Bioremediation of Wastewater

Factors and Treatments

Editor
Olga Sánchez, PhD

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About the Editor

OLGA SÁNCHEZ, PhD

Olga Sánchez received her PhD degree in Biological Sciences from the Universitat Autònoma de Barcelona (Spain) in 1996. Her research began with the study of the physiology of photosynthetic bacteria and derived to the utilization of complex microbial biofilms in packed reactors for the treatment of contaminated effluents. In 2007 she got a fixed position as aggregate teacher at the Department of Genetics and Microbiology of the Universitat Autònoma de Barcelona, and presently, her investigation focuses on the application of molecular techniques for the characterization of the diversity of different natural microbial communities, including marine environments or wastewater treatment systems. These methodologies include clone libraries, FISH (Fluorescence In Situ hybridization), fingerprinting techniques such as DGGE (Denaturing Gradient Gel Electrophoresis) and next-generation sequencing technologies.

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Acknowledgment and How to Cite

The editor and publisher thank each of the authors who contributed to this book. The chapters in this book were previously published in various places in various formats. To cite the work contained in this book and to view the individual permissions, please refer to the citation at the beginning of each chapter. Each chapter was read individually and carefully selected by the editor; the result is a book that provides a nuanced look at the bioremediation of wastewater. The chapters included are broken into three sections, which describe the following topics:

- In Chapter 1, while investigating the bacterial diversity and abundance in a Hong Kong wastewater treatment plant for sewage, Ye and Zhang analyzed the influent, activated sludge, digestion sludge, and effluent. They found that the bacterial diversities in the four samples varied significantly, but what is significant is that they introduce a method for differentiating between "gene percantage" and "cell percentage" when analyzing bacterial genera.
- In Chapter 2, Guo and colleagues offer helpful methodology for more precisely assessing microbial ecology in activated sludge and other complex environmental samples.
- The results of Chapter 3, performed by Fredriksson and colleagues, demonstrate that experimental comparisons of universal 16S rRNA primers can reveal differences not detected by theoretical comparisons. As with both the Ye and Guo articles, these discussions contribute to a more thorough and detailed understanding of how we can analyze and measure microbial communities in wastewater.
- The work of Wang and colleagues in Chapter 4 helpfully expands our knowledge about the microbial nitrification and denitrification processes and their underlying biological mechanisms for industrial wastewater treatment.
- Sánchez and colleagues were the first to analyze the activated sludge of a seawater-processing plant in Chapter 5. We concluded that the bacterial diversity was as high as has been observed in conventional wastewater treatment plants. However, the composition of the bacterial community differed greatly from other plants. Our results suggest that only a few populations of specialized bacteria (likely with high transcription activity) are responsible for removal of ammonia in seawater systems.

- The research conducted by Weissbrodt and colleagues in Chapter 6 investigated the dynamics of bacterial communities and the structures of bioaggregates during transitions from activated sludge flocs to early-stage nuclei and to mature granular biofilms. Their findings are helpful regarding the mechanisms of bacterial selection, granule formation, and biofilm maturation in relation to the evolution of process variables.
- In Chapter 7, Kandel and colleagues. offer a useful study that assesses the impact of predator bacteria on microbial processes.
- In Chapter 8, Wang and colleagues, while finding distinct similarities between microbial functional communities among activated sludge samples from four wastewater treatment plants, also reach helpful conclusions regarding correlations between microbial functional potentials and water temperature, dissolved oxygen, ammonia concentrations, and the loading rate of chemical oxygen demand.
- The investigation in Chapter 9 by Morató and colleagues of wastewater treatment plant design factors—such as granular media, water depth, and season effect—clarify our understanding of variables that affect the removal of microbial indicators in constructed wetlands.
- In Chapter 10, Tsuji and colleagues address concerns relating to dairy wastewater, reporting the effects of both lipase and a particular yeast strain on milk fat.
- In Chapter 11, Boboescu and colleagues implement a useful experimental design to investigate the process variables involved in batch-mode biohydrogen production, and offer implications that point to a greater optimization of this methodology on a larger scale.
- The comparison in Chapter 12 by Adrados and colleagues regarding the composition of the microbial communities of three different types of domestic wastewater treatment systems enlarges microbial analysis by analyzing both the bacterial and archaeal populations. They focused on the possible influence of the water influent composition, the design, and the bed filling of the treatment systems. They found no relation between the influent and effluent bacterial communities inside the same treatment system.

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Introduction

The quantity and quality of waste generated and discharged into natural water bodies is a topic of serious concern in our world today. Consequently, there is a need for different strategies to address wastewater treatment and subsequent reuse, especially in arid and semi-arid areas where water shortages are the rule. Biological treatment processes constitute crucial tools in the biodegradation of organic matter, transformation of toxic compounds into harmless products, and nutrient removal in wastewater microbiology.

