Biases during DNA extraction of activated sludge samples revealed by high throughput sequencing

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Abstract Standardization of DNA extraction is a fundamental issue of fidelity and comparability in investigations of environmental microbial communities. Commercial kits for soil or feces are often adopted for studies of activated sludge because of a lack of specific kits, but they have never been evaluated regarding their effectiveness and potential biases based on high throughput sequencing. In this study, seven common DNA extraction kits were evaluated, based on not only yield/purity but also sequencing results, using two activated sludge samples (two sub-samples each, i.e. ethanol-fixed and fresh, as-is). The results indicate that the bead-beating step is necessary for DNA extraction from activated sludge. The two kits without the bead-beating step yielded very low amounts of DNA, and the least abundant operational taxonomic units (OTUs), and significantly underestimated the Gram-positive Actinobacteria, Nitrospirae, Chloroflexi, and Alphaproteobacteria and overestimated Gammaproteobacteria, Deltaproteobacteria, Bacteroidetes, and the rare phyla whose cell walls might have been readily broken. Among the other five kits, FastDNA SPIN Kit for Soil extracted the most and the purest DNA. Although the number of total OTUs obtained using this kit was not the highest, the abundant OTUs and abundance of Actinobacteria demonstrated its efficiency. The three MoBio kits and one ZR kit produced fair results, but had a relatively low DNA yield and/or less Actinobacteria-related sequences. Moreover, the 50 % ethanol fixation increased the DNA yield, but did not change the sequenced microbial community in a significant way. Based on the present study, the FastDNA SPIN kit for Soil is recommended for DNA extraction of activated sludge samples. More importantly, the selection of the DNA extraction kit must be done carefully if the samples contain dominant lysing-resistant groups, such as Actinobacteria and Nitrospirae.

Keywords DNA extraction · Bacteria · Activated sludge · High throughput sequencing · Commercial kit

Introduction

Along with the development of the low cost, next generation high throughput sequencing techniques, the Earth Microbiome Project has been launched in 2011, aiming to reveal the gigantic, unexplored microbial genetic resource in soil, seawater, freshwater, the atmosphere, and other environments on our planet. At least 200,000 samples will be analyzed according to this ambitious plan. To maximize the comparability among the different studies, it needs standardized protocols for every operation step, including DNA extraction, PCR, sequencing, and data processing. Extraction of DNA of high quality is the first key step to profile microbial community with high fidelity (Martin-Laurent et al. 2001). However, the diversity of environmental sample types makes it impossible to simply apply one protocol or kit for DNA extraction. Unlike soil or other environmental samples, activated sludge (AS) is almost composed of bacterial cells or their products, mostly extracellular polymeric substances (EPS) (Liu and Fang 2003). Generally, 1 g of dry mass of AS contains over 1−10×10^{12} bacterial cells (about 1−10×10^9 cells per milliliter of working activated sludge (Nielsen and Nielsen 2001). This value is over 100-fold higher than the microbial density in soil samples. Its abundance guarantees that biomass is not a concern, and only hundreds of microliters to several milliliters of sludge are enough for DNA extraction.
treatments were conducted.

DNA examination

For DNA quantification, two methods were adopted, i.e., NanoDrop (NanoDrop-1000, Thermo Scientific,
USA) and Qubit (Invitrogen, USA, using the high sensitive DNA quantification kit), with the detection limits of 2 and 0.1 ng μl⁻¹ respectively. Two microliters of each sample for NanoDrop was loaded directly after extraction. For Qubit, the DNA was diluted 20–200 times in the working solution according to the concentration. After quantification, 8 μl DNA was loaded onto a 1.0% agarose gel containing 1× GelRed dye and 1× TAE buffer. DNA was allowed to run for 30 min under a voltage of 100 V. The gels were visualized in the Bio-Rad Gel DOC system (Bio-Rad Laboratories, Inc., USA).

