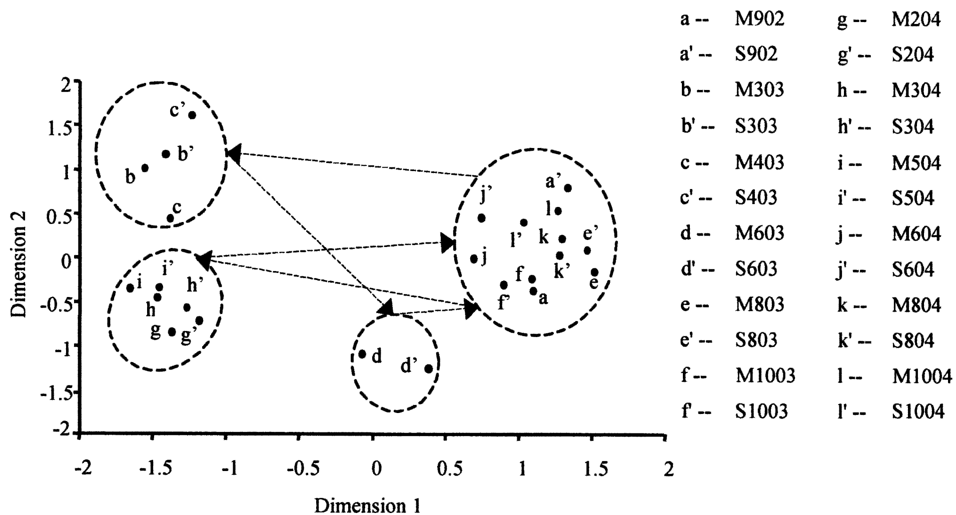

Fig. 7. MDS plots for DGGE banding patterns collected from 2002 to 2004. Each sample point is labeled with a letter. Letters a, a′ to l, and l′ correspond to sampling months and stations M902, S902 to M1004, and S1004 as shown in Fig. 6. Stress = 0.028.

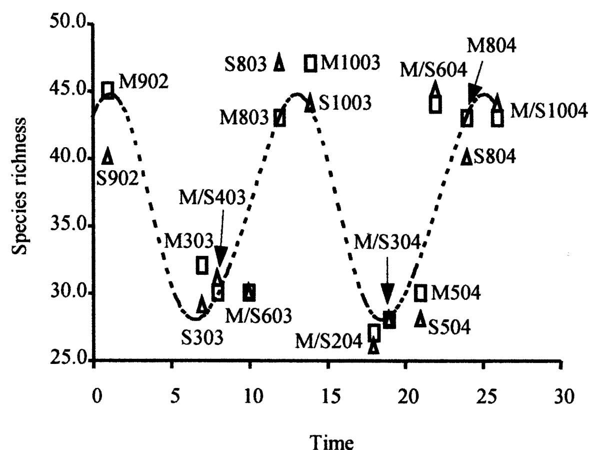

Fig. 8. Time series of DGGE band richness of Chesapeake Bay bacterioplankton from September 2002 to October 2004. Sampling months refer to Fig. 6. Open square: middle bay (M); open circle: southern bay (S).

surface-water–dissolved oxygen fluctuated remarkably over the seasons (Table 1). The annual spring inflow of freshwater initiates hypoxic and anoxic conditions in the bay by delivering nutrients, increasing stratification, lowering salinity, and affecting the residence time of the water (Boicourt 1992). Therefore, dissolved oxygen could be an important environmental factor that affects the temporal succession of bacterial communities in Chesapeake Bay.

Another important source of variation in CDF2 was viral-particle counts. Viruses cause prokaryotic mortality through host-specific cell lysis and can influence bacterial-community composition in various ways (Wommack and Colwell 2000). The seasonal correspondence of abundance and community patterns of both host and virus indicates that viruses hold the potential to structure the host-community compositions (Wommack unpubl. data). Apart from killing infected cells, viral lysis causes release of new materials, including cytoplasmic and structuring material from host cells, which can be important substrates that stimulate the growth of noninfected bacterial populations (Middelboe and Lyck 2002). Furthermore, gene swapping through transduction, transformation, and conjugation probably influences the host speciation and diversification (Paul 1999). Therefore, viruses can affect the host-community composition by "killing the winner" (Thingstad and Lignell 1997), stimulating noninfected bacteria (Middelboe and Lyck 2002), and generating genetic variability of bacteria through virus-mediated gene transfer (Weinbauer and Rassoulzadegan 2004).

