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Characterization of Microbial Community Structure Associated with Pollution in Xiaoqing River Sediment

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Abstract

To understand the impacts of anthropogenic activities on structure and composition of microbial communities and evaluate how microbial communities respond environmental gradients at river sediments, the composition of microbial communities in sediments from Xiaoqing River (in spring, summer and autumn) were assessed using DGGE and real-time PCR approaches. Meanwhile, 16S rRNA clone library construction of three sites was constructed to represent the composition and structure of microbial communities in the three distinct site-groups. The gene copy number was ranged from 10^7-10^8 cells/g that was most influenced by sample sediment density and medium grain size. Through analysis of DGGE gel profile, there were no distinct variation on richness, evenness and Shannon-Weaver index with all samples, which ranged 3.69-5.21, 1.73-2.30, and 0.56-0.76 respectively. The clustering result on the DGGE patterns showed that the microbial diversity of all samples were more similar than 40%, while the distinction was formed with three groups at a level of 46% similarity. Redundancy analysis revealed that the distribution of microbial composition seemed to be determined by the variables of nitrite, medium grain size and total carbon content. The clone library of three sites revealed that the *Proteobacteria* was the dominant phylum, which consistent with DGGE bands sequencing result. In addition, members of *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Planctomycetes* and *Verrucomicrobia* were recorded in all three libraries. The distribution of functional populations as denitrifier and anammox bacteria will be the focus of future research.

Keywords: microbial community structure, DGGE, diversity, pollution, Xiaoqing River sediments

Introduction

Microorganisms are ubiquitous and numerous life entities in natural ecosystems. Previous study using epifluorescence microscopy and flow cytometry revealed that the microbial abundance in sediment was as high as 10^3 to 10^9 cells per cubic centimetre (Duhamel and Jacquet, 2006; Kallmeyer *et al.*, 2012). Catalysing substance biogeochemical processes, microorganisms play important roles in decomposition of organic matter, nutrient cycling and biological nutrient availability. It is also clear that microorganisms hold extremely amount genetic diversity, which presents differences temporally and spatially with ecosystem function (Schäfer *et al.*, 2001; Fuhrman, 2009). In recent couple of decades, the use of genome sequences and related molecular techniques has overcome the need for cultivation to characterize microorganisms and measure

their comparison in nature (Dorigo *et al.*, 2005). Many studies were conducted to understand the distribution of microbial diversity and the environmental factors influence (Fierer and Lennon, 2011). However, identifying the underlying mechanisms causing differences in community composition, much more information are demanded than we have today.

River endures run-off occurring from the surrounding land with nutrients and pollutants. Those contaminations poses a threat for microorganism concentration and diversity settle in the sediment which can impact water quality later. Various studies have explored the microbial community structure in sediment of unpolluted, of eutrophic conditions and of heavy-metal polluted (Hamonts *et al.*, 2014). The results support the role of a variety of abiotic factors in determining and altering microbial community structure, but still confuse how vast microbial community assembly reflect or response to complex environmental drivers especially under impacts of anthropogenic activities (Nemergut *et al.*, 2013).

The Xiaoqing River is located in western Shandong Province and flows into the Laizhou Bay of Bohai Sea in China. It provides irrigation and cultivation water around 11,000 square kilometres in the region. However, rapid urbanization, industrial activities, wastewater drainage, and oil pollution have been causing heavy pollution in the river. In recent years, various contaminants have been injected into the Laizhou Bay via Xiaoqing River which caused seriously ecological deterioration (Luo *et al.*, 2013). As a result of pollution, sediments of some sites along the Xiaoqing River have become anoxic.

The purpose of the current investigation was to mapping the spatial and temporal distribution of microbial abundance and taxonomic diversity in surface sediment in the Xiaoqing River. We sampled four sites along the river during spring, summer and fall seasons in 2012. Population composition was followed by means of DGGE banding-pattern profiles of partial 16S rDNA amplicons. Using community ecological analyses, we examined how the microbial community in sediment varied in sediment and which properties from environmental and anthropogenic drivers are likely to control this variation.