Activated sludge in wastewater treatment plants (WWTPs), which contains a complex mixture of microbial populations dominated by bacteria, plays an essential role in the biological treatment in these facilities. Other natural wastewater treatment systems such as constructed wetlands, biological sand filters, and other decentralized solutions provide other valuable options to conventional methods due to their efficiency, low establishment costs, and reduced operation and management requirements. In all these systems, microbial processes are essential, since many reactions are microbiologically mediated.

The composition of the microbial community has been extensively studied in the past decades. Different methods have been used in order to deepen our understanding of the structure and functionality of the microorganisms involved. By applying culture-dependent techniques, many species have been isolated from systems, such as activated sludge, although most of the microorganisms cannot be obtained by these classical methods. However, molecular tecniques such as gene libraries, fingerprinting methodologies, fluorescence in situ hybridization, and the recently incorporated next-generation sequencing technologies have greatly expanded our knowledge on wastewater biodiversity, showing that a significant hidden diversity of unknown and uncultured microorganisms have the potential to act as degraders of environmental pollutants. Therefore, further attempts to isolate the key microorganisms involved will be essential in order to

explore the degradation capacity of the microbial communities developed in biological treatment processes. The articles in this compendium were chosen to represent an overview of the most current research into these facets of wastewater bioremediation. Ongoing research is essential to the future of human health, as well as the over all health of our planet.

Olga Sánchez, PhD

In Chapter 1, Ye and Zhang successfully demonstrated that 454 pyrosequencing was a powerful approach for investigating the bacterial communities in the activated sludge, digestion sludge, influent, and effluent samples of a full scale wastewater treatment plant treating saline sewage. For each sample, 18,808 effective sequences were selected and utilized to do the bacterial diversity and abundance analysis. In total, 2,455, 794, 1,667, and 1,932 operational taxonomic units were obtained at 3 % distance cutoff in the activated sludge, digestion sludge, influent, and effluent samples, respectively. The corresponding most dominant classes in the four samples were *Alphaproteobacteria, Thermotogae, Deltaproteobacteria,* and *Gammaproteobacteria*. About 67 % sequences in the digestion sludge sample were found to be affiliated with the *Thermotogales* order. Also, these sequences were assigned into a recently proposed genus *Kosmotoga* by the Ribosomal Database Project classifier. In the effluent sample, the authors found high abundance of *Mycobacterium* and *Vibrio,* which are genera containing pathogenic bacteria. Moreover, in this study, they authors proposed a method to differentiate the “gene percentage” and “cell percentage” by using Ribosomal RNA Operon Copy Number Database.

High throughput sequencing of 16S rRNA gene leads us into a deeper understanding on bacterial diversity for complex environmental samples, but introduces blurring due to the relatively low taxonomic capability of short read. For wastewater treatment plants, only those functional bacterial genera categorized as nutrient remediators, bulk/foaming species, and potential pathogens are significant to biological wastewater treatment and environmental impacts. Precise taxonomic assignment of these bacteria at

least at genus level is important for microbial ecological research and routine wastewater treatment monitoring. Therefore, the focus of Chapter 2, by Guo and colleagues, was to evaluate the taxonomic precisions of different ribosomal RNA (rRNA) gene hypervariable regions generated from a mix activated sludge sample. In addition, three commonly used classification methods including RDP Classifier, BLAST-based best-hit annotation, and the lowest common ancestor annotation by MEGAN were evaluated by comparing their consistency. Under an unsupervised method, analysis of consistency among different classification methods suggests there are no hypervariable regions with good taxonomic coverage for all genera. Taxonomic assignment based on certain regions of the 16S rRNA genes, e.g. the V1&V2 regions—provide fairly consistent taxonomic assignment for a relatively wide range of genera. Hence, it is recommended to use these regions for studying functional groups in activated sludge. Moreover, the inconsistency among methods also demonstrated that a specific method might not be suitable for identification of some bacterial genera using certain 16S rRNA gene regions. As a general rule, drawing conclusions based only on one sequencing region and one classification method should be avoided due to the potential false negative results.

Assessments of bacterial community diversity and dynamics are fundamental for the understanding of microbial ecology as well as biotechnological applications. In Chapter 3, Fredriksson and colleagues show that the choice of PCR primers has great impact on the results of analyses of diversity and dynamics using gene libraries and DNA fingerprinting. Two universal primer pairs targeting the 16S rRNA gene, 27F&1492R and 63F&M1387R, were compared and evaluated by analyzing the bacterial community in the activated sludge of a large-scale wastewater treatment plant. The two primer pairs targeted distinct parts of the bacterial community, none encompassing the other, both with similar richness. Had only one primer pair been used, very different conclusions had been drawn regarding dominant phylogenetic and putative functional groups. With 27F&1492R, *Betaproteobacteria* would have been determined to be the dominating taxa while 63F&M1387R would have described *Alphaproteobacteria* as the most common taxa. Microscopy and fluorescence in situ hybridization analysis showed that both *Alphaproteobacteria* and *Betaproteobacteria* were abundant in the activated sludge, confirming that

the two primer pairs target two different fractions of the bacterial community. Furthermore, terminal restriction fragment polymorphism analyses of a series of four activated sludge samples showed that the two primer pairs would have resulted in different conclusions about community stability and the factors contributing to changes in community composition. In conclusion, different PCR primer pairs, although considered universal, target different ranges of bacteria and will thus show the diversity and dynamics of different fractions of the bacterial community in the analyzed sample. The authors also show that while a database search can serve as an indicator of how universal a primer pair is, an experimental assessment is necessary to evaluate the suitability for a specific environmental sample.

