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ORIGINAL ARTICLE

Biofilm microbial community structure in an urban lake utilizing reclaimed water

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Abstract Analyses of biofilm community structure may potentially be employed for aquatic ecosystem health assessment, however, to date, biofilm diversity within urban lakes using reclaimed water has not been examined. Accordingly, the microbial community diversity and structure of biofilms from the surface of multiple matrices with varying roughness (0.1, 1.0 and 10.0 μm) were characterized using a suite of molecular techniques including scanning electron microscopy, genetic fingerprinting and phospholipid-derived fatty acid analyses. Samples were largely comprised of inorganic particles, algae and numerous bacterial species; 12 phospholipid-derived fatty acid (PLFA) types were identified, significantly less than typically associated with sewage. Both growth matrix surface roughness and biofilm growth phase were shown to concur with significantly different microbial quantity and community structures. Gram-negative bacteria bacillus i15:03OH and 18:0 were the dominant bacterial genera, collectively comprising ≈75 % of identified PLFA species content. Calculated species diversity (*H*) and species dominance (*D*) exhibited identical correlational patterns with measured water quality parameters; significant positive correlations were exhibited with respect to Mg^2, while significant negative correlations were found for NO^3, TP, BOD, COD, SP, PO^4, SO^4 and pH. Results indicate that analyses of biofilm formation and structure could be effectively used to undertake integrated assessments of the ecological health of lake systems using reclaimed water. Further work is required to elucidate the optimum conditions for sample collection and analytical interpretation.

Keywords Biofilm · Growth matrices · Microbial community structure · Reclaimed water · Urban Lake

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Introduction

The process of water reclamation is characterized by chemical, physical and biological treatment of domestic and/or commercial wastewater, in order that the treated water may be safely released to natural (e.g., aquifer, stream, lake) or man-made aquatic systems (e.g., lake, reservoir, park) (McClain and Williams 2014). Successfully reclaimed water may be used to reduce the instance of aquatic contamination via wastewater diversion and dilution (Juanico and Friedler 1999) and water supply augmentation (McClain and Williams 2014). Although reclaimed waters are required to satisfy national discharge standards, high concentrations of dissolved salts, organic constituents, suspended solids and microorganisms may remain, thus representing a potential source of environmental contamination and adverse human health effects in adjacent areas. For example, McClain and Williams (2014) have reported high levels of antibiotic resistance (lincomycin, ciprofloxacin and erythromycin) among bacteria found in reclaimed water samples from central Arizona. Similarly, Kinney et al. (2006) have previously found high levels of wastewater-derived pharmaceuticals in soil irrigated with reclaimed water in Colorado.

To date, >90 % of known microorganisms have been found to exist within solid matrix adherent biofilms; these biofilms comprise microorganisms (bacteria, protozoa and fungi) and inorganic minerals within a self-produced extracellular polymeric matrix, typically composed of polysaccharides, proteins and extracellular DNA (Hall-Stoodley et al. 2004). Numerous exploratory investigations pertaining to biofilm microbial communities in wastewater (Fernandez et al. 2008; Nozhevnikova et al. 2012), drainage water (Percival et al. 1999; Lear et al. 2009), irrigation water (Kinney et al. 2006), lakes (Dong et al. 2005), wetlands (Baty et al. 1996) and river sediments (Liang et al. 2013) have been undertaken. Lünsdorf et al. (2002) previously studied the formation and growth of composite biofilms in acidic (pH 2.5) mining lakes in Germany, noting that associated microbial communities may be effectively utilized for remedial purposes.

Biological status monitoring represents the most direct and effective method for diagnosis of aquatic ecosystem health status, with microorganisms playing a central role in ecological service function provision (i.e., environmental improvement, habitat provision, bioremediation, etc.) (Atlas and Cerniglia 1995; Bailey et al. 2007). Moreover, an improved understanding of ecosystem structure and maintenance permit enhanced impact analyses, hazard and exposure evaluations, in addition to other management and regulatory activities (Bailey et al. 2007). Biofilms associated with both natural and man-made waterbodies effectively reflect the long-term evolution of microbial community structure and function within the associated aquatic environment (Wu et al. 2010). With respect to these environments, biofilms exist at the water–sediment interface, and thus possess inherent potential for characterization of both the water matrix and underlying sediments (Wu et al. 2010). More specifically, microbial community structures associated with specific aquatic ecosystems may be used to characterize chemical, physical and biological features within this ecosystem i.e., variable microbial categories (and community structures) will dominate resident biofilms with ecosystem-specific properties (Morin et al. 2010). For example, Wu et al. (2010) analyzed biofilms from natural Chinese lakes for biomass production, chlorophyll concentration, algal composition, extracellular enzyme activity and polysaccharide content; findings suggested that biofilms could be employed as pollution biomarkers at sample sites.

Biofilm formation and growth are significantly influenced by multiple factors including hydrologic conditions, basement type, nutritional levels and light (Sekar et al. 2004). Matrix surface roughness is recognized as the principal physicochemical surface parameter influencing microbial attachment, thus exerting a direct impact on biofilm formation and growth (Lee and Powers 2007). To date, a paucity of data exists with respect to biofilm microbial community structure and varying matrix surface roughness. Similarly, few studies have focused on biofilms in lakes utilizing reclaimed water.

Accordingly, within the current study, an urban lake currently reusing reclaimed water and located in central Beijing (Olympic Lake) was selected as a representative study site for sampling and analyses. The structural and polymorphic characteristics of microbial communities on the surface of multiple biofilm matrices with varying surface roughness (0.1, 1.0, 10.0 μm) were investigated via a suite of biochemical and molecular techniques (e.g., scanning electron microscopy, PCR-DGGE genetic fingerprinting, PDFA biomarker analyses). The presented approach may be used to provide a theoretical basis for ecological health assessment of aquatic systems using reclaimed water.

Materials and methods

Sample collection and preparation

Olympic Lake is located in Beijing Olympic Park in the Chaoyang District (N 40°00′; E 116°24′), with a maximum depth of 2.24 m (Fig. 1). Lake construction was completed in May 2007 and it is currently employed as an urban artificial ecological water system. The lake has an area of 165,000 m^2, a depth range of 0.6–1.1 m and a total volume of 159,000 m^3 (Wang et al. 2014), thus making it the largest constructed wetland system in the world supplied entirely by reclaimed water (excluding direct rainfall and resulting localized runoff). Two primary reclaimed water sources are currently employed, namely, the Qinghe Reclaimed Water Treatment Plant [80,000 m^3/day, ANANOX (Anaerobic-Anoxic–OXic Process)] and the Beixiaohe Reclaimed Water Treatment Plant (60,000 m^3/day, Membrane Bioreactor + Reverse Osmosis).

Hills and Thomason (1998) and Scardino et al. (2006) have previously compared the effect of differing substrate materials (e.g., acrylic, organic glass and shell) and substrate roughness on the measured biofilm attachment points of acorn barnacles (Semibalanus balanoides) and diatoms, respectively. Both studies reported that the influence of substrate roughness on the biological attachment point was significantly greater than the influence of substrate material, with biofilm attachment on glass slides similar to those reported for other experimental materials. As glass slides may be simply and accurately processed to simulate variable surface matrices, the in situ glass sampling method previously employed by Liang et al. (2013) was used, with glass slides (25 mm × 75 mm) of roughness 0.1, 1.0 and 10.0 μm employed.