Materials and Methods

Sampling collection

Sediment samples were taken from four sites of the Xiaoqing River as shown in Figure 1 in April (spring), August (summer) and October (autumn). There has been a heavily agriculture contaminant with eutrophic drainage discharge to XQH4. XQH3 is the main location of chemical plant which receives various types of wastewaters. Furthermore, due to continuous over exploitation of coastal aquifers, the salinity of river basin is high affected by saline water intrusion (Meng *et al.*, 2002). Triplicate samples were taken from individual sites using sterile 20 ml syringes (luer end removed), homogenized and then subdivided for molecular analyses (10 ml) and environmental parameters (40 ml). The former were stored in liquid nitrogen containers and put in -80 ℃ refrigerator in the laboratory until use. And the latter were stored in cool box and processed immediately processed to analyse after transported to laboratory.

Temperature, pH and salinity of overlying water were recorded in situ with an YSI model 556MPS during sampling. Water content was determined based on the loss of weight of the sample after 48 h of freeze drying. Pore water was obtained by centrifugation (10 min at 5,000 ×g). The particle diameter was investigated by using a laser diffraction instrument (Malvern

Mastersizer 2000, Malvern, UK). Dissolved inorganic nutrients, including NH_4^+-N, NO_2^--N,

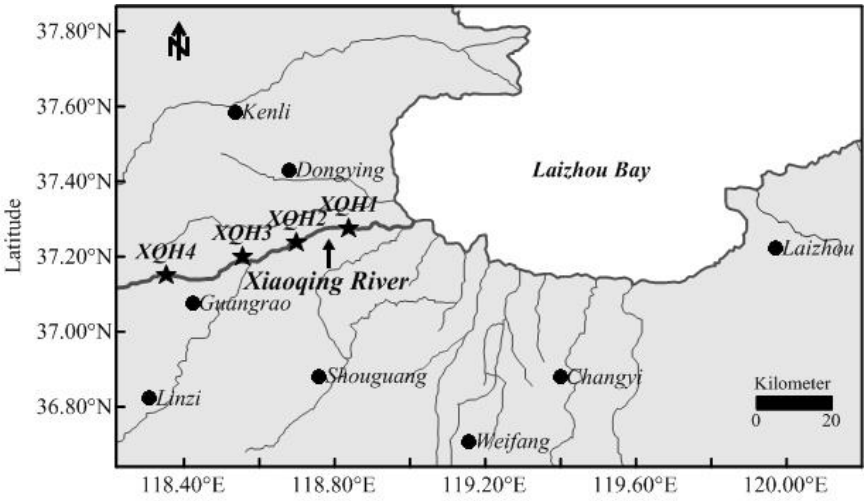

Figure 1. Location of sampling sites.

NO_3^--N andPO_4^{3-}-P were measured using standard colorimetric methods on an AAA3 segmented flow analyser (Seal Analytical GmbH, German). Total carbon and total nitrogen were determined with an elemental analyser (Elementar, Vario Micro). The mass spectrometer of Elan DRC II ICP-MS instrument (PerkinElmer Ltd., Hong Kong) was used for Pb, Cr, Mn, Cu, Zn and Cd measurements.

DNA extraction and PCR amplification

Genomic DNA was extracted from 0.5 g sediments using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., USA).

Amplification of 16S rDNA was performed with primers 341f-GC/534r for DGGE (Muyzer *et al.*, 1993), 331f/797r for quantitative PCR (Smith and Osborn, 2009) and 27f/1542R for clone library (Suzuki *et al.*, 1998). Reaction conditions were carried out using method as described in above references. Real-time (SYBR) quantitative PCR was performed by 7500 Fast Real-Time PCR system and data was analysed using the System detection software (Applied Biosystems, CA). The products were visualized by electrophoresis on a 1% (w/v) agarose gel and purified by MiniBEST Agarose Gel DNA Extraction Kit (Takara Biotechnology, China).

DGGE and statistical analysis

Community profiles of microbial community within the sediments were obtained using DGGE analysis as described by Muyzer *et al.* (1993). 6% polyacrylamide gels with denaturant gradient of 40-60% were used on D-code system (Bio-Rad Laboratories Ltd., UK). Electrophoresis was performed at a constant voltage of 100 V for 16 h at 60 ℃. Gel images were recorded using Gel Doc™ XR+ imaging system (Bio-Rad Laboratories Ltd., UK) after stained by SYBR green I nucleic acid gel stain (Life technology, USA). Major bands were excised, reamplified and then sequenced by a commercial company (Invitrogen, Beijing, China).

DGGE bands were scored as present (score = relative densities) or absent (score = 0). The data was analyzed by cluster analysis and principle component analysis (PCA) using the software program PRIMER 6 and Canoco 4.5 (described as Sahan and Muyzer, 2008; Li *et al.*, 2013). Shannon-Weaver diversity indices, evenness measure estimates and richness were also calculated to compare the relative complexities of microbial communities.