Biological nitrification/denitrification is frequently used to remove nitrogen from tannery wastewater containing high concentrations of ammonia. However, information is limited about the bacterial nitrifiers and denitrifiers and their functional genes in tannery wastewater treatment plants (WWTPs) due to the low-throughput of the previously used methods. In Chapter 4, Wang and colleagues used 454 pyrosequencing and Illumina high-throughput sequencing, combined with molecular methods, to comprehensively characterize structures and functions of nitrification and denitrification bacterial communities in aerobic and anaerobic sludge of two full-scale tannery WWTPs. Pyrosequencing of 16S rRNA genes showed that *Proteobacteria* and *Synergistetes* dominated in the aerobic and anaerobic sludge, respectively. Ammonia-oxidizing bacteria (AOB) *amoA* gene cloning revealed that *Nitrosomonas europaea* dominated the ammonia-oxidizing community in the WWTPs. Metagenomic analysis showed that the denitrifiers mainly included the genera of *Thauera, Paracoccus, Hyphomicrobium, Comamonas* and *Azoarcus*, which may greatly contribute to the nitrogen removal in the two WWTPs. It is interesting that AOB and ammonia-oxidizing archaea had low abundance although both WWTPs demonstrated high ammonium removal efficiency. Good correlation between the qPCR and metagenomic analysis is observed for the quantification of functional genes *amoA, nirK, nirS* and *nosZ*, indicating that the metagenomic approach may be a promising method used to comprehensively investigate the abundance of functional genes of nitrifiers and denitrifiers in the environment.

In Chapter 5, Sánchez and colleagues investigated the bacterial community composition of activated sludge from a wastewater treatment plant (Almería, Spain) with the particularity of using seawater by applying 454-pyrosequencing. The results showed that *Deinococcus-Thermus, Proteobacteria, Chloroflexi* and *Bacteroidetes* were the most abundant retrieved sequences, while other groups, such as *Actinobacteria, Chlorobi, Deferribacteres, Firmicutes, Planctomycetes, Spirochaetes* and *Verrumicrobia* were reported at lower proportions. Rarefaction analysis showed that very likely the diversity is higher than what could be described despite most of the unknown microorganisms probably correspond to rare diversity. Furthermore, the majority of taxa could not be classified at the genus level and likely represent novel members of these groups. Additionally, the nitrifiers in the sludge were characterized by pyrosequencing the *amoA* gene. In contrast, the nitrifying bacterial community, dominated by the genera *Nitrosomonas*, showed a low diversity and rarefaction curves exhibited saturation. These results suggest that only a few populations of low abundant but specialized bacteria are responsible for removal of ammonia in these saline wastewater systems.

Aerobic granular sludge (AGS) is based on self-granulated flocs forming mobile biofilms with a gel-like consistence. In Chapter 6, by Weissbrodt and colleagues, bacterial and structural dynamics from flocs to granules were followed in anaerobic-aerobic sequencing batch reactors (SBR) fed with synthetic wastewater, namely a bubble column (BC-SBR) operated under wash-out conditions for fast granulation, and two stirred-tank enrichments of *Accumulibacter* (PAO-SBR) and *Competibacter* (GAO-SBR) operated at steady-state. In the BC-SBR, granules formed within 2 weeks by swelling of *Zoogloea* colonies around flocs, developing subsequently smooth zoogloeal biofilms. However, *Zoogloea* predominance (37–79%) led to deteriorated nutrient removal during the first months of reactor operation. Upon maturation, improved nitrification (80–100%), nitrogen removal (43–83%), and high but unstable dephosphatation (75–100%) were obtained. Proliferation of dense clusters of nitrifiers, *Accumulibacter*, and *Competibacter* from granule cores outwards resulted in heterogeneous bioaggregates, inside which only low abundance *Zoogloea* (<5%) were detected in biofilm interstices. The presence of different extra-

cellular glycoconjugates detected by fluorescence lectin-binding analysis showed the complex nature of the intracellular matrix of these granules. In the PAO-SBR, granulation occurred within two months with abundant and active *Accumulibacter* populations (56 ± 10%) that were selected under full anaerobic uptake of volatile fatty acids and that aggregated as dense clusters within heterogeneous granules. Flocs self-granulated in the GAO-SBR after 480 days during a period of over-aeration caused by biofilm growth on the oxygen sensor. Granules were dominated by heterogeneous clusters of *Competibacter* (37 ± 11%). *Zoogloea* were never abundant in biomass of both PAO- and GAO-SBRs. This study showed that *Zoogloea*, *Accumulibacter*, and *Competibacter* affiliates can form granules, and that the granulation mechanisms rely on the dominant population involved.