PCR and Illumina high throughput sequencing

For Illumina high throughput sequencing, the highest yields of DNA extracted from each kit for each sample (both fixed and as-is) were evaluated. The V6 region of the 16S rRNA gene was amplified by the primer set of V6F and V6R (Sogin et al. 2006). The forward primer was added with 28 sample-specific, eight-base barcodes at its 5′ end, which allows the multiplexing during sequencing (Binladen et al. 2007). A final concentration of 0.5 ng μl⁻¹ genomic DNA was used as a template because some kits produced a very low concentration of DNA. PCR conditions were set as follows: 95 °C for 5 min, then 30 cycles of 95 °C for 15 s, 57 °C for 30 s, and 72 °C for 30 s, finally extending at 72 °C for 10 min. Three 50-μl PCR reactions were conducted for each DNA sample and then mixed and visualized in a 2 % agarose gel after electrophoresis (~100 bp). Then, the products were purified with a PCR production purification kit (PCR quick-spin™ kit, iNtRON Biotechnology, Inc., Korea). Finally, the PCR products from each treatment were mixed to obtain equal molar DNA for sequencing. About 18 μg PCR products were sent out to BGI (Shenzhen, China) for 101PE paired-end sequencing on the platform of Illumina Hiseq2000 (Illumina, USA).

Sequence processing

The raw paired-end sequence data analysis was performed as follows: (1) we combined each pair-end reads into one sequence and removed all sequences with any mismatches between the two reads (using a self-written python script); (2) we removed sequences without barcodes and obtained the tags containing barcode and primer (using a self-written python script); (3) we trimmed and cleaned the subsample and get operational taxonomic units (OTUs) from the tags through the Mothur software (see SI methods in detail) (Schloss 2009; Schloss et al. 2009); (4) we extracted one representative sequence from each OTU (using a self-written python script) and classified it through the GAST program (Huse et al. 2008).

Results

DNA quantification and qualification

The yields of extracted DNA are shown in Fig. 1a and b. The dry weights of sludge used in extraction were 2.3±0.4 and 3.5±0.6 mg for Stanley and Shatin samples, respectively. The highest yield of DNA was obtained by the FA-SS kit, which was more than two times that of the second highest kit (MB-PS). The three MoBio kits had moderate yields (0.7–4 μg). The DNA contents extracted by the other three kits were too low to be observed in the agarose gel (Figure S2). On the other hand, samples fixed in 50 % ethanol

Table 1 Seven DNA extraction kits evaluated in this study

| Kits | Abbreviation | Cell lysis | DNA purification | References |
|------|--------------|------------|------------------|------------|
| MoBio UltraClean® Fecal DNA Isolation Kit | MB-FE | BB and CLB | Spin filter | McGarvey et al. 2004 |
| MoBio PowerSoil® DNA Isolation Kit | MB-PS | BB and CLB | Spin filter | Zhang et al. 2009 |
| MoBio UltraClean® Soil DNA Isolation Kit | MB-US | BB and CLB | Spin filter | Gelder et al. 2005 |
| Qbiogene FastDNA® SPIN Kit for Soil | FA-SS | BB and CLB | Spin filter | Auerbach et al. 2007 |
| Qiagen QIAamp DNA Stool Mini Kit | QG-ST | CLB | Spin filter | Bonot et al. 2010 |
| Epicentre™ SoilMaster DNA extraction Kit | EP-SM | CLB | Spin filter | Roh et al. 2006 |
| ZR™ Soil Microbe DNA Kit | ZR-SM | BB and CLB | Spin filter | Wang et al. 2011 |

BB bead beating, CLB cell lysis buffer
produced significantly more DNA than the corresponding unfixed ones in FA-SS and MB-PS treatments \((P < 0.05)\).

