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Bacterial communities in water and sediment shaped by paper mill pollution and indicated bacterial taxa in sediment in Daling River

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ABSTRACT

Effluents from paper mills are highly toxic and are a major source of aquatic pollution. In this study, we collected water and sediment samples to examine the microbial communities using denaturing gradient gel electrophoresis and identified bacterial taxa greatly affected by paper mill pollution using next-generation sequencing data. Our results indicated that bacterial communities in downstream sediments were similar to those in paper mill discharge sites, indicating obvious effects of pollution, while bacterial communities in downstream water samples showed similar profiles to those in upstream sites, both being quite different from the bacterial communities in paper mill discharge sites. This was possibly because of the short contact period. In addition, bacterial communities in the estuary were quite different from those in other water and sediment samples, which was owing to the special habitat type. Considering the storage of paper mill pollutants in sediment and the significant effect on shifts in bacterial communities, we selected *Clostridia* and *Epsilonproteobacteria* at the class level and *Fusibacter* and *Desulfobulbus* at the genus level as bacterial indicators of paper mill pollution. To monitor the remediation of polluted aquatic environments, we propose *Sphingobacteria*, *Alphaproteobacteria*, *Actinobacteria*, *Subdivision3*, *Planctomycetacia* and *Verrucomicrobiae* at the class level and *Bacillus*, *Steroidobacter*, *Nocardioides*, *Terrimonas*, *Pirellula* and *Methylibium* at the genus level.

1. Introduction

There is growing concern about the effect of various forms of pollution, particularly that caused by industrialisation. Effluents from various factories along rivers are likely to have detrimental impacts on aquatic ecosystems. The pulp and paper making industry is very water intensive and ranks third in the world, after the primary metals and chemical industries, in terms of freshwater withdrawal (Thompson et al., 2001). The paper industry is a notorious source of air (Ali and Sreekrishnan, 2001), water and land pollution (Thompson et al., 2001). Paper manufacture generates significant quantities of wastewater (as much as 60 m^3/tonne of paper produced) (Thompson et al., 2001), which contains various types of pollutants, including chlorinated bisphenol A, resin acids, volatile sulphur compounds, nonylphenol ethoxycarboxylates, lignin and polychlorinated contaminants (Dsikowitzky and Schwarzbauer, 2014; Kuehl et al., 1987). The discharge of paper mill wastewater (PMW) into surrounding surface water sources serve as a direct threat to both macroflora and microflora and fauna (Oanh and Bengtsson, 1995). There is evidence that paper mill pollution (PMP) can disrupt the reproductive and neuroendocrine systems of fish (Kovacs et al., 2013; Orrego et al., 2006) as well as reduce seed germination rates (Iqbal et al., 2013).

Microorganisms play an important role in the geochemical cycles (Lim et al., 2011) and transformation of pollutants (Singh et al., 2008). Therefore, many types of bacteria have been isolated from PMW for degradation studies (Chandra et al., 2008; Singh et al., 2008). Of these, some isolates exhibit special characteristics such as cellulase production (Maki et al., 2011). However, to date, there have been few studies on the effect of PMP on river microbial communities both in water and sediment. The objectives of this study were (i) to study the characteristics of bacterial communities in these two different media by denaturing gradient gel electrophoresis (DGGE) and (ii) to study the structures of bacterial communities in the sediment samples by next-generation sequencing (NGS) of 16S rRNA genes to elucidate the influence of PMP on the bacterial communities of the Daling River and

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mine selected taxa for long-term monitoring and bioremediation of PMP.

2. Materials and methods

2.1. Sample collection sites

In this study, we mainly investigated a section of Daling River, which is in Jinzhou area, northeastern China, covering approximately 80 km. A paper mill built in 1939 is located in the centre of the selected area (Fig. 1). Water and sediment samples were collected from the Daling River (latitude N: 41.26–40.839; longitude E: 121.324 and 121.534) in May 2011. The volume of each water sample collected for analysis was 2 L, while 200 g sediment samples were collected from a depth of 2–10 cm using a grab sampler (Kanghua Apparatus factory, Jiangsu, China). The temperature of water in sampling sites ranged from 11 °C to 20 °C and salinity from 0.020% to 0.123%. PMW was drained into Daling River and a pungent odour spreaded in the draining site. PMW was the main point pollution in the study area, which brought various pollutants into the environment including sulphate and polychlorinated biphenyl. The water samples were divided into 400 ml aliquots and filtered through nitrocellulose filters with 0.22 μm pores (Sangon Biotech, Co., Ltd., Shanghai, China) under negative pressure on the day of collection to avoid shifting of bacterial communities and physicochemical parameters. Following this, all samples were transported in the dark at 4 °C in a sealed container to our laboratory within 5 days and stored at −80 °C until assay.