The most significant variable in CDF2 was water temperature. In temperate estuaries, temperature is considered to be an interactive limiting factor, coupled with substrate supply, to control bacterial biomass, growth, and respiration (Wikner and Hagström 1991; Shiah and Ducklow 1994; Pomeroy and Wiebe 2001). Correlation of water temperature and seasonality of *Gammaproteobacteria* implies that water temperature is also important in regulating bacterial-community structure (Heidelberg et al. 2002). Within a moderate range, temperature could affect that how bacteria respond to changes in DOM supply (Kirchman and Rich 1997) and consequently affect the bacterial composition. Our DGGE band patterns showed that seasonal changes in water temperature were paralleled by shifts in bacterioplankton compositions. In the CDA, temperature successfully discriminated winter and summer–fall communities (Fig. 9). Meanwhile, bacterial abundance, viral abundance, nitrite and nitrate, and dissolved oxygen correlated with water temperature to some extent (Table 5). This finding leads to the conclusion that water temperature may be an important environmental force that triggers the seasonal variation of bacterioplankton communities in Chesapeake Bay.

Surprisingly, no strong relation between bacterial community and salinity was observed. The salinity range of the transect was between 10 and 20 and varied with season. Previous studies suggested a relation between

Table 2. Harmonic regression parameter estimates for the annual pattern of bacterial species richness.

Bacterial species richness		$R^2 = 0.76$, df = 23, $p < 0.0001$		
Parameter	Estimates	SE	t value	p value
Intercept β_0	36	1.36	26.51	<0.0001
1st-order sine β_{11}	3.77	2.02	1.87	0.08
1st-order cosine β_{21}	7.38	1.60	4.62	0.0002
2nd-order sine β_{12}	0.66	1.90	0.35	0.73
2nd-order cosine β_{22}	−0.36	1.28	−0.28	0.78

Table 3. Canonical discriminant functions (CDFs) and their correlations.

CDF	Canonical correlation	Eigen value (proportion, cumulative)	Approximate F (numerator df, denominator df)	p*
1	0.96	10.53 (0.65, 0.65)	4.70 (27, 36)	<0.0001
2	0.92	5.5 (0.34, 0.99)	2.76 (16, 26)	0.01
3	0.33	0.12 (0.01, 1.00)	0.24 (7, 14)	0.97

* The significance of individual CDFs can be inferred from eigen value or p value <0.05.

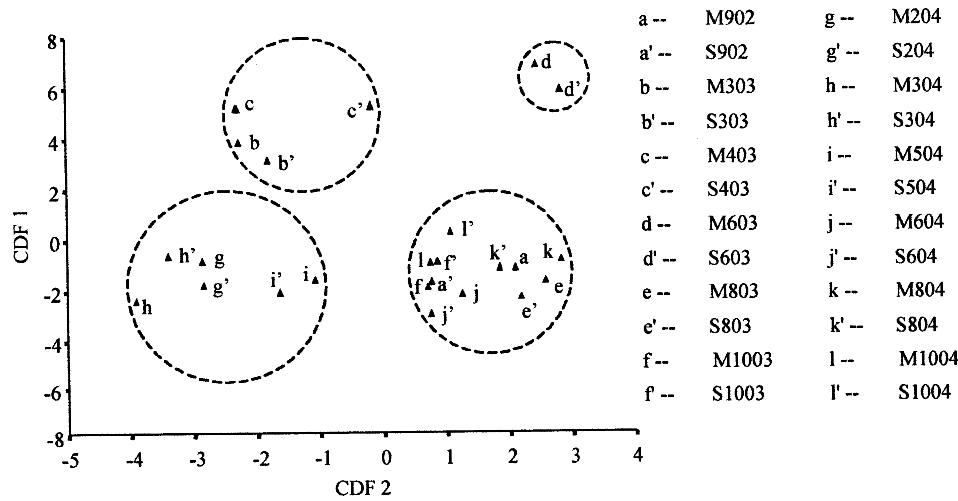

Fig. 9. Separation of the bacterioplankton communities collected from 2002 to 2004 on the basis of the first and second canonical discriminant functions (CDF1 and CDF2). Labels a to l′ are same as Fig. 7.

Table 4. Total canonical structure (TOC) and its significance (p) for three canonical discriminant functions.