The Qubit quantification results were usually lower than those obtained using NanoDrop, especially for DNA extracts of low quality, as shown in Fig. 1c. Qubit results based on fluorescence may be more reliable because the impurities in the DNA extract could also result in UV absorbance, whereas fluorescence-based quantification was more specific. According to the results of the Qubit method, the yield of the FA-SS kit was 2,241 to 4,741 \(\mu\)g/g dry mass, relatively higher than that in a previous report (Bonot et al. 2010).

Two kits, i.e., QG-ST and EP-SM, without bead-beating to disrupt cells, yielded very low DNA \((<0.7 \mu\text{g} \text{in all treatments})\), showing that robust mechanical homogenization is needed for DNA extraction of AS samples.

By contrast, the FA-SS and MB-FE kits produced the purest DNA indicated by the OD\(_{260/280}\) values of \(\sim 1.85\) in all treatments. The highly purified DNA extracted by the FA-SS kit implied that the purified method of this kit was more efficient and robust. The ZR-SM was very low in purity, with the ratio of OD\(_{260/280}\) around 1.0. It was in accordance with the significant difference between the DNA amounts determined by NanoDrop and Qubit. The two MoBio kits, MB-FE and MB-PS, obtained fairly pure DNA, with slight variations. The high ratio of OD\(_{260/280}\) of the DNA extracted from QG-ST and EP-SM may result from the low DNA concentration that causes imprecision in absorption measurements. Ethanol fixation did not affect the purity for all treatments \((P > 0.05 \text{ in all kits})\). The results of DNA electrophoresis are shown in Figure S2. DNA obtained after all the treatments (QG-ST, EP-SM, and ZR-SM were very weak) was smaller than 21 kb, typically around 10 kb.

**OTU-based analysis**

About 3.7 million raw reads were obtained by the high throughput sequencing. After processing, all treatments were subsampled at the same depth of 46,734 tags. The two treatments of Shatin AS extracted by the EP-SM kit with and without fixation were excluded because of the low read number. There were 29,553 OTUs for a total 1,215,084 tags (a total of 26 treatment groups). The OTU numbers were 17,872 and 15,079 OTUs for the Stanley and Shatin AS samples, respectively. The rarefaction curves for the unfixed and fixed treatments in each activated sludge sample are shown in Figure S3.
Table 2 lists the analysis of the number of OTUs of each DNA extraction treatment. The treatments with the highest number of OTUs were ZR-SM and MB-PS, whereas the treatments with the lowest number of OTUs were EP-SM and FA-SS for the Stanley and Shatin AS samples, respectively. The treatments with the least OTUs usually had smaller diversity indexes calculated based on the total OTUs. However, the numbers of abundant OTUs containing over 100 tags in each treatment, as well as the diversity indexes calculated based on the top 500 OTUs, were not obviously different among the five kits with the bead-beating step. The two kits without the bead-beating step were obviously lower in the numbers of abundant OTUs and diversity indexes based on the top 500 OTUs than the other five kits. The missed OTUs within the top 500 OTUs also indicated that the EP-SM and QG-ST kits were less efficient than the other five kits, whereas the five kits had little difference between them.

Table 2 Total OTUs and OTU-based diversity indexes of the different extraction treatments