Standard aquaculture generates large-scale pollution and strains water resources. In aquaculture using zero discharge systems (ZDS), highly efficient fish growth and water recycling are combined. The wastewater stream is directed through compartments in which beneficial microbial activities induced by creating suitable environmental conditions remove biological and chemical pollutants, alleviating both problems. Bacterial predators, preying on bacterial populations in the ZDS, may affect their diversity, composition and functional redundancy, yet in-depth understanding of this phenomenon is lacking. In Chapter 7, Kandel and colleagues analyzed the dynamics of populations belonging to the obligate predators *Bdellovibrio* and like organisms (BALOs) in freshwater and saline ZDS over a 7-month period using QPCR targeting the *Bdellovibrionaceae*, and the *Bacteriovorax* and *Bacteriolyticum* genera in the *Bacteriovoracaeae.* Both families co-existed in ZDS compartments, constituting 0.13–1.4% of total Bacteria. Relative predator abundance varied according to the environmental conditions prevailing in different compartments, most notably salinity. Strikingly, the *Bdellovibrionaceae*, hitherto only retrieved from freshwater and soil, also populated the saline system. In addition to the detected BALOs, other potential predators were highly abundant, especially from the *Myxococcales*. Among the general bacterial population, *Flavobacteria, Bacteroidetes, Fusobacteriaceae* and unclassified Bacteria dominated a well mixed but seasonally fluctuating diverse community of up to 238 operational taxonomic units, as revealed by 16S rRNA gene sequencing.

Biological WWTPs must be functionally stable to continuously and steadily remove contaminants which rely upon the activity of complex microbial communities. However, knowledge is still lacking in regard to microbial community functional structures and their linkages to environmental variables. Therefore, in Chapter 8, Wang and colleagues aimed to investigate microbial community functional structures of activated sludge in wastewater treatment plants (WWTPs) and to understand the effects of environmental factors on their structure. Twelve activated sludge samples were collected from four WWTPs in Beijing. A comprehensive functional gene array named GeoChip 4.2 was used to determine the microbial functional genes involved in a variety of biogeochemical processes such as carbon, nitrogen, phosphorous and sulfur cycles, metal resistance, antibiotic resistance and organic contaminant degradation. High similarities of the microbial community functional structures were found among activated sludge samples from the four WWTPs, as shown by both diversity indices and the overlapped genes. For individual gene category, such as *egl, amyA, lip, nirS, nirK, nosZ, ureC, ppx, ppk,aprA, dsrA, sox* and *benAB*, there were a number of microorganisms shared by all 12 samples. Canonical correspondence analysis (CCA) showed that the microbial functional patterns were highly correlated with water temperature, dissolved oxygen (DO), ammonia concentrations and loading rate of chemical oxygen demand (COD). Based on the variance partitioning analyses (VPA), a total of 53% of microbial community variation from GeoChip data can be explained by wastewater characteristics (25%) and operational parameters (23%), respectively. This study provided an overall picture of microbial community functional structures of activated sludge in WWTPs and discerned the linkages between microbial communities and environmental variables in WWTPs.

Constructed wetlands constitute an interesting option for wastewater reuse since high concentrations of contaminants and pathogenic microorganisms can be removed with these natural treatment systems. In Chapter 9, by Morató and colleagues, the role of key design factors which could affect microbial removal and wetland performance, such as granular media, water depth and season effect was evaluated in a pilot system consisting of eight parallel horizontal subsurface flow (HSSF) constructed wetlands treating urban wastewater from Les Franqueses del Vallès (Barcelona,

Spain). Gravel biofilm as well as influent and effluent water samples of these systems were taken in order to detect the presence of bacterial indicators such as total coliforms (TC), *Escherichia coli*, fecal enterococci (FE), *Clostridium perfringens*, and other microbial groups such as *Pseudomonas* and *Aeromonas*. The overall microbial inactivation ratio ranged between 1.4 and 2.9 log-units for heterotrophic plate counts (HPC), from 1.2 to 2.2 log units for total coliforms (TC) and from 1.4 to 2.3 log units for *E. coli*. The presence of fine granulometry strongly influenced the removal of all the bacterial groups analyzed. This effect was significant for TC ($p = 0.009$), *E. coli* ($p = 0.004$), and FE ($p = 0.012$). Shallow HSSF constructed wetlands were more effective for removing *Clostridium* spores ($p = 0.039$), and were also more efficient for removing TC ($p = 0.011$) and *E. coli* ($p = 0.013$) when fine granulometry was used. On the other hand, changes in the total bacterial community from gravel biofilm were examined by using denaturing gradient gel electrophoresis (DGGE) and sequencing of polymerase chain reaction (PCR)-amplified fragments of the 16S rRNA gene recovered from DGGE bands. Cluster analysis of the DGGE banding pattern from the different wetlands showed that microbial assemblages separated according to water depth, and sequences of different phylogenetic groups, such as *Alpha, Beta* and *Delta-Proteobacteria, Nitrospirae, Bacteroidetes, Acidobacteria, Firmicutes, Synergistetes* and *Deferribacteres* could be retrieved from DGGE bands.