Variable	TOC (p)*	
	CDF1	CDF2
Chl *a*	0.85 (<0.0001)	0.22 (0.3)
Temperature	−0.42 (0.05)	0.84 (<0.0001)
Salinity	0.02 (0.94)	0.02 (0.94)
Dissolved oxygen	0.66 (0.0004)	−0.52 (0.0093)
Ammonia	0.53 (0.0084)	−0.32 (0.13)
Nitrite and nitrate	0.25 (0.24)	−0.59 (0.0024)
Phosphate	0.06 (0.79)	−0.08 (0.7)
Bacterial abundance	−0.16 (0.47)	0.71 (0.0001)
Viral abundance	−0.12 (0.58)	0.62 (0.0012)

* CDF3 is not included because it has no significant role in discriminating bacterial communities.

estuarine salinity gradients and the composition of estuarine bacterial communities (Crump et al. 1999) and, in particular, Alphaproteobacteria and Betaproteobacteria (Bouvier and del Giorgio 2002). Compared with significant seasonal variations, our MDS analysis on bacterial communities in 2002–2003 showed moderate spatial variations along the bay. However, salinity did not play a significant role in discriminating the community structures over the seasonal variations. Because of the long residence time of Chesapeake Bay water, indigenous bacterioplankton communities may remain relatively stable along the salinity gradient. The dominant bacterial groups in Chesapeake Bay are probably able to resist changes in osmotic pressure with the adaptations of physiologic features. Another minor variable is phosphate concentration. Because phosphate concentration remains relatively high and stable in the bay, it is not considered to be a limiting factor for microbial communities.

As a quick fingerprint technique, DGGE biases toward the abundant populations within a community (Muyzer et al. 1993; Kan et al. 2005). The composition of the entire assemblage is not completely described by the representative bands selected for sequencing. The minor groups are undetectable or form smearing bands on the gel and, thus, escape further characterization. Many factors, including bias by PCR and other steps of molecular analysis, can influence the outcome of PCR, and, therefore, DGGE underestimates the diversity and complexity of natural

Table 5. Pairwise correlation coefficients between independent variables.

	Chl *a*	Temperature	Salinity	Dissolved oxygen	Ammonia	Nitrite and nitrate	Phosphate	Bacterial abundance	Viral abundance
Chl *a*									
Temperature	−0.18								
Salinity	−0.14	0.1							
Dissolved oxygen	0.27	**−0.78***	0.16						
Ammonia	0.31	**−0.45***	0.1	**0.4***					
Nitrite and nitrate	0.11	**−0.51***	**−0.55***	0.33	0.35				
Phosphate	−0.06	−0.18	0.19	0.093	**0.47***	−0.03			
Bacterial abundance	0.04	**0.7***	0.08	**−0.65***	−0.34	−0.4	−0.03		
Viral abundance	−0.01	**0.57***	0.21	−0.31	−0.19	-0.31	0.02	**0.56***	

* Bolds are significant at $p < 0.05$.

microbial communities. Our statistical analyses are mainly based on DGGE band patterns and, therefore, only provide a "snapshot" of the bacterioplankton dynamics in Chesapeake Bay. Bacterial activity in aquatic ecosystem is very complicated, and linking bacterial distribution to the environmental parameters is not straightforward. Limited by sampling size and cruise frequency, statistical analyses only provide a partial view of the "real world" or even "false-positive" information. For instance, CDA is able to identify the parameters that regulate the population patterns observed; however, the direct correlation is still missing. All these limitations point to the necessity of further studies that focus on specific groups with more frequent samples.

We have shown that Chesapeake Bay bacterioplankton communities experienced pronounced seasonal changes and repeatable annual patterns. Replacement of major phylotypes of bacteria from winter to summer–fall indicated that the dominant groups could not survive seasonal changes in environmental conditions. Covariations of the structure of bacterioplankton with environmental variables measured in this study were well constructed in MDS and CDA. We interpret the seasonal succession of bacterial-community structure primarily as an interactive consequence of variations in several environmental factors. Temperature, Chl *a*, dissolved oxygen, nutrients, and viruses all appear to play significant roles in structuring the bacterial communities in Chesapeake Bay. However, considering the substantial phylogenetic, physiologic, and metabolic diversity contained within these communities, they can be expected to contain organisms with the ability to adapt to a wide range of environmental stresses. Thus, further studies of significant factors that contribute to the success of defined groups of bacteria or the total community will increase our understanding of estuarine microbial processes.

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