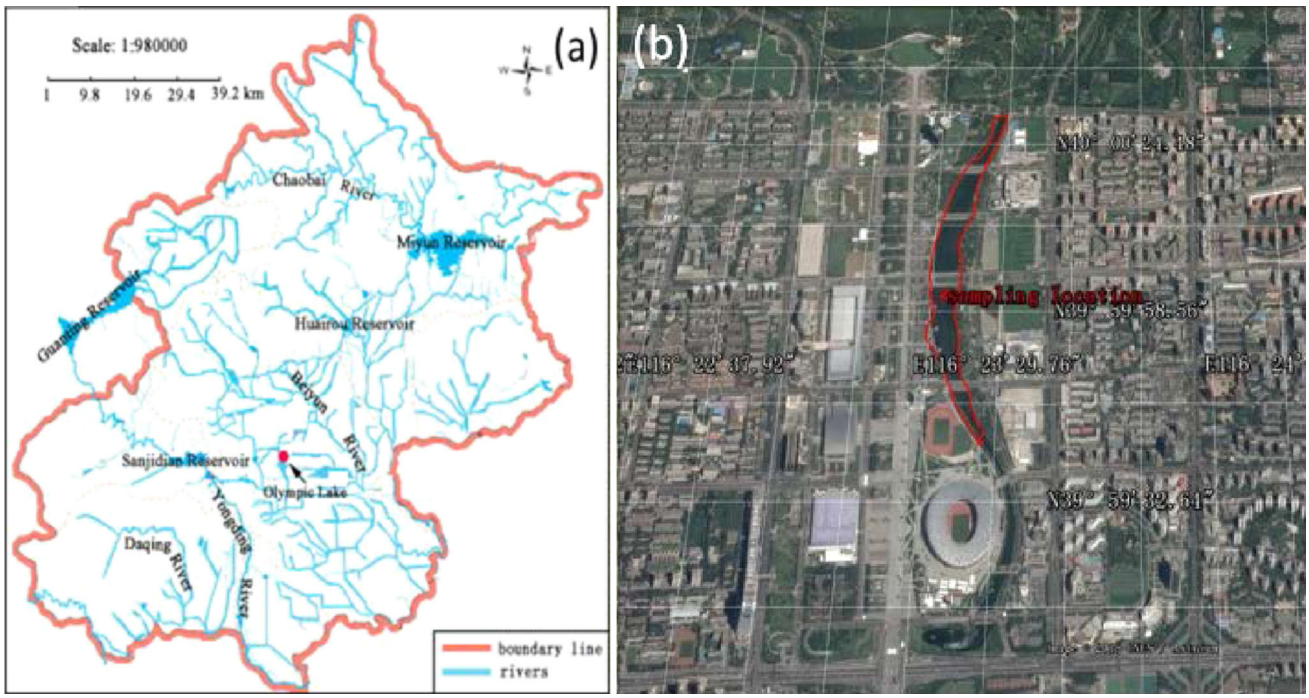

Fig. 1 **a** Location of Olympic Lake, Beijing; **b** ariel photograph with Olympic Lake and biofilm sampling location shown

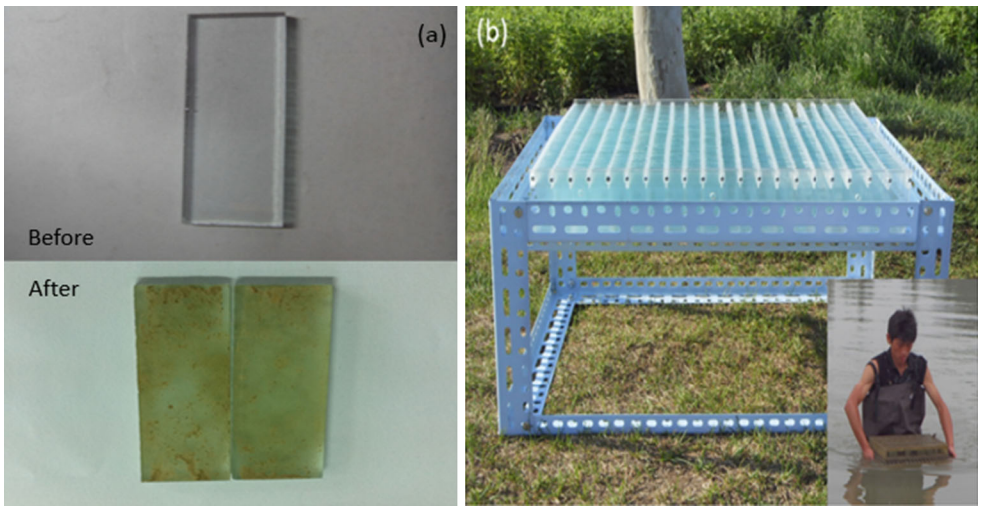

Fig. 2 **a** Photographs of glass slides (1.0 µm surface roughness) before and after biofilm growth; **b** one of three organic glass shelves used for biofilm collection in the current study; 1.0 µm surface roughness with 450 glass slides; 48 cm × 60 cm × 7 cm *10-day* 1.0 µm, *10-day* −10.0 µm, *25-day* −0.1 µm, *25-day* 1.0 µm, *25-day* 10.0 µm, *58-day* 0.1 µm, *58-day* 1.0 µm, *58-day* 10.0 µm

Slides were fixed on 48 cm × 60 cm × 7.5 cm organic glass shelves (constructed in College of Water Resources and Civil Engineering) with 450 analogous slides per shelf i.e., three shelves of 0.1, 1.0 and 10.0 µm roughness (Fig. 2). Shelves were adjacently placed on the lakebed of Olympic Lake approximately 50 m from the lakeshore, close to the lake centre, at a depth of 30 cm below the water surface. To date, this sampling method has not been used to investigate biofilms in urban lakes using reclaimed water. Biofilm samples were collected three times at 10, 25 and 58 days of biofilm growth, in order that the entire biofilm growth cycle was effectively included for analyses; 150 glass slides with attached biofilm were collected from each shelf per sampling event. Lakewater samples were collected from the biofilm sampling location (30 cm depth) during each biofilm sampling event and subsequently analyzed (APHA, AWWA, WEF 2005) (Table 1). Biofilm samples collected during the second sampling event underwent PCR-DGGE analyses; biofilm growth rate was adjudged to be greatest during this phase, thus presenting optimal conditions for community structure analyses. Glass slides with the highest visible levels of biofilm growth were

Table 1 Test results of water quality parameters for all three sampling events (mg L^{-1})

	NH_4	NO_3	TN	TP	BOD	COD	SP	Ca^2	Mg^2	PO_4	SO_4	HCO_3	pH
10-day	0.33	3.78	5.46	0.17	3.90	19.3	0.14	36.8	22.0	0.34	92.9	110	9.34
25-day	0.43	1.86	6.78	0.09	2.40	17.5	0.08	39.8	29.6	0.31	90.8	115	9.16
58-day	0.61	2.74	12.0	0.10	2.60	18.1	0.12	40.4	25.2	0.36	93.5	119	9.21

selected for surface topography analyses. Surface topography analyses were undertaken after each sampling phase and for each surface roughness. All biofilm samples were placed in a zip-lock bag and refrigerated as soon as possible after sampling. Biofilms attached to the glass slides were detached via sonication at 40 kHz frequency for 45 min; resulting suspensions were used for PLFA and PCR-DGGE analyses.