2.2. Physicochemical analysis

We measured the pH and dissolved oxygen (DO) content in the field using the GTB-607A and THB-1 portable metres (Shanghai INESA Scientific Instrument Co., Ltd., Shanghai, China). In addition, total nitrogen (TN), total phosphorus (TP) and chemical oxygen demand (COD) were assessed in the filtered water samples (Federation and Association, 2005). Triplicate samples were measured, and standard deviation was lower than 5%.

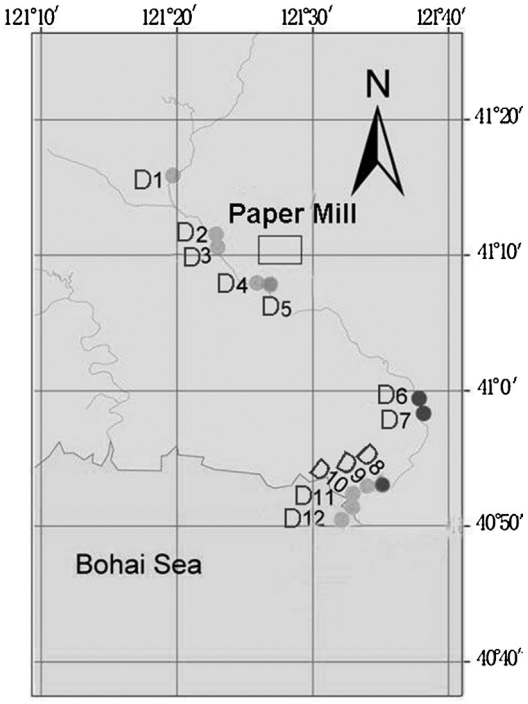

Fig. 1. Location of the sampling sites along the Daling River.

2.3. DNA extraction, polymerase chain reaction (PCR) analysis and DGGE analysis

The nitrocellulose filters containing trapped bacteria were cut into pieces, and genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method, as described in *Molecular Cloning: A Laboratory Manual* (Green and Sambrook, 2012). Genomic DNA of sediment samples was extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's recommendations. The V6–V8 region of the 16S rRNA gene was amplified in 25 μl PCR reaction volumes containing 0.5 μmol L^{-1} of each primer (Invitrogen Corporation, Carlsbad, CA, USA), 0.2 mmol L^{-1} dNTPs (TaKaRa Bio, Inc., Shiga, Japan), 1 U of *Taq* DNA polymerase with the recommended PCR buffer (TaKaRa Bio, Inc.) and 1 μl of DNA extract (5–10 ng DNA). Amplification of the 16S rRNA gene was performed using the primers U968-GC-F (5-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCAGGGGGGAACGCG AAGAACCTTAC-3) and L1401-R (5-CGGTGTGTACAAGACCC-3) (Watanabe et al., 2001). Samples were amplified using an initial denaturing step of 2 min at 94 °C, followed by 30 cycles consisting of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, elongation for 1 min at 72 °C and a final elongation step for 10 min at 72 °C. Each PCR reaction included a negative control. PCR products were separated on 1% agarose gels and purified using the Gel Extraction Kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China). The purified PCR products were quantified using the NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). DGGE analysis was performed using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) maintained at 60 °C and 120 V for 17 h in 0.5× TAE buffer (20 mM Tris–acetate, 0.5 mM EDTA, pH 8.0). PCR products (400 ng) were loaded onto 8% acrylamide/bis-acrylamide (37.5:1) gels with linear denaturing gradients from 45% to 65% (where 100% was 7 M urea and 40% (v/v) deionised formamide). The gels were stained with $AgNO_3$ for 10 min at room temperature, and images were captured using the GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Inc.). DGGE fingerprints were analysed with Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Inc.), and grey matrices of bands were exported for principle component analysis (PCA) of bacterial communities.

2.4. NGS sequencing

The V5–V6 region of the 16S rRNA gene was amplified in 25 μl PCR reactions containing 0.5 μmol L^{-1} of each primer (Invitrogen Corporation), 0.2 mmol L^{-1} dNTPs (Thermo Scientific), 40 ug of bovine serum albumin (Thermo Scientific), 4 mmol L^{-1} $MgCl_2$ (Thermo Scientific), 1 U of *Taq* DNA polymerase with the recommended PCR buffer (Thermo Scientific) and 1 μl of DNA extract (5–10 ng DNA). Amplification of the 16S rRNA gene was performed using the primers Bac_410-F (AGGATTAGATACCCTGGTA) and Bac_685-R (CRRCACGAGCTGACGAC) with seven barcode bases fused to the 5′ end of the Bac_410-F primer during synthesis.

Samples were amplified using an initial denaturing step of 2 min at 94 °C, followed by 23 cycles consisting of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, elongation for 1 min at 72 °C and a final elongation step for 10 min at 72 °C. Each PCR reaction included a negative control. PCR products were run on a 1% agarose gel and purified using the Gel Extraction Kit (Axygen Scientific, Inc., Salem, MA, USA). The purified PCR products were quantified using the Bio TekFlx800 with Quant-iT Pico Green dsDNA Assay Kit (Invitrogen