Clone library construction and phylogenetic mapping

Microbial library was constructed for two samples based on the cluster result of DGGE analyses by RFLP method. Phylogenetic tree was further analyses from the alignment sequences using MEGA 4.0 with evolutionary distances (Jukes-Cantor distances) followed by neighbour-joining method. Diversity within each microbial community (α-diversity) were analysed by Mothur 1.29 (Schloss *et al.*, 2009).

Results and Discussion

Environmental characteristics

For characterization of the Xiaoqing River sediments, a variety of parameters were analysed and summarized in Table 1 to 3. The sediment samples appeared visually greyish-black colour and densely packed with silk-sized particles. Samples from XQH3 had a noticeable odor of H_2S.

The nutrients concentrations detected in this study were consistent with those in other rivers influenced by urbanization and agriculture, such as the Po River (Tesi *et al.*, 2013) and the Pearl River (Huang *et al.*, 2004). Ammonium and nitrate concentration in pore water presented apparent temporal variation. Relative lower ammonium and nitrate concentration were detected in August and October respectively, which may be caused by different fertilizer discharged into river with irrigation water. The overall C/N stoichiometric ratio ranged from 18.13 (Apr of XQH2) to 44.33 (Aug of XQH1) is very low compared with the Redfield Ratio for phytoplankton as 53/8, which meant the N eutrophication in the sediment. Heavy metal contents of the sediment were found to be lower than that of study at the Xiaoqing Estuary (Zhuang and Gao, 2014). The higher Mn and Zn concentrations were found in the samples and this result were similar to previous study of other highly polluted sediment by heavy metals (Varol and Şen, 2012; Lokhande *et al.*, 2011). The physical and chemical results indicated heavily polluted situation in the Xiaoqing River due to anthropogenic activities.

Table 1. Physical characteristics of the sediments.

Samples		T (℃)	Salinity	pH	Density (g/cm^3)	Water (%)	Clay (%)	Silk (%)	Sand (%)	Median grain size (ø)
	Apr	16.99	5.26	7.57	1.96	21.24	5.14	70.04	24.83	4.73
XQH1	Aug	26.57	5.60	7.89	2.01	21.10	6.35	56.06	37.60	4.46
	Oct	17.28	4.71	7.72	1.85	33.10	5.95	76.49	17.57	5.21
	Apr	18.80	2.53	7.74	1.58	35.03	6.24	78.92	14.83	5.24
XQH2	Aug	28.36	1.69	7.73	1.71	35.19	5.62	47.78	46.60	4.17
	Oct	17.70	2.75	7.83	2.13	26.28	3.72	46.07	50.21	3.99
	Apr	19.43	1.93	7.66	1.42	34.47	4.94	66.39	28.67	4.81
XQH3	Aug	28.48	1.71	7.73	1.52	43.92	6.56	64.71	28.73	4.66
	Oct	18.07	2.75	7.78	1.91	21.08	6.43	72.88	20.69	4.91
XQH4	Apr	19.92	1.67	7.64	2.05	23.51	6.27	72.51	21.22	5.03

	Aug	27.42	1.78	7.76	1.52	34.54	6.83	80.88	12.29	5.62
	Oct	17.91	2.91	7.78	1.61	44.12	8.18	82.33	9.48	5.72

Table 2. Nutrients in the pore water and TC/TN concentration of sediments.

Samples		NH_4^+-N (μM)	NO_2^--N (μM)	NO_3^--N (μM)	PO_4^{3-}-P (μM)	TC (%)	TN (‰)
	Apr	53.54	3.94	53.16	0.21	4.79	3.04
XQH1	Aug	3.64	1.73	31.43	0.02	1.33	0.35
	Oct	34.47	0.26	1.04	0.34	2.35	1.26
	Apr	11.11	9.98	45.78	0.32	4.89	2.82
XQH2	Aug	8.79	0.42	23.56	0.01	1.73	0.58
	Oct	29.39	0.18	4.02	0.18	1.33	0.36
	Apr	24.19	14.60	55.07	0.69	4.46	2.87
XQH3	Aug	0.43	0.25	6.02	0.02	1.20	0.39
	Oct	0.22	1.24	7.50	0.11	0.59	0.35
	Apr	8.18	61.45	39.14	0.32	1.65	0.93
XQH4	Aug	8.50	2.91	9.89	0.04	4.83	3.03
	Oct	0.25	1.40	7.92	0.12	4.93	2.69

Table 3. Heavy metal contents of the sediments.