Scanning electron microscopy

Biofilm surface topography analyses were undertaken using scanning electron microscopy. A Leo (ZEISS) 1450 VP scanning electron microscope (200–300 V) was used for all SEM analyses, with a working resolution of 3.5 nm. Prior to analyses, biofilm samples were prepared using an analogous method to that described in Liang et al. (2013) and carbon coated. Briefly, biofilm samples were divided into 0.6 cm^2 portions of approximately 0.1 cm thickness, followed by fixing in a 2.5 % glutaraldehyde solution and three 15 min rinses with 0.1 M sodium phosphate buffer solution (Na_2HPO_4; pH 5.8–8.0). Subsequently, samples were fixed in 1 % O_8O_4 solution for 3 h, followed by the same 3 × 15 min Na_2HPO_4 rinse procedure. Samples were dehydrated via 15 min rinses in ethanol solutions of increasing concentration (30, 50, 70, 85 and 95 %) and three final 15 min rinses in 100 % ethanol. Finally, dehydrated samples were dried using a BAL-TEC CPD030 CO_2 critical point drying process. Carbon coating was used due to the transparency of glass slides (i.e., gold sputtering was not deemed necessary). SEM was undertaken in the presence of argon and a low vacuum (1 Pa), in order to avoid carbon heating/burning, with the working stage distance systematically adjusted to prevent sample scorching.

Polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) gene fingerprinting

Microbial genomic DNA was extracted via chemical cleavage; an extended reaction time was employed (60 min) due to lower levels of DNA in biofilm samples than typically present in natural soil samples. Genomic DNA was purified using a glass bead DNA gel extraction kit according to manufacturer's instructions (SoilGen DNA Kit (SK111); ComWin Biotech Co. Ltd., Shanghai) and subsequently amplified by PCR (Sandhu et al. 2009). PCR was performed using 20–80 ng template DNA with the primers, PRBA338f and PRUN518r, located at the V3 region of the 16S rRNA genes. PCR mixtures for the bacterial 16S rDNA sequence amplification contained 10 pmol of each primer, 4 μg of bovine serum albumin and sterile distilled water up to a final volume of 25 μl. The PCR cycles used for amplification were: 92 °C for 2 min, followed by 30 cycles of 92 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 6 min. Amplified products were mapped via DGGE; DGGE was performed with 8 % (w/v) acrylamide gels containing a linear chemical gradient ranging from 40 to 60 % denaturant with 100 % defined as 7 M urea and 40 % formamide. Gels were run for 12 h at 60 V with a DCode Universal Mutation System (Bio-Rad Laboratories, Hercules, CA, USA), followed by band recovery and sequencing. Sequences were submitted to the National Center for Biotechnology Information (http://www.ncbi.nih.gov) nucleic acid sequence database for GenBank sequence analyses using the BLAST program. The highest sequence homologies of known species with respect to reference samples were found. Phylogenetic analyses were undertaken using *ClustalX1.81* and *Mega3* via the N-J method, and a phylogenetic tree subsequently developed (Kumar et al. 2004). Bootstrap re-sampling of 1000 duplicate detections (iterations) was used to validate the developed phylogenetic tree (Felsenstein 1985).

Phospholipid-derived fatty acid biomarkers

Phospholipid-derived fatty acid (PLFA) biomarker analyses were undertaken using a slightly modified approach to that previously described by Pennanen et al. (1999). Biofilm suspensions were extracted by a one-phase chloroform, methanol and 0.15 mol/L citrate buffer extractant (1:2:0.8 *V/V/V*) by shaking for 4 h at 20 °C. Solvents were separated by centrifugation (4000 rotations/min). Silicic acid chromatography was used to separate neutral, glycol and polar (phospho-) lipids; neutral lipids were eluted with 5 mL of chloroform, glycolipids with 10 mL of acetone and phospholipids with 10 mL of methanol (Minna et al. 2004). The methyl ester of nonadecanoic acid (19:0) was added as an internal standard immediately prior to separated phospholipids fatty acids

being trans-esterified into fatty acid methyl esters via a mild alkaline methanolysis reaction. Fatty acid methyl esters were analyzed using a GC–MS system (Hewlett Packard HP 6890, GMI, USA), with total quantities (nmol/mm^3) determined using methyl esters of nonadecanoic acid as an internal standard.

Microbial community diversity indices

A range of ecological diversity indices are available for the evaluation of bacterial species richness/abundance and evenness/dominance (Hill et al. 2003). In the current study, three diversity indices have been employed in concurrence with PLFA biomarker results ("Polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) gene fingerprinting"), to calculate microbial community polymorphism associated with biofilm samples from Olympic Lake.

Shannon-Wiener's diversity index H

Shannon-Wiener's diversity index, also known as the index of Shannon's information diversity, is used to describe species number within a community, the proportion of individuals in the community and the associated degree of homogeneity (Hill et al. 2003). The index is based upon the degree of entropy (uncertainty) associated with species number prediction, which is proportional to the weighted geometric mean of proportional species abundances (equivalent to the logarithm of true diversity). Accordingly, the greater the index is, the more information contained; it is calculated as follows:

$$H = -\sum_{i=1}^{s} (N_i/N) \times \ln(N_i/N) \quad (1)$$

where S represents species number based upon PLFA results and N_i/N represents the proportion of each PLFA type.

Pielou's evenness index J

Species evenness equates to the relative level of equality with respect to species number within a specific microbial community (Rousseau et al. 1999; Hill et al. 2003). When the number of each individual species approaches equality (i.e., $N_1 \approx N_2 \approx \ldots\ldots \approx N_S$), the evenness index of the community (J) will be high. Pielou's evenness was calculated as follows (Rousseau et al. 1999):

$$J = \frac{H}{\ln S} \quad (2)$$

where H is derived from Eq. 1 and S is the total species number

Simpson's dominance index D

Simpson's dominance index (D) represents the relative level of superiority attributed to individual species within a specific microbial community (Sun and Liu 2004). A greater dominance index score equates to a more (proportionately) dominant species position with a specific microbial community; D was calculated as follows (Sun and Liu 2004):

$$D = 1 - \sum_{i=1}^{s} (N_i/N)^2 \quad (3)$$

Statistical analyses

One-way analysis of variance (ANOVA) with associated post hoc Bonferroni multiple comparisons analysis were employed to examine the existence of mean differences between categorical variables with three or more levels of classification (i.e., biofilm growth phase, matrix surface roughness). Non-parametric (Spearmann) rank correlations with bootstrap re-sampling (1000 iterations) were employed to investigate the existence of correlations between continuous variables (i.e., diversity indices, water quality parameters). STATA 13 software was used for all statistical analyses.

Results

Biofilm surface topography

The identified surface topography characteristics of attached biofilm samples on multiple surfaces via SEM are presented in Fig. 3. Biofilm samples were characterized as typical porous matrices comprising surface pore channels of variable aperture. As shown (Fig. 3), biofilm surfaces adsorb large volumes of particles including inorganic particles and multiple microbial categories (diatoms, bacilli, cocci, fungi and spirilla). Intra-particle pores were filled with significant volumes of viscous extracellular polymeric substances (EPS), as typically secreted by bacteria. Due to the adhesive properties of EPS, microbial communities were shown to form stable and heterogeneous porous structures (Liu and Fang 2002). Biofilm samples associated with a growth matrix surface roughness of 1.0 μm exhibited the highest particle density (Fig. 3b, e, h), as characterized by small interior pores and relatively few macropores. Conversely, biofilms from the 0.1 μm growth surface exhibited significantly reduced particle density (Fig. 3a, d, g), with a notable proportion of surface macropores. The steric configuration and pore size of samples associated with 10.0 μm surface roughness were relatively uniform (Fig. 3c, f, i), with some holes or ditch-like structures observed on these sample surfaces.