Milk fat curdle in sewage is one of the refractory materials for active sludge treatment under low temperature conditions. For the purpose of solving this problem by using a bio-remediation agent, in Chapter 10, Tsuji and colleagues screened Antarctic yeasts and isolated SK-4 strain from algal mat of sediments of Naga-ike, a lake in Skarvsnes, East Antarctica. The yeast strain showed high nucleotide sequence homologies (>99.6%) to *Mrakia blollopis* CBS8921T in ITS and D1/D2 sequences and had two unique characteristics when applied on an active sludge; i.e., it showed a potential to use various carbon sources and to grow under vitamin-free conditions. Indeed, it showed a biochemical oxygen demand (BOD) removal rate that was 1.25-fold higher than that of the control. The authors considered that the improved BOD removal rate by applying SK-4 strain was based on its lipase activity and characteristics. Finally, the authors purified the lipase from SK-4 and found that the enzyme was quite

stable under wide ranges of temperatures and pH, even in the presence of various metal ions and organic solvents. SK-4, therefore, is a promising bio-remediation agent for cleaning up unwanted milk fat curdles from dairy milk wastewater under low temperature conditions.

Biohydrogen production through dark fermentation using organic waste as a substrate has gained increasing attention in recent years, mostly because of the economic advantages of coupling renewable, clean energy production with biological waste treatment. An ideal approach is the use of selected microbial inocula that are able to degrade complex organic substrates with simultaneous biohydrogen generation. Unfortunately, even with a specifically designed starting inoculum, there is still a number of parameters, mostly with regard to the fermentation conditions, that need to be improved in order to achieve a viable, large-scale, and technologically feasible solution. In Chapter 11, Boboescu and colleagues applied statistics-based factorial experimental design methods to investigate the impact of various biological, physical, and chemical parameters, as well as the interactions between them on the biohydrogen production rates. By developing and applying a central composite experimental design strategy, the effects of the independent variables on biohydrogen production were determined. The initial pH value was shown to have the largest effect on the biohydrogen production process. High-throughput sequencing-based metagenomic assessments of microbial communities revealed a clear shift towards a *Clostridium* sp.-dominated environment, as the responses of the variables investigated were maximized towards the highest H_2-producing potential. Mass spectrometry analysis suggested that the microbial consortium largely followed hydrogen-generating metabolic pathways, with the simultaneous degradation of complex organic compounds, and thus also performed a biological treatment of the beer brewing industry wastewater used as a fermentation substrate. Therefore, the authors have developed a complex optimization strategy for batch-mode biohydrogen production using a defined microbial consortium as the starting inoculum and beer brewery wastewater as the fermentation substrate. These results have the potential to bring us closer to an optimized, industrial-scale system which will serve the dual purpose of wastewater pre-treatment and concomitant biohydrogen production.

In Chapter 12, Adrados and colleagues analazed the prokaryotic microbial communities (*Bacteria* and *Archaea*) of three different systems operating in Denmark for the treatment of domestic wastewater (horizontal flow constructed wetlands (HFCW), vertical flow constructed wetlands (VFCW) and biofilters (BF)) using endpoint PCR followed by Denaturing Gradient Gel Electrophoresis (DGGE). Further sequencing of the most representative bacterial bands revealed that diverse and distinct bacterial communities were found in each system unit, being γ-*Proteobacteria* and *Bacteroidetes* present mainly in all of them, while *Firmicutes* was observed in HFCW and BF. Members of the *Actinobacteria* group, although found in HFCW and VFCW, seemed to be more abundant in BF units. Finally, some representatives of α, β and δ-*Proteobacteria*, *Acidobacteria* and *Chloroflexi* were also retrieved from some samples. On the other hand, a lower archaeal diversity was found in comparison with the bacterial population. Cluster analysis of the DGGE bacterial band patterns showed that community structure was related to the design of the treatment system and the organic matter load, while no clear relation was established between the microbial assemblage and the wastewater influent.

PART I

MICROBIAL COMMUNITIES FOR WASTEWATER TREATMENT

CHAPTER 1

Bacterial Communities in Different Sections of a Municipal Wastewater Treatment Plant Revealed by 16S rDNA 454 Pyrosequencing

LIN YE AND TONG ZHANG

1.1 INTRODUCTION

Biological treatment processes are the most widely used approach for treating municipal and industrial wastewater in wastewater treatment plants (WWTPs) due to their high efficiency for various organic/nutrient matters removal and low operational cost. The microbial community, which is dominated by bacteria (Wagner et al. 2002), plays an essential role in the biological treatment reactors and has been studied for several decades by both isolation (Neilson 1978) and molecular methods, such as polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (Muyzer et al. 1993; Ye and Zhang 2010), terminal restriction fragment length polymorphism (Liu et al. 1997), cloning (Schuppler et al. 1995), and fluorescent in situ hybridization (Erhart et al. 1997). The culturing