Samples		Pb (ppb)	Cr (ppb)	Mn (ppb)	Cu (ppb)	Zn (ppb)	Cd (ppb)
	Apr	69.35	167.91	1759.00	54.73	268.11	0.83
XQH1	Aug	171.36	526.43	1018.91	141.19	1669.21	1.92
	Oct	151.24	561.23	845.97	78.52	1542.31	1.32
	Apr	130.57	698.87	1986.62	170.21	2430.33	2.07
XQH2	Aug	176.40	698.35	1486.40	177.96	1487.10	2.46
	Oct	158.75	643.24	1345.21	154.87	1648.54	2.43
	Apr	96.31	563.64	1603.95	99.86	1332.92	1.75
XQH3	Aug	146.68	456.30	1130.23	141.69	1336.25	1.90
	Oct	101.80	378.94	1345.21	132.48	1451.46	1.94
	Apr	43.95	31.14	1869.98	29.55	90.72	0.51
XQH4	Aug	69.02	17.42	904.88	19.67	47.27	0.31
	Oct	72.56	26.54	1123.59	20.94	56.78	0.64

Microbial abundance

The quantitative PCR achieved 104.9 % efficiency with the regression coefficient of 0.995. Results revealed that the microbial abundance varied from 8.26×10^8 to 4.14×10^7 cells/g. No significant differences were observed for sediment microbial abundance in four sites or in three seasons from the control at the $P < 0.05$ level (Table 4). The sediment density had a significant positive relationship with microbial quantities ($r = 0.66$, $P = 0.003$, $n = 12$), furthermore the median grain size had a negative relationship ($r = 0.49$, $P = 0.028$, $n = 12$). It was indicated that the physical characteristics of sediment rather than the chemical ones determined the microbial abundance.

Table 4. 16S gene transcript numbers in surface sediment (cells/g).

Samples	Numbers of gene		
	Apr	Aug	Oct
XQH1	$2.58 \times 10^8 \pm 6.86 \times 10^6$	$8.26 \times 10^8 \pm 1.33 \times 10^7$	$1.66 \times 10^8 \pm 4.00 \times 10^6$

XQH2	$4.72 \times 10^7 \pm 4.24 \times 10^6$	$1.72 \times 10^8 \pm 2.16 \times 10^7$	$4.58 \times 10^8 \pm 5.56 \times 10^7$
XQH3	$7.94 \times 10^7 \pm 5.20 \times 10^6$	$2.40 \times 10^8 \pm 1.02 \times 10^5$	$1.73 \times 10^8 \pm 2.44 \times 10^6$
XQH4	$4.72 \times 10^8 \pm 4.60 \times 10^6$	$4.14 \times 10^7 \pm 1.46 \times 10^6$	$1.08 \times 10^8 \pm 7.22 \times 10^6$

DGGE analysis of microbial composition

To reveal the microbial community structure, approximately 200-base pair fragments were used in DGGE to obtain the profile (Figure 2) which gave adequate information about similarity matrix, diversity indices and dominant phylotypes. Cluster analyses showed all samples groups together at a level of 40% similarity. At a level of 46% similarity, three assemblages were distinguished. The most similar DGGE band patterns belonged to XQH3 and XQH4 in April with the similarity of 70%. As seen in Table, species richness measured by Margalef's and microbial diversity as measured by the Shannon-Weaver index, ranged between 3.69-5.21 and 1.73-2.30, respectively. The diversity indices results appeared to be stable and little difference was detected from various sites or seasons.