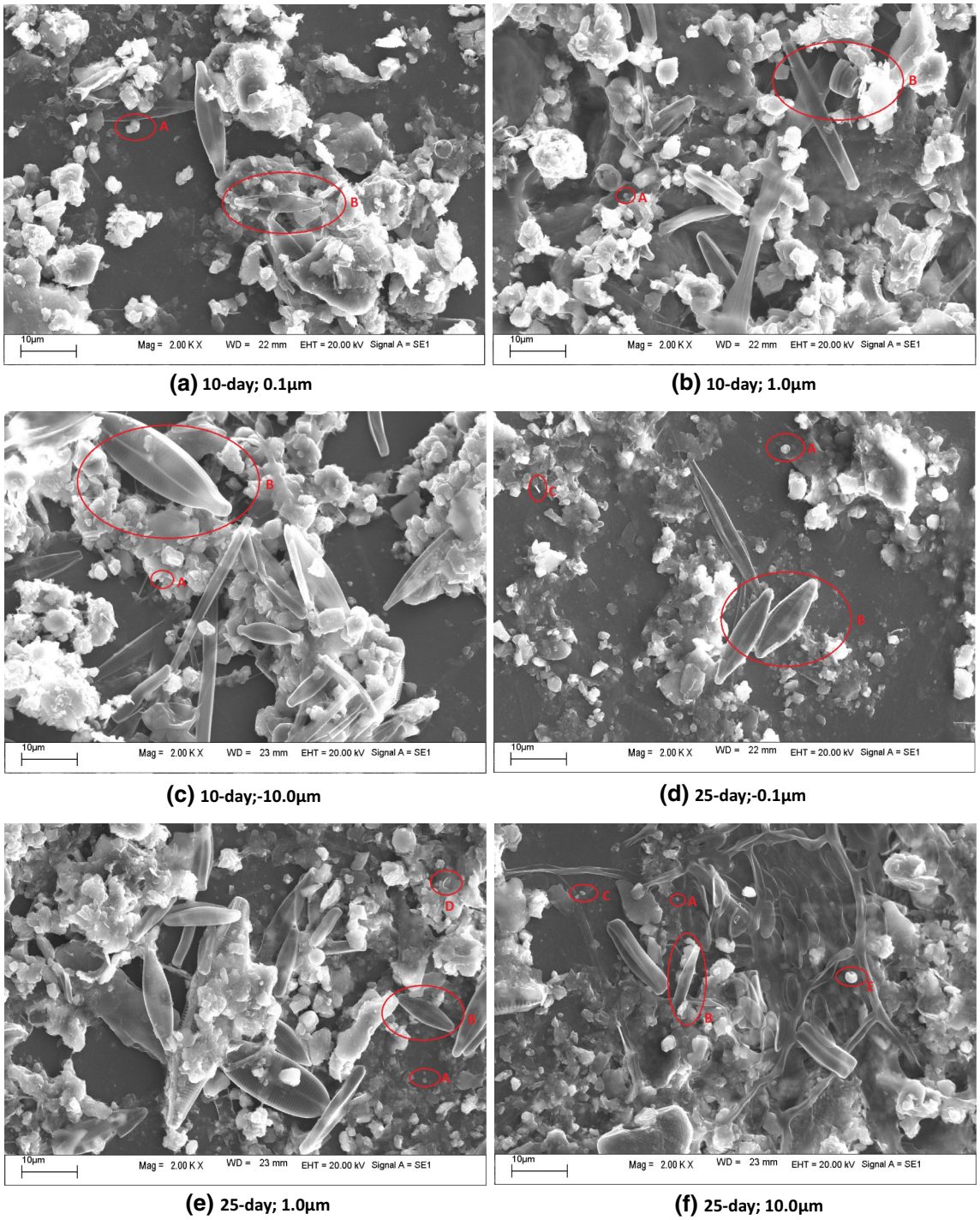

Fig. 3 Scanning electron microscopy imagery of biofilm samples from the current study; *A* Cocci, *B* Diatoms, *C* Bacilli, *D* Fungi, *E* Spirilla

Biofilm microbial community structure

DGGE band patterns of 16S rDNA from biofilm samples after 25 days of growth are presented in Fig. 4, with significant differences apparent between the three biofilm samples. The biofilm associated with matrix surface roughness 1.0 μm exhibited 6 DGGE bands, while the 0.1 and 10.0 μm samples exhibited three and four bands, respectively. Due to the efficacy of PCR-DGGE as an indicator of associated microbial community diversity (Kumar et al. 2004), it may be thus concluded that the species number associated with the surface roughness 1.0 μm biofilm sample was significantly greater than that present in the other two samples.