Bacterial Communities in Different Sections of a Municipal Wastewater Treatment Plant Revealed by 16S rDNA 454 Pyrosequencing. © *Ye L and Zhang T.* Applied Microbiology and Biotechnology ***97,****6 (2013), doi: 10.1007/s00253-012-4082-4. Licensed under a Creative Commons Attribution License, http://creativecommons.org/licenses/by/3.0.*

methods have been a very direct and effective way to characterize the microbial community. However, most of the bacteria in the natural environment cannot be cultured in an artificial medium in the laboratory (Giovannoni et al. 1990; Hugenholtz et al. 1998) and the diversity of the uncultured bacteria is quite considerable (Whitman et al. 1998). The molecular methods greatly promoted our understanding of the microbial community. But for complex environmental samples (such as soil and activated sludge) with overwhelming genetic diversities, these methods are still far away from revealing the panorama of the bacterial community and can only investigate the most abundant population in these samples (Claesson et al. 2009).

The next generation sequencing technologies originated several years ago made the high throughput sequencing easy to be implemented with low cost (Glenn 2011). 454 Pyrosequencing is one of the popular high throughput sequencing systems, which can generate more than 400,000 effective reads with average read length up to several hundred base pairs and average quality of greater than 99.5 % accuracy rate (Droege and Hill 2008; Glenn 2011). By incorporating barcode sequences on primers, a certain number of DNA samples can be sequenced at the same time in one run. This technology has been successfully used in investigating microbial diversity and abundance in various samples, such as marine water (Qian et al. 2010), soil (Roesch et al. 2007), and human distal intestine (Claesson et al. 2009). There are also several studies applying pyrosequencing in exploring the microbiota in WWTP (Kim et al. 2011; McLellan et al. 2010; Ye et al. 2011); however, these results are very limited and preliminary.

In this study, to investigate the bacterial diversity and abundance in a full scale WWTP treating saline sewage in Hong Kong, we systematically analyzed 16S rRNA gene in the influent, activated sludge, digestion sludge, and effluent by using 454 high throughput pyrosequencing. It was found that the bacterial diversities in the four samples are quite different. Besides, in this study, we also introduced a method to consider and compare the difference of 16S rRNA gene percentage and bacteria cell number percentage in these samples. The results showed that some genera, especially the abundant genera, may be underestimated or overestimated when using 16S rRNA gene to reflect their abundances.

1.2 MATERIALS AND METHODS

1.2.1 WWTP DESCRIPTION AND SAMPLING

In this study, the activated sludge, digestion sludge, influent, and effluent samples were taken from Shatin WWTP, which is a full scale municipal wastewater treatment plant in Hong Kong. Because seawater has been used extensively in the toilet flushing system in Hong Kong, this WWTP treats saline sewage (salinity ~1.2 %) containing about 30 % seawater with activated sludge system. It treats about 216,000 m^3 wastewater per day with a COD concentration of 226 ~ 491 mg l^{-1}. The aeration tank was partitioned into two zones (anoxic zone and aerobic zone) for carbon and nitrogen removal but no special facilities for phosphate removal. The activated sludge sample analyzed in this study was taken from the aerobic zone. To reduce sludge volume, both the primary sludge and the surplus activated sludge are digested in mesophilic anaerobic digesters. Detailed information about the WWTP and the samples were summarized in Table S4 and Fig. S3. When taking samples, the activated sludge (mixed liquid containing both flocs and suspended bacteria in the aerobic zone of the aeration tank) and the digestion sludge were taken from the reactor and mixed thoroughly and then fixed on site by mixing with 100 % ethanol at a volume ratio of 1:1 and kept in an ice box for transportation and then stored in our laboratory at −20 °C before DNA extraction. Influent and effluent samples were kept in plastic containers and delivered to our laboratory within 3 h. Immediately after arriving at the laboratory, 12 ml influent was centrifuged at 4,000 rpm for 10 min at 4 °C and 400 ml effluent was filtrated using a 0.45-μm glass fiber filter to collect the bacteria cells. The collected residue was used for DNA extraction.

1.2.2 DNA EXTRACTION AND PCR

For all samples in this study, DNA was extracted using FastDNA® SPIN Kit for Soil (MP Biomedicals, Illkirch, France). Before pyrosequencing, the above DNA of each sample was amplified with a set of primers tar-

geting the hypervariable V4 region of the 16S rRNA gene (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp). The forward primer is 5′-AYTGGGYDTAAAGNG-3′ and the reverse primers are the mixture of four primers, i.e., 5′-TACCRGGGTHTCTAATCC-3′, 5′-TACCAGAGTATCTAATTC-3′, 5′-CTACDSRGGTMTCTAATC-3′, and 5′-TACNVGGGTATCTAATCC-3′ (Claesson et al. 2009). Barcodes that allow sample multiplexing during pyrosequencing were incorporated between the 454 adaptor and the forward primers. The PCR amplification was conducted in a 100-μl reaction system using MightyAmp polymerase (TaKaRa, Dalian, China). The amplification was conducted in an i-Cycler (BioRad, Hercules, CA, USA) under the following conditions: 98 °C for 2 min, 28 cycles at 98 °C for 15 s, 56 °C for 20 s and 68 °C for 30 s, and a final extension at 68 °C for 10 min. The PCR products were purified by using PCRquick-spinTM PCR Product Purification Kit (iNtRON Biotechnology, South Korea) and then mixed equally before conducting pyrosequencing.

