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Bacteria and bacteriophage communities in bulking and non-bulking activated sludge in full-scale municipal wastewater treatment systems

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ABSTRACT

Bacteriophages can influence the bacterial community through specific infection and lysis. However, there are no studies on the viral community composition or its relationship with the bacterial community in bulking activated sludge (AS). In this study, a fluorescence staining counting method, high throughput and metagenomic Illumina sequencing techniques were used to analyze the changes in bacterial and viral communities in bulking and non-bulking AS in three separate full-scale municipal wastewater treatment plants (WWTPs). The results indicated that during AS bulking, the balance of bacteria and bacteriophages was obviously altered with the ratios of bacteria to virus-like particles increasing 5.7–17.5-fold; the diversity of both the bacterial and viral communities was low; the abundance of some bacterial genera significantly increased including bulking causative bacteria such as *Flavobacterium*, *Candidatus Microthrix*, and *Haliscomenobacter* increasing 10.7–48.7-, 3.5–37.7- and 2.5–5.8-fold, respectively. Viral metagenomic analysis indicated that high diversity and a large number of unknown viruses existed in the WWTPs, while bacteriophages, *Podoviridae*, *Siphoviridae*, and *Myoviridae*, dominated the classified viruses, of which the abundance of *Podoviridae* and *Myoviridae* increased sharply by 3.8–4.7- and 1.3–3.1-fold, respectively, in the bulking AS. In addition to the dominant bacteriophages, a large number of human and animal viruses were present in the WWTPs, especially in the autumn non-bulking AS.

1. Introduction

Bacteriophages are viruses which infect bacteria and play an active role in the ecology of natural environments, influencing prokaryotic population dynamics and mediating lateral gene transfer between diverse bacterial species [1]. Analyses of viral communities have been reported in natural marine ecosystems, oceanic ice, sediments, soil and in engineered systems such as drinking water distribution systems, wastewaters and activated sludge (AS) bioreactors [2]. With the development of metagenomics and sequencing technologies, our understanding of viral abundance and diversity in various environments has improved. There is general consensus that viruses are the most abundant and diverse biological entities on Earth and are difficult to classify and assess, especially in natural environments. They range from 10^4 ml^{-1} to more than 10^8 ml^{-1}, which is typically 3–10 times greater than bacterial counts in the aquatic environment [3]. It has been speculated that the presence of bacteriophages may be a major factor in the bacterial population dynamics of such ecosystems and the potential of bacteriophages to control bacterial infections has been identified in cultured fish [4], plants [5] and in the control of cyanobacterial blooms [6].

Wastewater infrastructure is central to sustainable development in every modern society and provides a treated effluent that decreases pollution and waterborne disease risks prior to discharge and in many cases for reuse. However, sludge bulking, a problem in

Abbreviations: AS, activated sludge; WWTP, wastewater treatment plants; LTW, Luotuowan wastewater treatment plant; XSZ, Xiaoshangzhuang wastewater treatment plant; A/A/O, anaerobic-anoxic-aerobic process; A/O, anaerobic-aerobic process; TP, total phosphate; TN, total nitrogen; NH_3-N, amino nitrogen; SS, suspended solids; SV30, sludge settling ratio; MLSS, mixed liquid suspended solids; SVI, sludge volume index; nr, non-redundant protein sequence database; COG, cluster of orthologous groups of proteins; eggNOG, Evolutionary genealogy of genes; CAZy, carbohydrate-active enzymes database; ARDB, antibiotic resistance genes database; KEGG, Kyoto encyclopedia of genes and genomes.

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WWTPs, frequently occurs resulting in poor sludge settleability and even system collapse. In northern China, this problem occurs in over 50% of AS in WWTPs each year [7]. The relationship between the bacterial community and sludge bulking has been studied in many lab-scale or full-scale wastewater treatment systems, and the main bulking causative bacteria differed in different systems and localities [7,8]. In general, biomass bulking in AS processes is due to the over-development of filamentous bacteria and/or by substantial accumulation of high viscosity polysaccharide substances secreted by *Zoogloea*, among which filamentous bulking is the main problem. Several filamentous bacteria have been isolated and identified from WWTPs such as *Microthrix parvicella*, *Sphaerotilus natans*, *Eikelboom* type 1702, *Haliscomenobacter hydrossis*, *Nocardia*, *Thiothrix* spp., *Meganema perideroedes*, and *Leucothrix mucor* [2,7].

Compared to bacterial community analysis, research on viral communities in wastewater or wastewater treatment systems is limited. A few recent studies based on viral metagenomic clone sequencing, pyrosequencing and metagenomic analysis using Illumina technology, respectively, indicated that wastewater harbored marked viral diversity that was yet to be discovered and the viral community can be used as bioindicators to assess wastewater treatment quality and the potential impact of dairy operations [1,9,10]. Other reports have also indicated that bacteriophages may play roles in pathogen control, improving dewaterability during the sludge dewatering process of wastewater sludge treatment, improving digestibility of excess biological sludge, control of filamentous bacteria to overcome the problems of bulking and foaming of AS and control of non-phosphate accumulating bacteria in biological phosphate removal [2,3,11,12]. However, to date, there are no reports on viral community structure in the bulking AS, or studies on the relationships between sludge bulking and bacterial and viral communities in full-scale WWTPs.