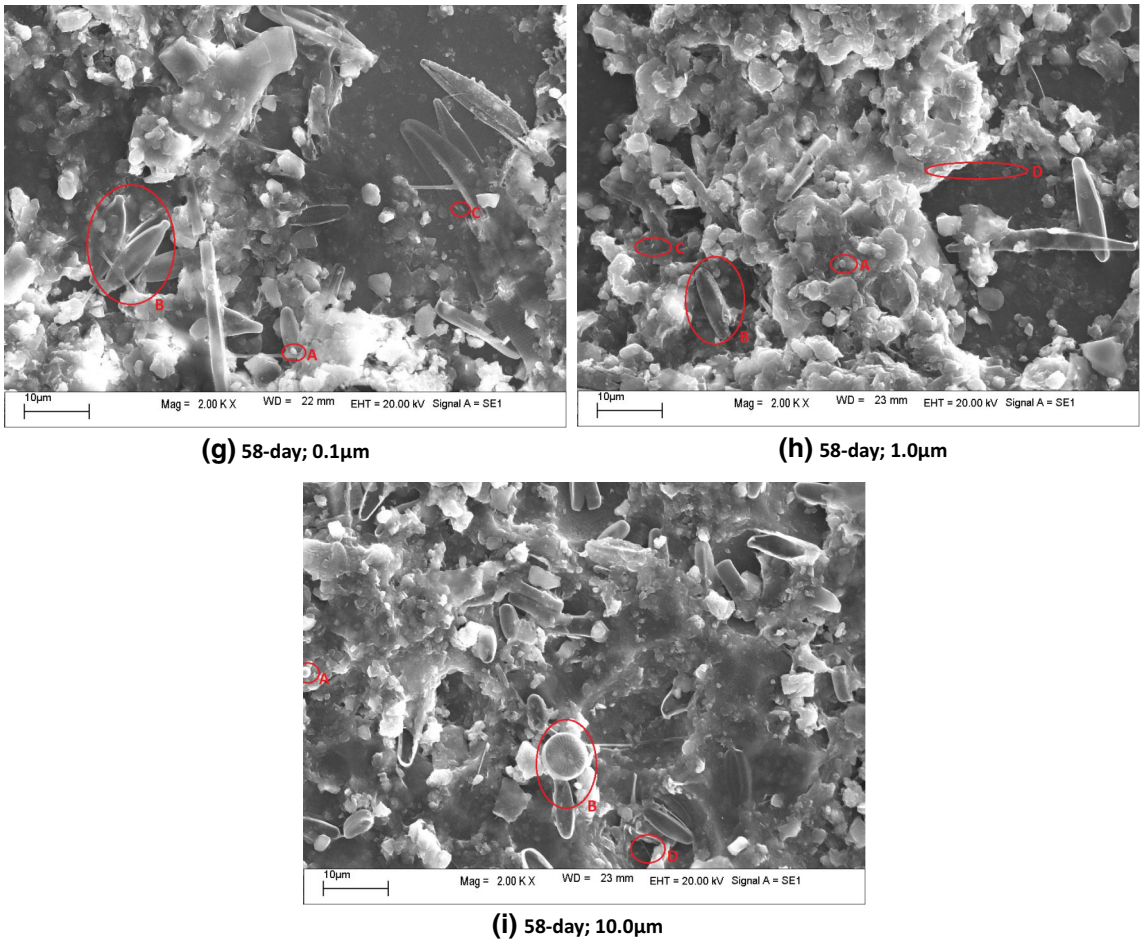

Fig. 3 continued

The developed phylogenetic tree for illustration of the dominant bacterial species among differing biofilm samples is given in Fig. 5. Based upon data from DGGE (Fig. 4; Table 2) and phylogenetic tree composition (Fig. 5), 11 bacterial types belonging to 5 categories were found among the three samples. Bacteria associated with phylogenetic bands 1, 3, 7 and 11 belonged to *Gammaproteobacteria*; band 2 bacteria belonged to *Deltaproteobacteria*; bands 4, 6, 8 and 10 belonged to *Alphaproteobacteria*; band 5 bacteria belonged to *Sphingobacteria* and band 9 bacteria belonged to *Flavobacteriia.* Thus, all samples comprised gram-negative proteobacteria (Fig. 4), with *Gammaproteobacteria* and *Alphaproteobacteria* the most frequently identified proteobacteria. Biofilm samples contained varying bacterial species, for example, the biofilm associated with the matrix of surface roughness 0.1 μm included *Deltaproteobacteria*, *Sphingobacteria* and *Flavobacteria*, while the other two biofilms (1.0 and 10.0 μm) included *Gammaproteobacteria* and *Alphaproteobacteria. Sphingobacteria* was also found in the 10.0 μm biofilm (Table 2).

Phospholipid-derived fatty acid biomarker diversity

Biofilm PLFA type and content were qualified and quantified, respectively; 12 PLFA species were found among the three analyzed samples, comprising gram-negative bacteria (i15:03OH, 16:1ω7c and 18:1ω7t), gram-positive bacteria (i16:0), pseudomonas (16:0), heavy pyrolysis hydrogen bacillus (18:0), general bacteria (12:0, 13:0 and 14:0) and (fungi 18:2ω9t, 18:2ω10t and 18:2ω12c) (Fig. 6) (Hill et al. 2000; Kaurl et al. 2005). PLFA species diversity associated with the 1.0 μm roughness biofilm sample was greater than that observed among the other two samples. A maximum of 7 PLFA species were found in biofilm samples associated with a surface roughness of 1.0 μm, while biofilm samples associated with surface roughness of 1.0 and 10.0 μm comprised a maximum of three and four PLFA species, respectively.

Gram-negative bacteria i15:03OH and heavy pyrolysis hydrogen bacillus 18:0 were evenly distributed within the majority of samples (Fig. 6), with i15:03OH comprising sample proportions of 36.8–52.1 %, thus representing the

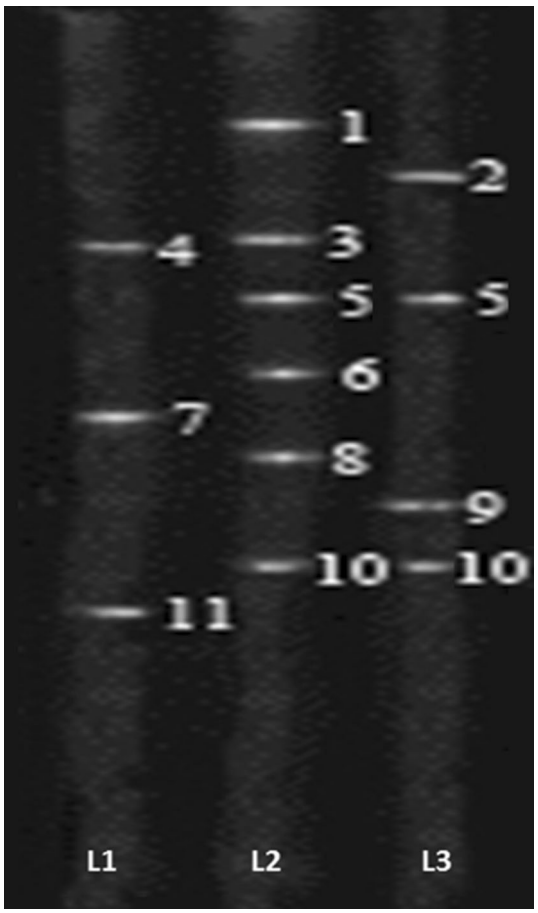

Fig. 4 PCR-DGGE 16S rDNA profiles of biofilm samples after 25-day growth on three differing matrices at Olympic Lake, Beijing. Lanes: *1* 10.0 μm surface roughness, *2* 1.0 μm surface roughness, *3* 0.1 μm surface roughness

most significant proportion of PLFA among all three samples (matrix surface roughness 0.1, 1.0 and 10.0 μm). Several unique PLFAs were detected, for example, general bacteria 12:0 and gram-negative bacteria 18:1ω7t were found within biofilms associated with matrix surface roughness 0.1 μm. Similarly, pseudomonas 16:0 and general bacteria 14:0 were unique to biofilms grown on surface roughness 1.0 and 10.0 μm. Gram-positive i16:0, gram-negative 16:1ω7c and fungi 18:2ω10t and 18:2ω12c were unique to biofilms grown on surface roughness 1.0 μm, while general bacteria 13:0 and fungi 18:2ω9t were exclusively found in biofilms associated with surface roughness 10 μm.

Microbial community diversity parameters were calculated using PLFA biomarker analyses findings (Fig. 7), as outlined in "Microbial community diversity indices". The range of calculated Shannon-Wiener's diversity index (*H*), Pielou's evenness index (*J*) and Simpson's dominance index (*D*) were 0.62–1.44 (Mean 0.89; Std. Dev. 0.243), 0.72–1.0 (Mean 0.81; Std. Dev. 0.109) and 0.43–0.69 (Mean 0.55; Std. Dev. 0.074), respectively. Calculated diversity (1.44) and dominance indices (0.69) were both highest in the biofilm associated with surface roughness of 1.0 μm, while evenness index (1.0) was highest in the 10.0 μm biofilm. One-way ANOVA with Bonferroni multiple comparisons analyses indicates that no statistical association existed between any diversity indices and biofilm matrix surface roughness, however, similar analyses indicates a statistically significant relationship between both calculated diversity (*H*) and biofilm growth phase (i.e., 10-day, 25-day and 58-day) (F (2) = 5.936, p = 0.038) and species dominance (*D*) and biofilm growth phase (F (2) = 6.573, p = 0.031). In both cases, biofilm samples after 25 days of growth exhibited significantly higher species diversity and dominance indices (Figs. 7 and 8).

