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Notes

Diversity and distribution of free-living and particle-associated bacterioplankton in Sandusky Bay and adjacent waters of Lake Erie Western Basin

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ABSTRACT

Studies on the bacterial communities in Lake Erie have been largely focused on cyanobacteria. To characterize the community structure of the other bacteria, we examined the 16S rRNA gene content of free-living (FL) and particle-associated (PA) bacterioplankton populations in Sandusky Bay (SD) and adjacent waters in the Western Basin (WB) of Lake Erie. A comprehensive survey of nutrients and other limnological variables was also performed in parallel. A total of 18,569 of 16S rRNA V6 pyrotag sequences were recovered, which were affiliated with 64 unique bacterial orders within 14 phyla. FL bacteria were composed differently from PA ones and contained significantly more *Actinomycetales*. In addition, FL bacteria were taxonomically more diverse. Despite the distinct environmental conditions, compositional variation was insignificant between bacteria in the SD and WB samples.

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Introduction

Sandusky Bay (SB), the largest bay that is contiguous with Lake Erie, serves as a corridor to receive and transport high loads of terrestrial materials from the Sandusky River to the Western Basin (WB) of Lake Erie. Much of the imported material is retained and processed in Sandusky Bay (Heath, 1992; Hwang and Heath, 1999). As a result, water in the bay and adjacent areas in Western Basin are typically hyper-eutrophic and eutrophic, respectively (Conroy et al., 2011). Consequently, these areas are hot spots of cyanobacterial harmful algal blooms (CyanoHABs) during summer (Budd et al., 2002; Vincent et al., 2004).

Extensive efforts have been made to examine structures and activities of bloom-forming cyanobacteria in Lake Erie (Millie et al., 2009; Ouellette et al., 2006; Rinta-Kanto et al., 2005; Wilhelm et al., 2006). However, studies on the rest of the bacterial communities are very limited. Little is known about non-cyanobacteria (referred as bacteria hereafter for simplicity), including those that are colonizing particles or living freely in the water, despite their potential roles in nutrient recycling, cyanotoxin degradation (Ramani et al., 2012) and bacterial endotoxin production (Rapala et al., 2002). Moreover, among the sparse number of studies of bacteria in Lake Erie (Dutka and Kwan, 1983; Dutka et al., 1974; Ward and Frea, 1980), many were either culture-dependent or have adopted classic "black box" approaches, which treat the bacterial community as a taxonomically and functionally homogenous unit (Hoostal and Bouzat, 2008). However, it is widely accepted that only less than 1% of bacterial species can be readily cultured (Amann et al., 1995) and dynamics and interactions among individual bacterial taxa within the microbial loop are critical for energy flux and nutrient cycling in environments (Pomeroy, 1974).

To help fill the knowledge gap on bacteria in Lake Erie, this study employed high throughput pyrosequencing techniques to characterize the *in situ* 16S rRNA gene content of free-living (FL) and particle-associated (PA) bacteria in Sandusky Bay and adjacent Western Basin areas in Lake Erie. Potential variations in bacterial taxonomic composition and diversity were also examined among sampling sites.

Materials and methods

Sample collection and processing

Surface water samples (~0.5 m below the air-water interface) were collected from six sites in Lake Erie on June 24, 2009, using the Ohio State University Stone Laboratory's *R/V Erie Monitor*. Three sampling sites were selected inside or at the mouth of Sandusky Bay, whereas the remaining three sites were in the Western Basin (Fig. 1). The geographical coordinates of each site and its water depth are provided in Table S1. At each sampling site, standard *in situ* limnological parameters were measured using a Hach Hydrolab multi-parameter sonde, including temperature (T), specific conductivity