| Sludge | Kit | Fixation | Total OTUs | >100 tags OTUs | Diversitya | Diversityb | Number of undetected Top 500 OTUs (the highest rank of the missing OTUs)c |
|--------|-----|----------|------------|----------------|------------|------------|-------------------------------------------------------------------|
| Stanley | MB-FE | UF        | 4,359      | 85             | 6.107      | 5.156      | 2 (339)                                                           |
|        | MB-FE | F         | 3,895      | 89             | 6.206      | 5.306      | 1 (339)                                                           |
|        | MB-PS | UF        | 4,243      | 86             | 6.116      | 5.282      | 1 (339)                                                           |
|        | MB-PS | F         | 3,884      | 88             | 6.123      | 5.172      | 3 (280)                                                           |
|        | MB-US | UF        | 4,173      | 86             | 6.289      | 5.242      | 0                                                                 |
|        | MB-US | F         | 4,451      | 83             | 5.925      | 5.124      | 1 (339)                                                           |
|        | FA-SS | UF        | 3,814      | 81             | 6.145      | 5.27      | 2 (339)                                                           |
|        | FA-SS | F         | 4,140      | 82             | 5.811      | 4.84      | 18 (212)                                                          |
|        | QG-ST | UF        | 3,916      | 70             | 5.638      | 4.805      | 31 (119)                                                          |
|        | QG-ST | F         | 3,579      | 77             | 5.716      | 4.909      | 21 (119)                                                          |
|        | EP-SM | UF        | 3,382      | 76             | 5.763      | 4.924      | 29 (265)                                                          |
|        | EP-SM | F         | 3,513      | 75             | 6.427      | 5.377      | 0                                                                 |
|        | ZR-SM | UF        | 4,722      | 88             | 6.252      | 5.297      | 1 (500)                                                           |
| Shatin  | MB-FE | UF        | 3,943      | 92             | 6.069      | 5.276      | 1 (290)                                                           |
|        | MB-FE | F         | 3,751      | 92             | 6.121      | 5.256      | 1 (290)                                                           |
|        | MB-PS | UF        | 3,996      | 86             | 6.142      | 5.289      | 1 (290)                                                           |
|        | MB-PS | F         | 3,972      | 82             | 6.13       | 5.285      | 1 (290)                                                           |
|        | MB-US | UF        | 3,902      | 88             | 5.906      | 5.125      | 3 (290)                                                           |
|        | MB-US | F         | 3,519      | 92             | 5.914      | 5.208      | 2 (290)                                                           |
|        | FA-SS | UF        | 3,429      | 93             | 5.933      | 5.234      | 1 (290)                                                           |
|        | FA-SS | F         | 3,410      | 89             | 5.846      | 4.97      | 20 (164)                                                          |
|        | QG-ST | UF        | 3,515      | 77             | 5.956      | 5.112      | 15 (152)                                                          |
|        | QG-ST | F         | 3,639      | 82             | 5.975      | 5.168      | 1 (480)                                                           |

- Shannon diversity indexes calculated on the basis of total OTUs
- Shannon diversity indexes calculated on the basis of the top 500 OTUs
- Top 500 OTUs were determined based on the total abundance in all treatments for each sample.
were obviously unreliable because of the large numbers of blue blocks in high-ranked OTUs.

Taxonomy-based analysis

The bacterial community structure at the phylum level for each treatment is shown in Fig. 3. The most abundant phyla (here and below, the Alpha, Beta, Gamma, and Delta classes in Proteobacteria were treated as phyla) were Betaproteobacteria and Gammaproteobacteria for the Stanley and Shatin AS samples, respectively. The treatments of QG-ST and EP-SM without the bead-beating step resulted in a significantly low abundance in Gram-positive Actinobacteria, Nitrospirae, Alphaproteobacteria, and Chloroflexi, especially for the former two phyla. The other Gram-positive phylum, the Firmicutes, was not obviously different among all the treatments. This indicated that only chemical or enzymic lysis could not disrupt efficiently the Gram-positive Actinobacteria and that robust mechanical homogenization is needed. However, it seemed that Gammaproteobacteria, Deltaproteobacteria, Bacteroidetes, and many rare phyla were usually overestimated in the QG-ST and EP-SM kits with gentle cell lysis.

Moreover, the 50 % ethanol fixation slightly changed the community structure. A detectable bias was Chloroflexi in the Stanley sample. Fixation decreased the abundance of this phylum. This phylum usually has a filamentous shape, and the reason for such a decrease is not clear.