Non-parametric (Spearmann) rank correlations with bootstrap re-sampling (1000 iterations) were employed to investigate the presence of associations between calculated diversity indices (Table 3). As shown, calculated species evenness (*J*) did not have any statistical association with measured water quality parameters, while species diversity (*H*) and species dominance (*D*) exhibited identical correlational patterns. Significant positive correlations were exhibited with respect to Mg^2, while significant negative correlations were found for NO^3, TP, BOD, COD, SP, PO^4, SO^4 and pH.

Discussion

The health of aquatic ecosystems may be effectively monitored via biofilm evaluation, due to biofilm structure and subsequent diversity being directly affected by myriad local environmental factors. Moreover, bacterial diversity is considered to reflect anthropogenic impacts on aquatic ecosystems; thus diversity indices have become an important indicator of ecosystem health (Guasch et al. 2003). Advances in molecular methods (SEM, PLFA analysis, PCR-DGGE) over the past decade permit thorough and rapid examination of biofilm microbial community structure. Overall, results of the current study indicate that comprehensive analyses of biofilm formation, structure and pollutant purification capability may be effectively used to undertake integrated assessments of the ecological health and contamination status of lake systems using reclaimed water. With respect to the study site (Olympic Lake, Beijing), results of the analytical suite suggest that current ecological health of an acceptable standard in the context of its current function.

Scanning electron microscopy (SEM) has been successfully used to provide high resolution imagery of the surface morphology and structure of microbial biofilms

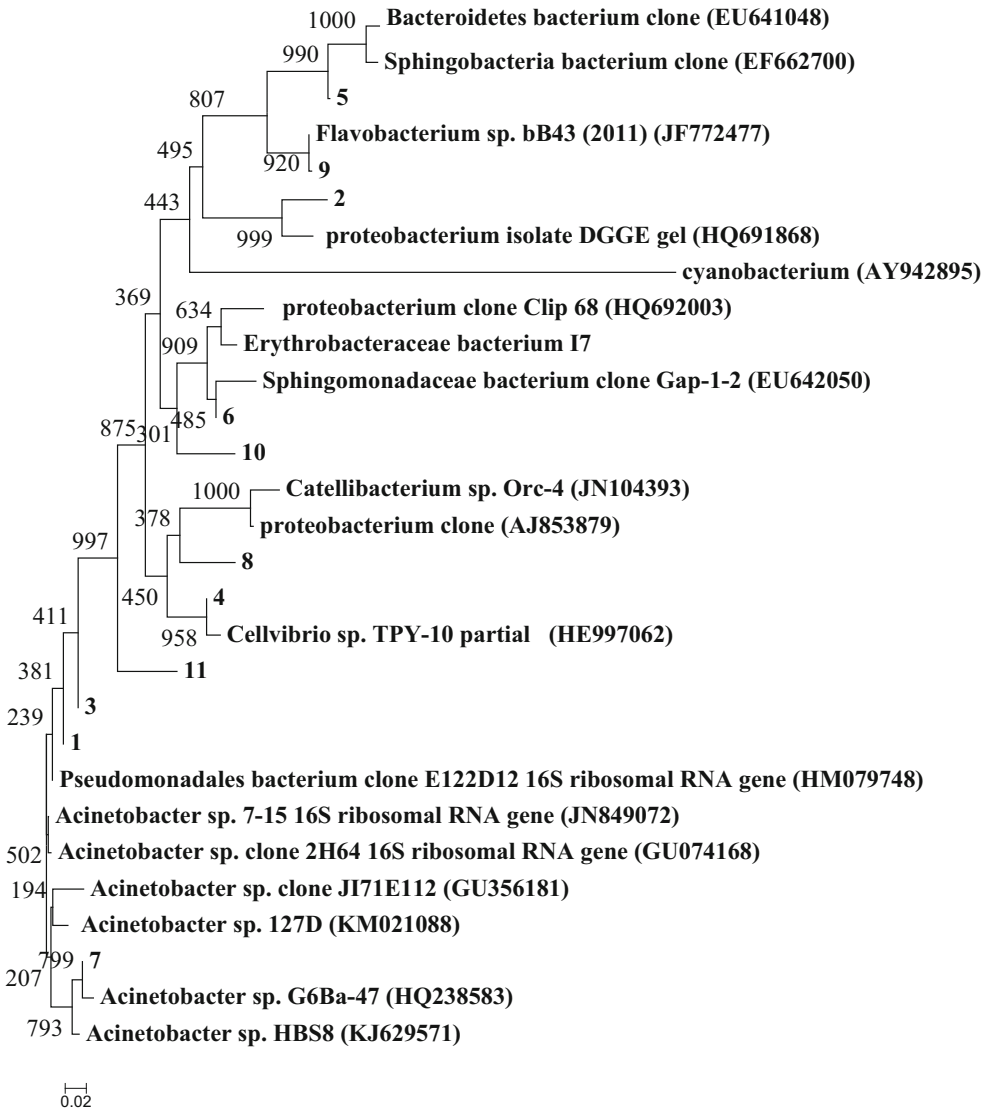

Fig. 5 Constructed biofilm phylogenetic tree showing predominant bacterial species and associated genomic positioning

(Marrie and Costerton 1984). In the current study, numerous SEM images were processed and both visually and digitally analysed. It is considered that the presence of abnormal microbial phenomena (e.g., pathogens, indicator organisms or other contaminants) would be clearly recognizable and therefore indicative of variable and/or adverse ecological health. None of the aforementioned constituents were found via visual or digital analyses of SEM imagery from Olympic Lake, thus suggesting that these were not present and that the current ecological health of at this site is of an acceptable standard.

Differing microorganisms contain distinct phospholipid-derived fatty acid species types, combinations and quantities, particularly with respect to fatty acids within the microbial cell membrane (Pennanen et al. 1999). Phospholipids are characterized by rapid decomposition upon cell death and, as such, are indicative of cell activity (Kinnunen et al. 2012). Accordingly, PLFAs may be effective biomarkers for microbial biomass and indicators of community structure variability, with previous studies having demonstrated method efficacy with respect to dynamic monitoring of microbial communities (Pennanen et al. 1999; Waldrop and Firestone 2004; Dong et al. 2005).