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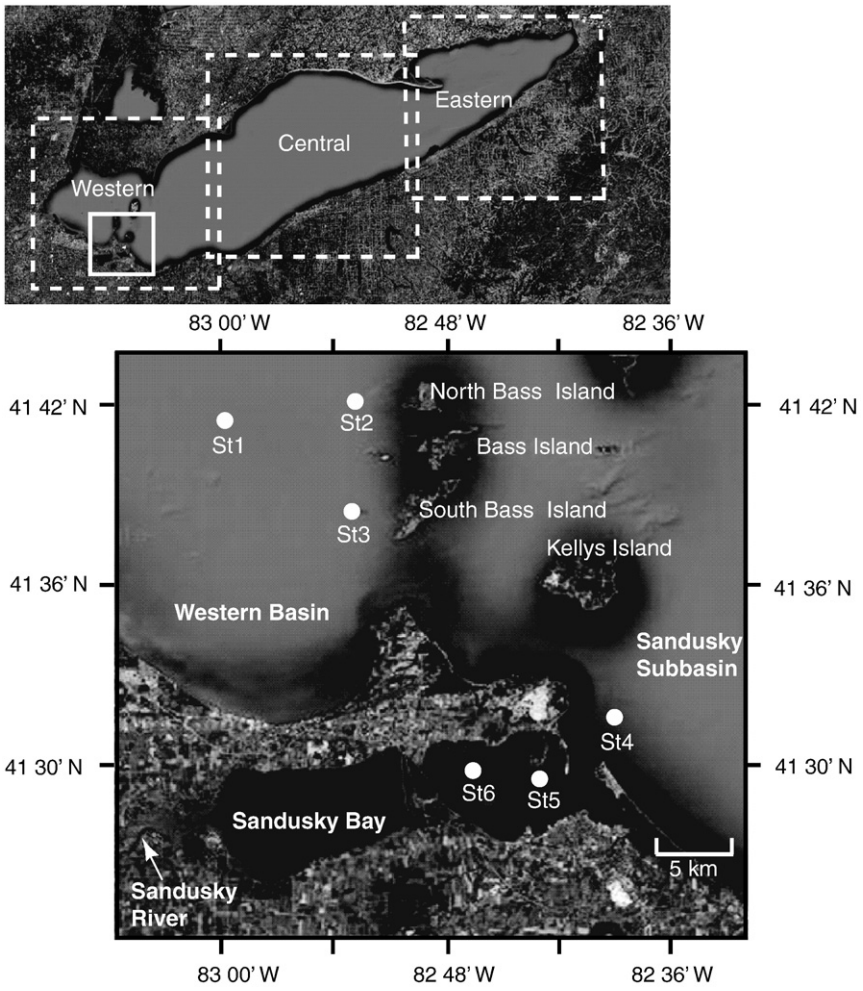

Fig. 1. Sampling stations in Sandusky Bay and the Western Basin of Lake Erie. Satellite image was generated using Google Earth. Geographic coordinates and water depth of sampling stations are provided in Table S1.

(Cond), pH, total dissolved solid (TDS), oxidation–reduction potential (ORP), turbidity (Turb), dissolved oxygen in concentration and percent (DO, DO%), and chlorophyll *a* (Chl) and phycocyanin (Pcy) concentrations. All sensor probes were calibrated using standards prior to use and data of these *in situ* measurements have been reported elsewhere (Ali, 2011; Ali et al., 2012).

Immediately after sampling, surface water was sequentially filtered through 3.0-μm-pore-size and 0.2 μm-pore-size membrane filters (Pall Life Sciences, Port Washington, NY) to collect fractions of particle-associated (PA) and free-living (FL) bacterioplankton, respectively. Filters with bacterial cells were washed three times with 50 ml sterile PBS solution and immediately placed on ice before being transported back to the lab and frozen at −80 °C. Filtrates that had passed 0.2 μm-pore-size membranes were collected in 50 ml sterile centrifuge tubes and kept on ice for transport back to the lab and stored at 4 °C for subsequent nutrient analyses. All samples were collected in triplicates and processed for nutrient analysis and DNA extractions within 2 days of collection.