In the present study, high-throughput sequencing and viral metagenomic sequencing were used to investigate the changes in bacterial and viral community structures in AS during biomass bulking in three different full-scale municipal WWTPs, to analyze the bacterial and viral ecology in non-bulking and bulking sludge and to determine the relationship between sludge bulking and the bacterial and viral communities in wastewater treatment systems.

2. Materials and methods

2.1. Characteristics of municipal wastewater treatment systems and wastewater samples

Wastewater samples (mixture of wastewater and AS) were collected from the aerobic basins of three processes in two municipal WWTPs (Luotuowan and Xiaoshangzhuang) in Xinxiang, China. Luotuowan wastewater treatment plant (LTW) conducts a typical anoxic-aerobic (A/O) process treating 150,000 m^3 of wastewater (around 40% combined industrial wastewater and 60% municipal wastewater) per day. Xiaoshangzhuang wastewater treatment plant (XSZ) receives approximately 1:1 municipal wastewater and combined industrial wastewater, and includes two treatment systems, a typical anaerobic-anoxic-aerobic (A/A/O) process (XSZ1) and a typical anaerobic-aerobic (A/O) process (XSZ2) with each process treating around 120,000 m^3 of wastewater per day. Samples of mixed wastewater and AS were continuously collected from the above three aerobic tanks three times in May (spring) and October (autumn), respectively, in 2014. Each sample was a mixture of wastewater and sludge collected from at least five areas of the aerobic tank. The samples were placed in sterile flasks and transported to the laboratory on ice.

The characteristics of the wastewater in the above three systems including COD, BOD_5, pH, total phosphate (TP), total nitrogen (TN), amino nitrogen (NH_3-N) and suspended solids (SS) were assayed using standard methods [13] and are shown in supplementary Table 1. The characteristics of sludge in the systems were reflected by three parameters, the sludge settling ratio (SV30), mixed liquid suspended solids (MLSS) and the sludge volume index (SVI). SV30 was determined by the percent of sludge volume in the mixture of wastewater and sludge after settling for 30 min in a 100 ml cylinder. MLSS was assayed using a standard method [13], and SVI was calculated as follows: SVI = SV30 × 10 ÷ MLSS.

2.2. Enumeration of bacteria and bacteriophage-like particles

Quantification of the total bacterial community was conducted by enumerating cells stained with DAPI (4, 6-diamidino-2-phenylindole) after fixing with 4% paraformaldehyde (mt/vol) solution [14]. After concentration on black polycarbonate filters pore size 0.22 μm (Millipore, USA), total cells were enumerated under an epifluorescence microscope. At least 10 fields of view per sample were enumerated using oil immersion at 1000 times magnification. An average of the total cells in each wastewater treatment period was obtained from three parallel samples.

For enumeration of virus-like particles, aliquots of the samples were diluted 10000 times with fresh sterile water and filtered through membrane filters (pore size, 0.22 μm). The filters were stained with SYBR Gold in dark conditions for 10 min and then filtered through a membrane filter (pore size, 0.02 μm) (Anodisc 25 mm, Whatman). The bacteriophages trapped on the membrane were mounted on a glass slide and then covered with a cover glass. A 100 × oil objective lens was used to count the above samples within 30 min using a positive epifluorescence microscope (EFM, Olympus BX63, Japan). The number of bacteriophages in each sample was counted from at least 10 randomly selected fields [15].

2.3. Analysis of the bacterial community in wastewater treatment systems

For analysis of the total bacterial community, sludge samples were washed three times with phosphate-buffered saline (PBS, pH 8.0) and centrifuged at 4 °C and 10,000 rpm for 15 min for total genomic DNA extraction. The total genomic DNA of the microbial community was extracted using a Soil DNA kit (Omega, Solon, OH, USA) following the manufacturer's instructions. DNA extraction was confirmed using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific) and by performing agarose gel electrophoresis (60 V, 2 h). The purified DNA was sent to Novogene (Beijing, China) for Illumina HiSeq 2500 sequencing. The V4 region of 16S rRNA gene (primer pair of 515 F/806 R, GTGCCAGCMGCCGCGGTAA and GGACTACHVGGGTWTCTAAT from 5′ to 3′) was used for sequencing and bacterial diversity analysis.

2.4. Concentration and purification of viruses from the sewage of municipal wastewater treatment systems

For bacteriophage community analysis, the wastewater samples (each about 20 l) were first allowed to settle for approximately 1 h to separate the supernatant and sludge. The sludge was washed three times, dissolved and thoroughly stirred in phosphate buffer (pH 7.0) to obtain the absorbed viruses from the sludge. The eluents were combined with the supernatants and underwent a series of graded filtrations through a membrane (pore sizes, 5 μm, 1 μm, 0.45 μm and 0.22 μm), respectively, to remove any remaining bacterial and eukaryotic cells. The filtrates were concentrated to approximately 200 ml using a 100 kDa ultrafiltration membrane package (Millipore, USA) on an ultrafilter (Millipore, USA).

For purification of viruses, the concentrates were first treated with DNase 1 and RNase at 37 °C for 30 min and then mixed with