For clone library construction, the DNA of another digestion sludge sample taken from the sample anaerobic digester was amplified by PCR using universal bacterial 16S rRNA primer set EUB8F (5′-AGAGTTTGATCMTGGCTCAG-3′) (Heuer et al. 1997) and UNIV1392R (5′-ACGGGCGGTGTGTRC-3′) (Ferris et al. 1996). The vector used for ligation was pMD®18-T (TaKaRa). The purified plasmid was sequenced on an ABI 3730xl capillary sequencer (Applied Biosystems, Foster City, CA, USA).

1.2.3 HIGH THROUGHPUT PYROSEQUENCING

The PCR products of the V4 region of 16S rRNA gene were sequenced using the Roche 454 FLX Titanium sequencer (Roche, Nutley, NJ, USA). Samples in this study were individually barcoded to enable multiplex sequencing. The results are deposited into the NCBI short reads archive database (accession number: SRA026842.2).

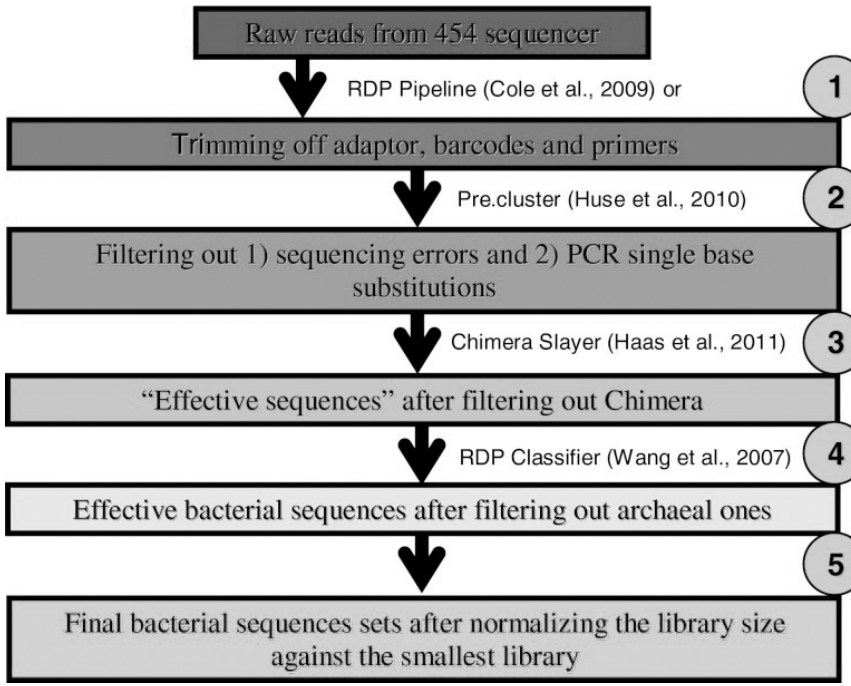

FIGURE 1: Sequences quality trimming flow chart

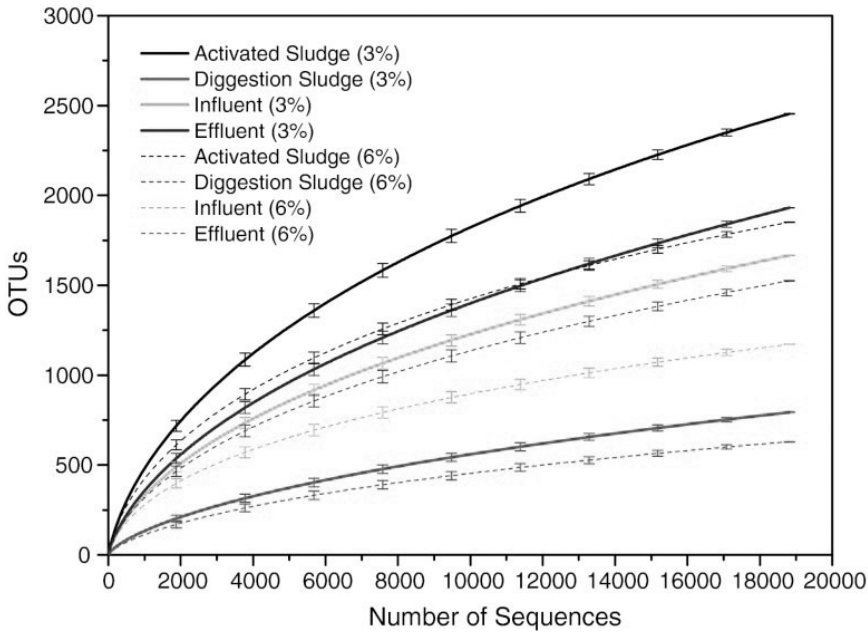

FIGURE 2: Rarefaction curves of the four samples at cutoff levels of 3 % (solid lines) and 6 % (dash lines) created by using RDP's pyrosequencing pipeline. The error bars show 95 % confidence of upper and lower limits