Nutrient analysis

Concentrations of organic and inorganic nutrients were measured using standard methods for chemical analysis for water and wastewater (APHA, 1999). Specifically, concentrations of dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were measured using a Shimadzu TOC/TN analyzer by combustion oxidation/infrared detection and combustion chemiluminescence detection methods, respectively. Nitrate and nitrite ($NO_3^- + NO_2^-$) concentrations were determined by the cadmium reduction method followed by colorimetric determination of nitrite, ammonium (NH_4^+) concentrations were measured fluorometrically; and soluble reactive phosphorus (SRP) concentrations were measured based on the standard colorimetric molybdenum blue method using a flow injection protocol.

DNA extraction and PCR amplification

DNA was extracted from frozen filters using the PowerMax Soil DNA Isolation Kits (MO BIO Laboratories Inc, Carlsbad, CA) following the manufacturer's instruction. Extracted DNA was examined on ethidium bromide-stained 1% agarose gels using gel electrophoresis and purified using the QIAquick Gel Extraction Kit (QIAGEN Group, Valencia, CA). Concentrations of purified DNAs were quantified by picogreen assay using the Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes Inc., Eugene, OR).

The V6 hyper-variable region of 16S rRNA genes was PCR amplified from extracted DNAs with Illustra PuRe Taq Ready-to-go PCR beads (GE Healthcare, Piscataway, NJ) using primers that were designed specifically for 454 high throughput pyrosequencing. The forward primers were 5′-(sequencing adaptor A)-(4-nt sample tag)-967F-3′ and the reverse primers were 5′-(sequencing adaptor B)-1046R-3′ (Sogin et al., 2006). The PCR program consisted of one initial 3-min denaturation (at 94 °C), 25 amplification cycles and one final 2-min extension (at 72 °C). Each amplification cycle included denaturing (at 94 °C for 30 s), annealing (at 57 °C for 45 s), and extension (at 72 °C

for 60 s) steps. For each sample, triplicate PCR amplifications were performed and the resulting amplicons were pooled and subsequently examined by gel electrophoresis. PCR amplicons were excised from the gels and double purified first with the QIAquick gel extraction kits (QIAGEN) and then with the AMpure XP systems (Beckman Coulter Genomics, Brea, CA). After quantification, equal quantity of the PCR amplicons from each sample were combined and sequenced using a Roche GS FLX sequencer.

The 16S rDNA V6 pyrotag sequences were deposited to the CAMERA database (http://camera.calit2.net/) under project id CAM_P_0000908.

Sequencing annotation and library coverage calculation

Pyrotag sequences of each sample were sorted based on unique sample tags. Then the nucleotides of the sequencing adaptors and sample tags were trimmed off from each sequence. Low quality sequences were removed following procedure described previously (Sogin et al., 2006). Remaining sequences were clustered at a 95% identity threshold (*c.a.* 3 bp average difference per read) using the cd-hit program (Li and Godzik, 2006). Clusters with a single sequence were removed (c.a. 32% of total clusters) in order to prevent potential overestimates of the bacterial diversity (Kunin et al., 2010).

The longest sequence within each cluster was assigned as the representative sequence by the cd-hit program (Li and Godzik, 2006) and its order-level taxonomic identification was determined using the BLASTn analysis against the near full length (> 1200 bp) 16S rRNA gene sequences in the RDP database based on cutoffs at 90% sequence overlap and 90% sequence identity match.

Richness of unique non-cyanobacterial orders was estimated according to the Chao1 estimation using the equation $S_{chao} = S + (A^2/2B)$, where S is the total number of orders, A is the number of orders with only one sequence and B is the number of orders with only two sequences. The sequence library coverage (LC%) was calculated by dividing the observed number of orders by the estimated Chao1 order richness in each sample (Table 1).

Order-level diversity rank assignment

The initial ranking (R_I) was made based on average values of three commonly used diversity indices, including Shannon-Weiner index, Fisher's alpha diversity index and Simpson's diversity index. The second independent ranking (R_R) was made based on rarefaction curve analysis (Hughes et al., 2001) with consideration of both taxon evenness and richness (Olszewski, 2004) (Fig. S1). This procedure has been proven to be useful to compare communities with different sample sizes (Youssef and Elshahed, 2008). For both methods, diversity ranks were assigned between 1 (the least diverse) and 12 (the most diverse). The final diversity ranks (R) were assigned to each sample based on the average values of the R_R and R_I.