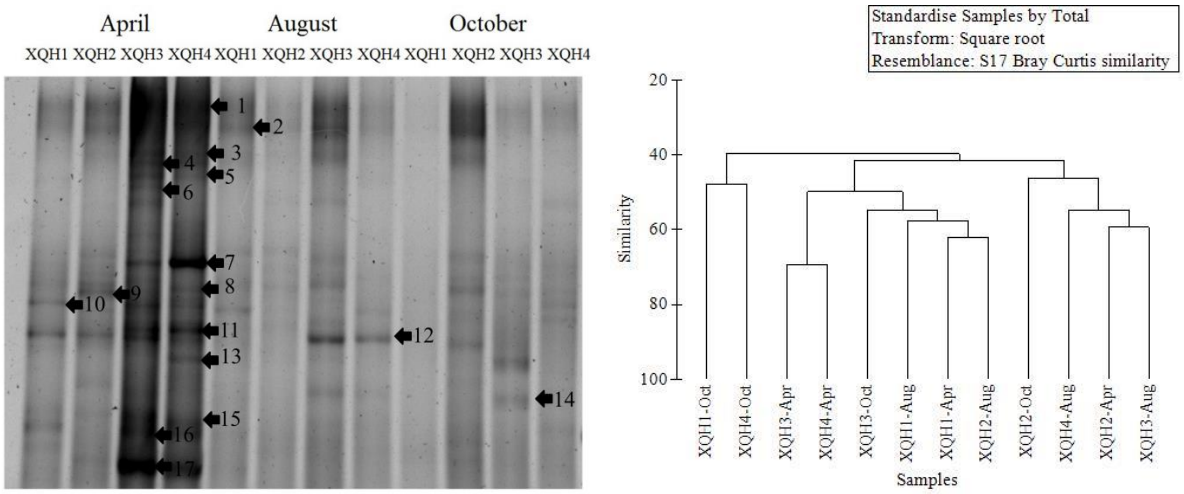

Figure 2. DGGE profile and similarity matrix result

Table 5. Diversity indices of different samples based on DGGE profiles.

Index	XQH1			XQH2		
	Apr	Aug	Oct	Apr	Aug	Oct
Richness	4.34	3.69	4.56	4.13	5.21	4.56
Evenness	0.71	0.60	0.81	0.76	0.72	0.56
Shannon-Weaver	2.15	1.73	2.50	2.28	2.30	1.73
Index	**XQH3**			**XQH4**		
	Apr	Aug	Oct	Apr	Aug	Oct
Richness	4.13	4.56	4.56	3.69	3.91	5.21
Evenness	0.62	0.72	0.74	0.74	0.72	0.71
Shannon-Weaver	1.85	2.24	2.28	2.15	2.11	2.27

Seventeen bands were sequenced and affiliated to *Proteobacteria*, *Bacteroidetes* and uncultured bacterial clones (Figure 2 and Table 6). *Proteobacteria* is the dominant phylum with 53%

sequences in 75% confidence threshold. Half of sequences showed similarity of low than 97% with known sequences in NCBI database which indicated the novel species still exist in the research sediments. Most of these sequences had their closest matches originally received from sea sediment, lake water, industrial sewage, and anaerobic sludge.

Table 6. Microbial phylotypes detected by PCR-DGGE.

Phylogenetic affiliation	Band No.	Closest match (accession number)	Identity (%)	Alignment (bp)
Proteobacteria	8	*Massilia* sp. TSO8 (AB545620)	99	188
	13	*Oxalobacteraceae* bacterium RDB-069 (AB730241)	98	186
	16	*Vibrio* sp. LAW003.06 (HM012728)	97	184
Bacteroidetes	7	*Flavobacterium* sp. AKB-2008-TE31 (AM988933)	100	182
	9	*Flavobacterium* dongtanense strain LW30 (NR_117428)	99	179
	12	*Myroides* sp. SCU-B239 (KJ000848)	96	178
	14	*Sejongia* sp. M_Sw_oHS_07/10_6_2 (KF777435)	97	184
Bacteria	1	Uncultured *Lysobacter* sp. clone RUGL1-274 (GQ421125)	95	169
	2	Uncultured bacterium isolate DGGE gel band HY09 (EU670682)	94	151
	3	Uncultured *Ralstonia* sp. isolate DGGE gel band 21TAT (KC800900)	92	174
	4	Uncultured *gamma proteobacterium* clone M3-052 (KF183270)	95	166
	5	Uncultured *Undibacterium* sp. isolate DGGE band PW-14 (JX191910)	94	163
	6	Uncultured bacterium isolate DGGE gel band X9 (EU670856)	95	148
	10	Uncultured *Lysobacter* sp. isolate DGGE gel band VW-16 (JX228211)	96	142
	11	Uncultured *gamma proteobacterium* clone 4299-27F (FR647869)	93	178
	15	Uncultured *Bacteroidetes* bacterium clone 175 (HE803917)	97	172
	17	Uncultured *Firmicutes* isolate DGGE band OTU76 (KC142274)	99	176

PCA of DGGE profiles and correlation analysis with environmental variables

CCA analysis of bacterial classes was used to reveal their relationship with environmental variables (Figure 3). The sum of all canonical eigenvalues indicated the total variation explained by environmental variation of 59.5%. Concerning the variance of bacterial class data, the first two CCA axes explained 56.0% of the total variance in the bacterial composition and 66.0% of the cumulative variance of the bacteria-environment relation. Correlations of bacterial classes and environment variables were 99.9% and 98.5% for axis CCA 1 and 2 respectively.