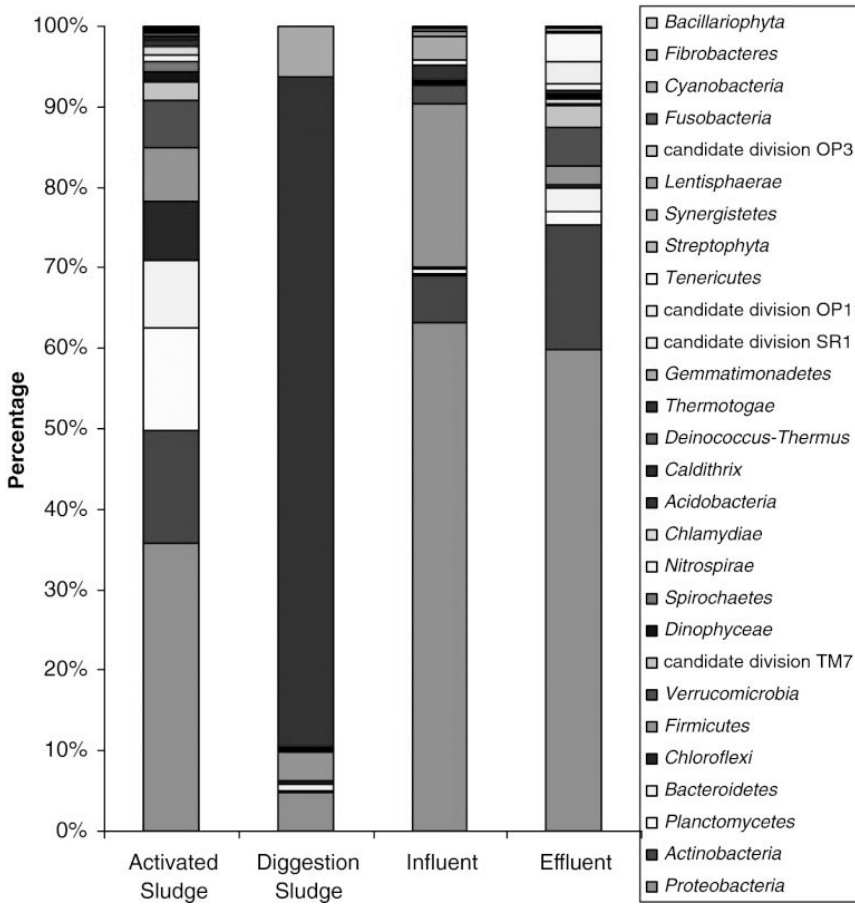

FIGURE 3: Relative abundances of different phyla in the four samples (the results were obtained by using BLASTN combined with MEGAN)

1.2.4 SEQUENCE PROCESSING AND BACTERIAL POPULATION ANALYSIS

Following pyrosequencing, Python scripts were written to (1) remove sequences containing more than one ambiguous base ('N'), (2) check the completeness of the barcodes and the adaptor, and (3) remove sequences shorter than 150 bp.

454 Sequencing noises were removed by Pre.cluster (Huse et al. 2010) tool in Mothur package (Roeselers et al. 2011). Chimeras introduced in the PCR process were detected using ChimeraSlayer (Haas et al. 2011) in Mothur package. Because all sequences flagged as chimeras are not recommended to be discarded blindly (http://microbiomeutil.sourceforge.net/), so the reads flagged as chimeras were submitted to Ribosomal Database Project (RDP) classifier (Wang et al. 2007; Cole et al. 2009). Those being assigned to any known genus with 50 % confidence threshold were merged with the non-chimera reads to form the collection of "effective sequences" for each sample.

Although the primers used in this study are bacteria-specific primers, a few archaeal sequences might be obtained. The same situation was also observed in another study (Qian et al. 2010). To remove these archaeal sequences, the effective sequences of each sample were submitted to the RDP classifier again to identify the archaeal and bacterial sequences, and the archaeal sequences were filtered out using a self-written Python script. The average length of all effective bacterial sequences without the primers was 207 bp. The above quality trimming process was summarized in Fig. 1.

After that, the "RDP Align" tool in RDP's Pyrosequencing Pipeline was used to align the effective bacterial sequences of each sample. A cluster file was generated for each sample with "RDP Complete Linkage Clustering" tool. With the cluster file, the rarefaction curves were generated using the "RDP Rarefaction" tool.

All effective bacterial sequences obtained from pyrosequencing in this study were compared with Greengenes 16S rRNA gene database (DeSantis et al. 2006) annotated with NCBI taxonomy using NCBI's BLASTN tool (Altschul et al. 1990) and the default parameters except for the maximum hit number of 100 (Claesson et al. 2009). Then, the sequences were assigned to NCBI taxonomies with MEGAN (Huson et al. 2007) by using