Statistical analysis

All statistical calculations were performed using the PRIMER v5 software package (Plymouth Marine Laboratory, Plymouth, UK) unless otherwise mentioned.

The distribution of bacterial orders among samples were normalized and square-root-transformed and then used for Bray–Curtis similarity matrix calculation. This similarity matrix was used subsequently for MDS (non-metric multidimensional scaling), ANOSIM (analysis of similarity) and SIMPER (similarity percentages) analyses. MDS analysis was performed to examine and visualize similarity between samples. The distance between two samples on the MDS plot positively reflects their degree of similarity. The robustness of MDS results was accessed by ANOSIM, a rough analogue of the standard univariate ANOVA test. ANOSIM index r_{ANOSIM} was reported on a scale of 0 to 1. The sample groups were reported as well-separated when $r_{ANOSIM} > 0.75$, overlapping but clearly different when $0.5 < r_{ANOSIM} < 0.75$, or barely separable when $r_{ANOSIM} < 0.25$ (Clarke and Warwick, 2001). SIMPER analysis was then performed to identify taxa that contributed the most to the observed difference between sample groups.

Raw data of environmental variables were log transformed (for DOC, TDN, NO_3^-/NO_2^-, NH_4^+, SRP, DO, DO%, Turb, Chl, Pcy, Cond, TDS and ORP) or untransformed (for pH) and then analyzed using principle component analysis (PCA) based on the Euclidean distance matrix. Potential variations of environmental parameters between sampling sites were analyzed using the t test and significant differences were reported when $P < 0.05$.

Results

Environmental conditions of sampling sites

A total of 15 limnological variables were measured to describe environmental conditions of each sampling site. PCA analysis of these variables ordinated sampling sites into two general groups (Fig. 2): the Western Basin sites (St1-3, referred as the WB sites hereafter) and the Sandusky Bay and Sandusky Sub-basin sites (St 4–6, referred as the SD sites hereafter). Generally, the SD sites had higher DOC, SRP, NH_4^+, Chl, T, SpCond, TDS, Turb and pH but lower DO%, TDN and $NO_3^- + NO_2^-$ than the WB sites (t test, $P < 0.05$) (Fig. 2; Table S2). Concentration of phycocyanin (Pcy), a signature pigment of cyanobacteria and oxidation-reduction potential (ORP) showed no significant difference between the SD and WB sites.

Bacterial diversity and composition

A total of 18,569 high quality 16S rRNA gene V6 region pyrotag sequences were recovered and had taxonomic assignments at the order or more specific levels. Rarefaction curves built based on order level bacteria distribution reached or closely approached saturation for all 12 samples (Fig. S1). The Chao1 index estimated 44–80 orders in

Table 1
Taxon richness, diversity and evenness values of free-living (FL) and particle-associated (PA) bacterioplankton based on order level taxonomic identification of 16S rDNA V6 partial sequences. *N*: number of total sequences; *S*: number of total unique orders; S_{chao}: estimated number of total unique orders by Chao1 estimator; LC%: library coverage percentage; $\%_{cyano}$: percent of cyanobacterial sequences; R: diversity ranks.