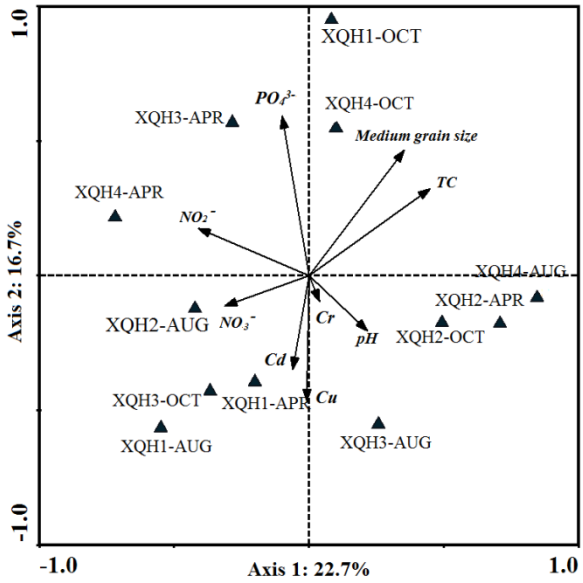

Figure 3. RDA ordination plots for the first two dimensions of the relationship between the sampling sites and environmental parameters. For environmental factor vectors, correlations are represented by the length and angle of arrows.

CCA1 represented a gradient due to the TC in positive and nitrite in negative, indicated by the correlation coefficient of 0.46 and -0.42. Based on the 5% level in a partial Monte Carlo permutation test, nitrite and medium grain size in combination with total carbon content contributed significantly ($P < 0.05$, F = 2.48) to the bacteria-environment relationship, providing 37.0% of the total CCA explanatory power.

While medium grain size and total carbon content were positively correlated with microbial PC1, those two geochemical variables were also significantly correlated ($r = 0.614$, P = 0.034). Their relationship with microbial composition supports the idea that medium grain size and carbon content are the main physical and chemical variables, respectively, which influence the microbial distribution in the Xiaoqing River. Medium grain size represents the sedimentary components and total carbon content indicates the available carbon resource for microbial metabolism. The relationship of the medium grain size with microbial genotype assemblages could be related directly or indirectly to sedimentological characteristics, via their differences on sediment source, anoxic level, sorting coefficient and other, as yet unknown, physico-chemical, sedimentological or geochemical factors. Nitrite was also the main variables to impact the microbial compositions instead of other nutrients, which is the unexpected result. This situation may be explained by the biogeochemical process of anaerobic methane oxidation coupled to denitrifatication in which nitrite is the electron acceptor. Certainly, care must be taken that causal connection cannot be concluded from statistical analysis alone as correlation values obtained might be the result of other vatiables. However, because of the covariability and complexity of the environmental variables, the exact influence and contribution of possible environmental factor need further precise experiments on isotope labelling incubation using native community in the future (Cetecioğlu *et al.*, 2009).

Phylogenetic construction and tree topologies

Samples of XQH1-OCT, XQH3-APR and XQH3-AUG were selected to construct clone library representing three site-groups at 45% similarity. Their phylotype distribution and maximum likelihood phylogenetic radual tree were shown in Figure 4.

The 16S rRNA sequences were grouped mainly with *Proteobacteria* and *Chloroflexi* (27.4%, 42.9% and 21.4% of the total clones, respectively). Both of them showed a definitely different percentage composition in the microbial communities compared. The *gamma Proteobacteria* dominated in XQH1-Oct sample with occupation of 29.1%, while the *Anaerolineae* dominated in XQH3-Apr sample with occupation of 39.2%. No obviously dominant class was found in XQH3-Aug.