In the current study, the diversity of microbial species in biofilm samples from Olympic Lake were significantly less than those typically associated with sewage; biofilm samples from Olympic Lake comprised a total of 12 microbial species; Qiu and Ma (2005) previously identified 35 microbial species in sewage-associated biofilms, while a summary of 16S rRNA-based diversity surveys of wastewater treatment plants and reactors reported the minimum number of bacterial species present in analysed systems ranged from 17 to 268 (Wagner and Loy 2002). This review found that sequences affiliated with the *Beta*-,

Table 2 Closest matched strains and similarity percentage of DGGE bands in the current study

DGGE band	Type strain	Closest matched strain	Similarity (%)
1	*Acinetobacter*	Acinetobacter sp. clone 2H64 16S rRNA gene (GU074168)	99
2	*Deltaproteobacteria*	Proteobacterium isolate DGGE gel (HQ691868)	94
3	*Moraxellaceae*	Acinetobacter sp. 7-15 16S rRNA gene (JN849072)	99
4	*Cellvibrio*	Cellvibrio sp. TPY-10 partial (HE997062)	99
5	*Sphingobacteria*	Sphingobacteria bacterium clone (EF662700)	99
6	*Sphingomonadaceae*	Sphingomonadaceae bacterium clone Gap-1-2 (EU642050)	99
7	*Acinetobacter*	Acinetobacter sp. G6Ba-47 (HQ238583)	99
8	*Catellibacterium*	Catellibacterium sp. Orc-4 16S rRNA gene (JN104393)	100
9	*Flavobacterium*	Flavobacterium sp. bB43(2011) 16S rRNA gene (JF772477)	98
10	*Erythrobacteraceae*	Erythrobacteraceae bacterium I7	100
11	*Acinetobacter*	Acinetobacter sp. clone JI71E112 (GU356181)	100

Table 3 Results of non-parametric correlation analyses between calculated diversity indices (Shannon-Wiener's diversity index *H*; Pielou's evenness index *J*; Simpson's dominance index *D*) and measured water quality parameters

Index	NO_3	TP	BOD	COD	SP	Mg^2	PO_4	SO_4	pH
Diversity	−0.738	−0.738	−0.738	−0.738	−0.738	0.738	−0.685	−0.685	−0.738
	0.012	0.012	0.012	0.012	0.012	0.012	0.021	0.021	0.012
Evenness	–	–	–	–	–	–	–	–	–
Dominance	−0.738	−0.738	−0.738	−0.738	−0.738	0.738	−0.685	−0.685	−0.738
	0.012	0.012	0.012	0.012	0.012	0.012	0.021	0.021	0.012

Only significant correlations have been presented, with test statistic (r_{sp}) displayed directly above associated *p* value

NH_4, TN, Ca^2 and HCO_2 have been excluded due to the absence of any significant correlations ($p > 0.05$)

Alpha- and *Gammaproteobacteria* as well as the *Bacteroidetes* and the *Actinobacteria* were most frequently encountered (Wagner and Loy 2002). PCR-DGGE gene fingerprinting showed that bacteria in biofilm samples predominantly belonged to the proteobacteria, thus concurring with results of PLFA analyses i.e., gram-negative bacteria comprised the highest PLFA proportion. *Gammaproteobacteria* and *Alphaproteobacteria* were the most frequently identified proteobacteria. Again, this contrasts with sewage-associated biofilms, which are typically dominated by ammonia-oxidizing and nitrite-oxidizing bacteria (Fdz-Polanco et al. 1996). Nitrification and degradation are characteristic reactions of the biological sewage treatment process, resulting in increased competition and distribution of dominant bacteria during short-term nitrification/de-nitrification and/or simultaneous nitrification/de-nitrification (Fdz-Polanco et al. 1996). Neither ammonia-oxidizing nor nitrite-oxidizing bacteria were present in biofilm samples from Olympic Lake; accordingly, when considered in concurrence with low microbial diversity, it may be concluded that no sewage-based contamination had occurred at Olympic Lake immediately prior to or during the study sampling period.

Biofilm formation and structure has been found to significantly correlate with numerous factors, including hydrologic conditions, basement type, nutrient level and light conditions (Percival et al. 1999; Lee and Powers 2007). Growth matrix surface characteristics, including surface roughness and free energy, are also important physicochemical factors for biofilm formation, due to their effect on microbial adhesion (Percival et al. 1999; Liang et al. 2013); surface roughness has been identified as the most influential physicochemical feature (Lee and Powers 2007). Previous studies have investigated biofilms on the surface of aquatic plants, gravels and stainless steel (Baty et al. 1996; Percival et al. 1999; Dong et al. 2005; Qin 2008). For example, Liang et al. (2013) previously examined biofilm attachment to the surface of gravels and aquatic plants in reclaimed waters, showing that microbial diversity of biofilms associated with aquatic plants was significantly greater than that on gravels, primarily due to differing surface roughness. In the current study, results indicate that PLFA species number and calculated diversity indices associated with biofilms grown on surface roughness 1.0 μm were greater than those grown on surface roughness 0.1 μm and 10.0 μm. Rougher matrices surfaces have been shown to provide favorable microbial

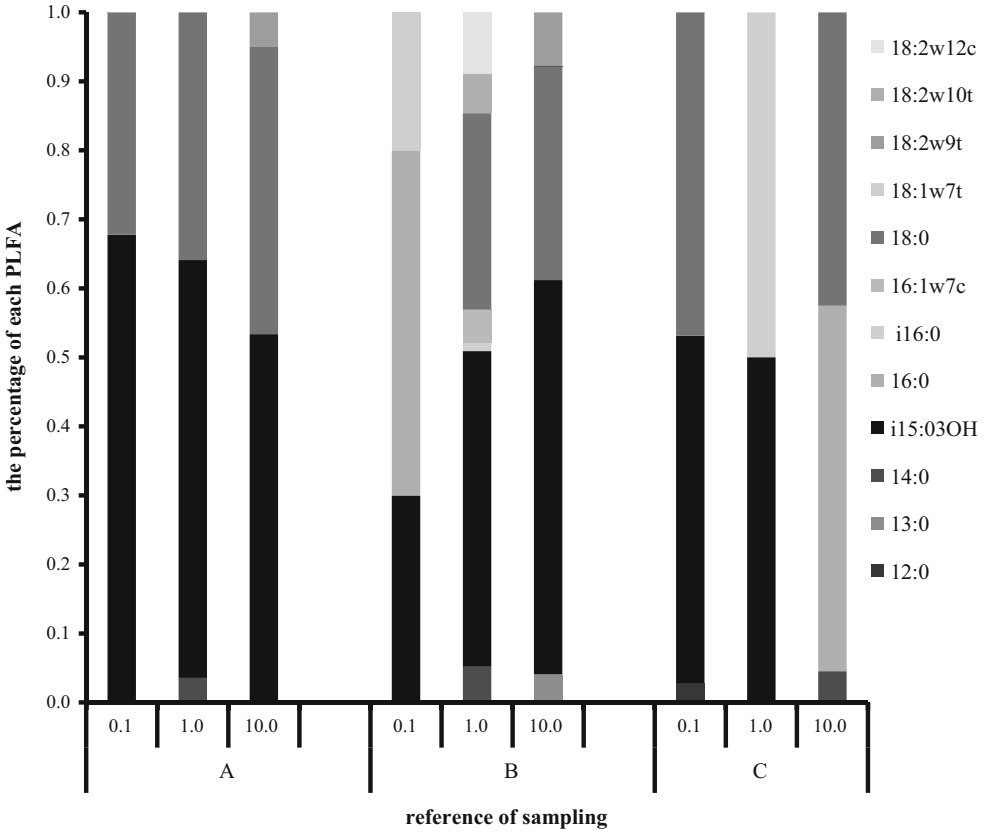

Fig. 6 Results of PLFA analyses of nine biofilm samples from Olympic Lake, Beijing. *A* 10-day samples, *B* 25-day samples, *C* 58-day samples