Index	WB Stations						SD stations					
	St1		St2		St3		St4		St5		St6	
	FL	PA	FL	PA	FL	PA	FL	PA	FL	PA	FL	PA
N	2504	2112	1207	1292	2515	1354	1553	1213	2237	871	911	880
S	66	62	57	57	56	54	46	47	50	35	43	36
S_{chao}	80	80	74	62	71	57	60	67	63	47	49	44
LC%	82.5	77.5	77.0	91.9	78.9	94.7	76.7	70.1	79.4	74.5	87.8	81.8
$\%_{cyano}$	0.6	0.8	0.2	0.3	0.2	0.7	0.6	8.3	1.3	12.7	3.8	13.8
R	12	11	10	9	7.5	7.5	3.5	5.5	3.5	1	5.5	2

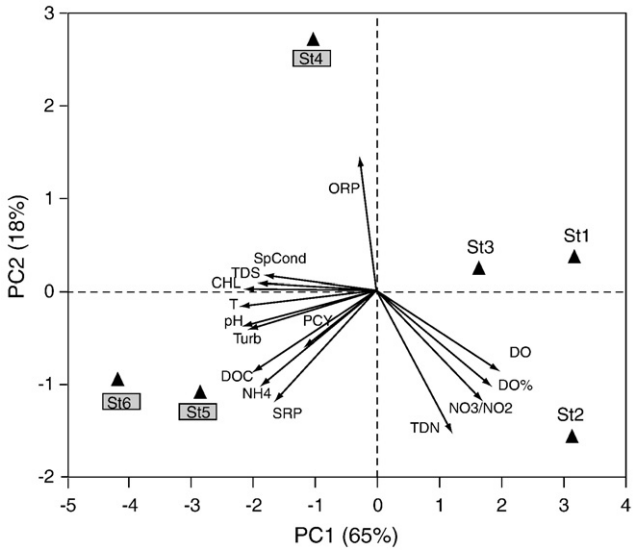

Fig. 2. PCA ordination of environmental conditions in the SD (shaded) and WB sites. The amount of variability accounted for by each principle component (PC) is shown. Measured environmental variables are represented by vectors.

each sample, which indicated that each sequence library had over 70% coverage (Table 1). The occurrence of cyanobacterial sequences were only 0.2–0.8% of total sequences for WB samples, but were much more abundant in the SD samples ($\%_{cyano}$ = 0.6–13.8 % of total sequences), especially for the particle-associated populations ($\%_{cyano}$ = 8.3–13.8%; *t* test, $P < 0.05$; Table 1). Over 90% cyanobacterial sequences were affiliated with *Planktothrix*; the rest were affiliated with *Microcystis*.

The pyrotag sequences of bacteria were diversely affiliated with a total of 64 orders and 14 phyla, indicating a highly heterogeneous bacterial community in the studied samples. Over 75% of the bacterial sequences, however, were affiliated with only 10 bacterial orders (Fig. 3). These orders included *Actinomycetales* (*Actinobacteria*), *Burkholderiales* (*Betaproteobacteria*), *Bacteroidales* and *Sphingobacteriales* (*Bacteroidetes*), *Caldilineales* (*Chloroflexi*), *Clostridiales* (*Firmicutes*), *Spirochaetales* (*Spirochaetes*), *Planctomycetales* (*Planctomycetes*), *Rhodobacterales* (*Alphaproteobacteria*) and *Verrucomicrobiales* (*Verrucomicrobia*).

WB samples (St1-3) were generally more diverse (R = 7.5–12) than the SD samples (St4-6; R = 1–5.5). In addition, except for St3 and St4, the FL populations consistently had higher diversity ranks than their corresponding PA populations. Diversity ranks of FLs were tied with or lower than corresponding PAs for St3 and St4 samples, respectively (Table 1).

The degree of compositional similarity between samples was examined using nonmetric multidimensional scaling (MDS) analysis, which generally grouped bacterial communities based on their population types (Fig. 4). Except for St1-FL and St6-PA, the distances between the FL and PA samples were longer than within the FL or PA samples. ANOSIM coefficient between FL and PA populations was between 0.5 and 0.75 (r_{ANOSIM} = 0.58), which statistically confirmed the separation between the FL and PA samples, although with low level of overlapping (Fig. 4). SIMPER analysis revealed that over half of the variance between FL and PA compositions was from varying distributions of 13 orders (Table 2). *Actinomycetales* contributed the most (11%) to the FL-PA differences and they were overrepresented in FL (38.8% of total sequences in average) in relative to PA (13.3%) samples (Fig. 3, *t* test, $P < 0.05$). On the other hand, no clear grouping patterns of bacteria were found based on sampling locations alone (SD and WB) by either MDS or ANOSIM analyses (r_{ANOSIM} <0.25), despite the distinct environmental conditions found between these two sites.