Conclusions

The microbial population compositions were outlined along the sediments of the Xiaoqing River in this research. Their abundance ranged from 10^7 to 10^8 cells/g. Cluster analyses revealed that the microbial diversity shared similarity with more than 40% level based on the analysis of DGGE gel profiles. *Proteobacteria*, were the dominant phylum in the clone library. The influence from geographic variation and seasonal shift is very little. Meanwhile, the physical parameters as density and medium grain size determined the quantities of microorganism in the sediment. And nitrite, medium grain size and total carbon content are the main environmental factors to influence the microbial constitutes. Future investigation should focus on what common rules of natural selection impact the microbial communities and how bacteria act in the biogeochemical cycles.

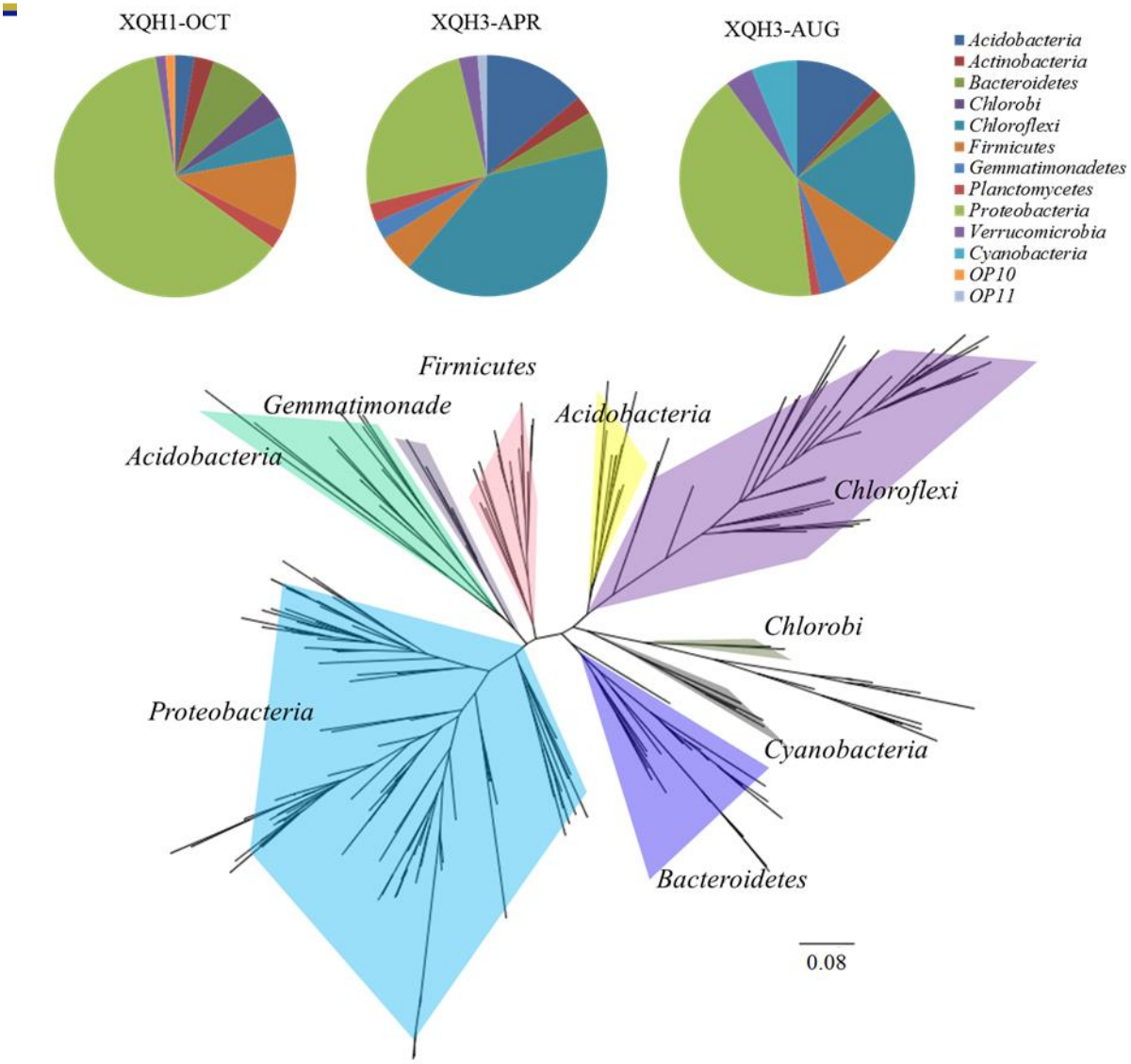

Figure 4. Phylotype distribution and comparison of the clone libraries and Maximum likelihood phylogenetic radual tree.

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