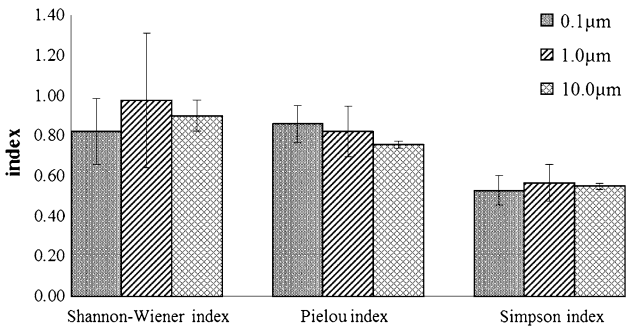

Fig. 7 Calculated diversity indices of biofilm microbial communities and growth matrix surface roughness; presented index figures are 3-sample temporal means with inset 95 % C.I. i.e., mean index from 10-day, 25-day and 58-day samples

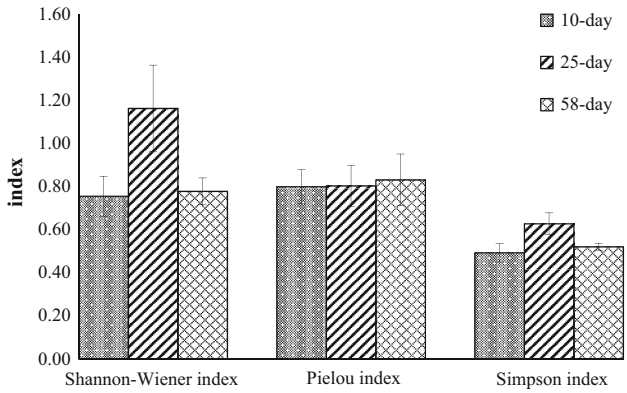

Fig. 8 Calculated diversity indices of biofilm microbial communities and biofilm growth phase; presented index figure are 3-sample means with inset 95 % C.I. i.e., mean index of growth matrix surface roughness 0.1, 1.0 and 10 μm

habitats; attached organic compounds offer increased nutrition for microbial survival and growth, in concurrence with increased adhesive capacities for both nutrients and microorganisms due to increased effective contact area (Percival et al. 1999; Liang et al. 2013). Conversely, increased biofilm growth rates have been shown to result in increased sloughing (Zhou et al. 2013), thus resulting in decreasing microbial diversity.

More specifically, previous studies have shown that matrices characterised by a large degree of surface roughness will likely enlarge the effective contact area with associated microorganisms. Accordingly, the adhesive ability of nutrients and microorganisms to the matrix surface generally exhibits a relatively linear increase in concurrence with incremental increases in matrix surface roughness. Thus, it is considered likely that higher quantities of nutrients and microorganisms adhered to glass slides characterised by a higher surface roughness coefficient, leading to the formation of an increasingly diverse microbial community in these biofilm samples. The same is

likely true with respect to the relationship between organic constituent type and diversity and matrices surface roughness. Conversely, more rapid biofilm growth rates may lead to higher rates of sloughing; spaces of biofilm absence/avoidance are more likely associated with surfaces characterised by higher roughness coefficients, thus "protecting" attached microorganisms and associated biofilm formation. Moreover, this phenomenon has been shown to reduce the impacts of flows and currents on biofilm sloughing. Accordingly, due to the relatively simple living environment and subsequent low biofilm growth and sloughing rates, the formation of highly diverse microbial communities was significantly impeded within Olympic Lake, leading to higher levels of microbial abundance on matrices characterised by moderate surface roughness (1.0 μm) (Gibbs et al. 1993; Schumacher et al. 2007, 2008).

As previously outlined ("Sample collection and preparation"), the water in Olympic Lake originates from the Qinghe and Beixiaohe reclaimed water treatment plants, utilising Anaerobic-Anoxic–Oxic and Membrane Bioreactor + Reverse Osmosis treatment processes, respectively. Both treatment processes typically result in a BOD removal rate of >80 % (Gan and Bai 2010). Wang et al. (2014) have shown that characteristic levels of nitrogen, total nitrogen, total phosphorus, COD_{CR} and bicarbonate in reclaimed water are approximately twice those typically encountered in natural waters, while the levels of ammonia nitrogen are up to five times greater. Mg^{2+} can potentially influence bacterial adhesion directly through effects on electrostatic interactions and indirectly by affecting physiology-dependent attachment processes. Accordingly, the positive correlation noted between measured aquatic Mg^2 and both calculated species diversity (H) ($p = 0.012$) and dominance (D) ($p = 0.012$) is likely due to an increased abundance of attached cells (i.e., increased surface colonization and depth); Song and Leff (2006) have previously reported that increasing Mg^{2+} concentrations resulted in increased initial attachment and altered subsequent biofilm formation and structure among *P. fluorescens*. Conversely, negative correlations between bacterial diversity and other water quality parameters (Table 3) may be elucidated via bacterial processes i.e., bacterial consumption and respiration resulting in reduced levels of both COD_{cr} and BOD_5 (Zhang et al. 2007), conversion of NO_3^--N to organo-nitrogen compounds and N_2 by denitrifying bacteria (denitrification) (Robertson and Kuenen 1983). Thus, specific bacteria proliferated within defined water chemistry ranges, leading to a reduction in bacterial diversity i.e., higher levels of microbial diversity typically occur in concurrence with higher organic loads, primarily due to nutrient availability.

Overall, study results indicate that while comprehensive analyses of biofilm formation, structure and the pollutant purification capability could be effectively used to undertake integrated assessments of the ecological health of lake systems using reclaimed water. However, as shown, both growth/sampling matrix roughness and biofilm growth phase have a significant effect on the microbial community structure of biofilm samples, thus, further work is required to elucidate these effects and determine optimum conditions for biofilm collection for ecological assessment purposes.

Conclusions

This study is the first to examine the microbial community structure of biofilms occurring in an urban lake reusing reclaimed water. Accordingly, results may be used to provide a baseline for biofilm microbial community structure and microbial diversity indices in similar aquatic sources. Three biofilm growth phases and matrix surface roughness were examined using a suite of molecular and biochemical approaches. The principal findings were:

1. In-situ glass sampling was successfully used in the current study to examine the community structure of biofilms in an urban lake utilizing reclaimed water. This method offers some distinct advantages including provision of undisturbed biofilm samples, sampling convenience and a high level of control over sampling location. Moreover, this method may be used to provide reference data in studies where other natural sampling matrices (plants, sediments, etc.) are employed.
2. Biofilms associated with the sample site were characterized as typical porous matrices comprising surface pore channels of variable aperture, leading to significant adsorption of inorganic particles and organisms, including myriad bacterial species.
3. The diversity of microbial species comprising biofilm samples from Olympic Lake were significantly less than those associated with sewage; overall, 12 PLFA types were detected among biofilm samples. Microbial community structure differed significantly with both growth matrix roughness and growth phase. Both calculated diversity index and dominance index of biofilm microbial communities associated with growth matrix roughness 1.0 μm were greater than those of the other two samples. Additionally, calculated diversity and dominance indices were greatest after 25-day growth. Moreover, several PLFAs were uniquely associated with specific growth matrices.
4. Gram-negative bacteria i15:03OH and bacillus 18:0 were the dominant bacterial species in biofilm samples, collectively comprising ≈75 % of identified bacterial

content; additionally identified bacteria included *Deltaproteobacteria, Gammaproteobacteria*, *Alphaproteobacteria*, *Sphingobacteria* and *Flavobacteria*.

5. Biofilm species diversity could represent an effective indicator of waterbody ecological health, however, as both growth matrix surface roughness and growth phase were found to affect microbial community structure, further work is required in order to ascertain the most suitable conditions for collection of biofilms for this purpose.

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