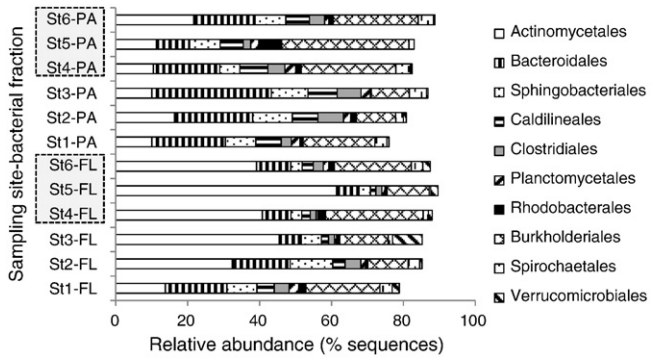

Fig. 3. Distribution of the top ten most abundant orders in the FL and PA populations in the SD (shaded fonts) and WB samples based on 16S rDNA V6 region partial sequences.

Discussion

As one of the first efforts to characterize bacterial community in Lake Erie, this study identified the diversity and composition of free-living (FL) and particle-associated (PA) non-cyanobacteria in Sandusky Bay and adjacent waters in the Western Basin of Lake Erie.

About 84% of recovered sequences in all SD and WB samples were affiliated with ten bacterial orders (Fig. 3) and the majority (80%) of them consisted of *Actinomycetales* (*Actinobacteria*), *Bacteroidales*, *Sphingobacteriales* (*Bacteroidetes*) and *Burkholderiales* (*Betaproteobacteria*). High abundances of these taxa are common to freshwater, especially those that are eutrophic (Eiler and Bertilsson, 2004; Newton et al., 2011; Oh et al., 2011; Zwart et al., 2002), which can be partly explained by their broad selection of substrates among phytoplankton- (alagal and cyanobacterial) derived dissolved organic carbon (Zeder et al., 2009). In addition, the success of free-living *Actinobacteria* in freshwater epilimina has been attributed to their potential ability in harvesting sunlight as an alternative energy source (Newton et al., 2011). Members of *Bacteroidetes* have been shown to effectively transform DOC released from particles of terrestrial origins, a source which typically contains a large amount of humic substances that are generally unavailable to most of the other bacterial taxa (Eiler et al., 2003; Hutalle-Schmelzer et al., 2010). Sandusky Bay and adjacent waters in Lake Erie receive high loads of allochthonous DOC from the input of Sandusky River (Heath, 1992; Hwang and Heath, 1999), and accordingly were found to harbor high relative abundance of *Bacteroidetes*.

Bacteria of the other orders were at low abundance in the identified bacterial communities (individually < 5% of total sequences).

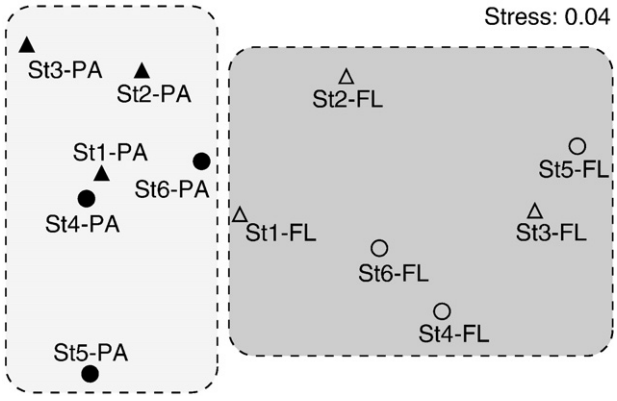

Fig. 4. MDS ordinations of FL (solid symbols within the light grey box) and PA (open symbols within the dark grey box) non-cyanobacterial compositions in the SD (circle symbols) and WB (triangle symbols) sites.

Table 2
Result of SIMPER analyses. Values of estimated individual (δ_i%) and cumulative ($\sum\delta$%) contribution of bacteria taxa to the differences observed between FL and PA bacterioplankton are shown. $AvgD_{FL\text{-}PA}$: average dissimilarity between FL and PA communities AvgD/SD: ratio of average dissimilarity with standard deviation.

Taxa	$AvgD_{FL\text{-}PA}$	AvgD/SD	δ_i%	$\sum\delta$%
Actinomycetales	3.09	1.98	11.01	11.01
Bacteroidales	1.61	1.46	5.75	16.75
Caldilineales	1.21	2.70	4.32	21.07
Burkholderiales	1.2	1.33	4.28	25.35
Verrucomicrobiales	1.09	1.23	3.87	29.22
Sphingobacteriales	0.96	1.62	3.43	32.65
Lactobacillales	0.89	2.12	3.17	35.82
Clostridiales	0.74	1.43	2.64	38.46
Aeromonadales	0.67	1.28	2.37	40.83
Spirochaetales	0.66	1.27	2.35	43.18
Rhodospirillales	0.62	1.38	2.22	45.40
Pseudomonadales	0.61	1.24	2.19	47.59
Flavobacteriales	0.6	1.19	2.16	49.75
Rhodobacterales	0.6	1.03	2.14	51.89

These taxa have been typically found as minor members in various freshwater environments (Allgaier and Grossart, 2006; Eiler and Bertilsson, 2004; Newton et al., 2011) and their ecological functions are largely understudied. Recent studies have suggested that members of *Planctomycetes* can perform anaerobic ammonium oxidation (anammox), therefore, are potentially important in the nitrogen cycle (Strous et al., 1999).

Environmental conditions have been recognized as important factors in shaping bacterial community structures and diversity (Yannarell and Triplett, 2005). Based on physio-chemical parameters and nutrient concentrations, SD and WB sites provided distinct environmental conditions. However, this did not concur with different bacterial compositions between the two sites. Bacterial compositions have been found to be affected more by the composition of phytoplankton than environmental conditions in ambient lake water (Eiler and Bertilsson, 2004). The cyanobacterial communities in the SD and WB samples were dominated by a single taxon i.e., *Planktothrix* (>90% cyanobacterial sequences in each sample), indicating relatively uniform phytoplankton compositions, which may underscore the similarity between the bacterial compositions in the two types of sampling sites.

Despite non-distinguishable community compositions, bacteria in WB sites, which had lower concentrations of chlorophyll *a* and nutrients, were more diverse than those in the SD sites. This indicated that diversity might be more sensitive than the bacterial composition to environmental conditions. The observed negative correlation between bacteria diversity and primary productivity/nutrient supply has been reported in other aquatic environments (Hewson and Fuhrman, 2004), and can be explained by the widely observed "humpback" relationship between diversity of organisms (plants, animals and microbes) and environmental gradient in temperate environments (Graham and Duda, 2011; Pärtel et al., 2007). This pattern suggests that maximum diversity of bacteria may be reached at intermediate level of nutrient supply and hyper-eutrophic conditions, which were found in the SD sites, may inhibit bacterial diversity due to decreases of niches (Hewson and Fuhrman, 2004).

Free flowing water and particles provide distinct environments to microorganisms, therefore, it is typically expected that the free-living and particle-associated bacteria are composed differently (Allgaier and Grossart, 2006), which agrees with our study and others (Acinas et al., 1999; Bidle and Fletcher, 1995; Zhang et al., 2007). Distribution of *Actinomycetales* contributed the most to the differentiation of FL and PA bacterioplankton. *Actinomycetales* were more abundant in the FL populations in both SD and WB samples (Fig. 3); similar phenomena have been repeatedly found in freshwater lakes with nutrient conditions ranged from oligotrophic to eutrophic (Allgaier and Grossart, 2006; Li et al., 2011; Rösel et al., 2012).

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