To further investigate the efficiencies of cell lysis of the various kits, the abundances of the top 5 Gram-positive genera were investigated, and the results are shown in Fig. 4. First, the treatments without the bead-eating step (i.e., QG-ST and EP-SM) had very low abundances of the top 5 Gram-positive genera in both the samples. Second, among the five kits with the bead-beating step, the FA-SS kit exhibited the best capability for cell lysis among the top 5 Gram-positive genera. The ZR-SM and MB-US kits also worked well. However, the MB-FE and MB-PS did not perform very efficiently, as indicated by the low detected abundances of these genera. Interestingly, the third abundant Gram-positive genus in the Stanley sample, Oscillospira, was richer in the treatment with QG-ST and EP-SM than that with the other five kits.

Discussion

Unlike other environmental samples, activated sludge is composed of nearly all microbial cells and their products (Frølund et al. 1996). The cells cluster together and are enclosed with EPS, which can protect them from shear forces and chemical reagents including sodium dodecyl sul fate (Davies et al. 1998). In terms of productivity and diversity, results from this study showed that the mechanical homogenization (the bead-beating step) is obviously necessary for DNA extraction from sludge samples.

The factors that affect DNA yield for a kit are mainly the efficiency of the cell lysis step and the subsequent losses during purification. The five kits with the bead-beating step have minor differences in the lysis process except that the FA-SS kit contains glass beads with different sizes (0.1–1 mm in diameter). The big glass beads may be efficient for dispersing cells from clusters, and the small ones are dedicated to crush the cells. However, the FA-SS kit also
contains a unique matrix that specifically binds DNA, whereas all of the other six kits just adopt a spin column to bind DNA. The two unique designs in the FA-SS kit may promote the quantity and quality of the extract DNA from AS, as indicated by the result.

The quantity of DNA is usually not of great concern for PCR-based community analysis because even as low as 10–100 ng DNA (equals to about 10⁶–10⁷ cells) is already enough for amplification and then sequencing. However, for the current metagenomic study sequenced by the Illumina platform, 3–10 μg virginal, highly pure DNA is needed. This makes the FA-SS and MB-PS the only two candidate kits. Dramatically, our result showed that the low quantity and even the low quality of the extracted DNA could also provide a fair profile of the bacterial community. For example, the ZR-SM kit produced very low concentrations of DNA with low quality (ratio of OD₂₆₀/OD₂₈₀ around 1.0), but the OTU-based and taxonomic analysis indicated that the results reflected reasonably the major bacterial community profile, with only a slight difference from the three MoBio kits and FA-SS kit. However, the QG-ST and EP-SM kits that also extracted low quality and quantity of DNA showed much higher biases on the community profile compared with the other five kits. This suggests that the ZR-SM kit may be efficient in cell lysis, but loses much DNA during the subsequent purification steps, which is a random event. Thus, it does not affect the community structure. It is noteworthy, however, that all the kits could be utilized efficiently by changing some of the operations. For example, the EP-SM kit performs the centrifugation at 1,000–2,000 g in some cases, which may be fair for soils (the density is much higher than activated sludge), but unsuitable for sludge samples, and could cause loss of sample. Increasing the strength of the centrifugation may increase the yield for this situation.

Other than yield and fidelity, a co-existing problem is that the DNA extract from commercial kits are usually small in segment size. This may be the result of the high shear force during the bead-beating or vortex processing. Small pieces of DNA are not suitable for construction of the fosmid, cosmid, and BAC libraries that prefer genomic DNA fragments over 25 kb, which are usually extracted by lab-developing methods (Robe et al 2003). Moreover, if the extracted DNA is used in full-length 16S rRNA gene (~1.5 kb) amplification, ~10 kb-sized fragments theoretically lose about 15 % of the genes. However, the current high throughput sequencing will be little affected because of the short amplified regions (mostly <400 bp).
For environmental samples, especially for those containing bulking water, fixation is needed before long-term transportation and storage. DNA may be altered in two different ways without fixation: (1) The bacterial community may change rapidly during transportation and storage because of the change of environment, and (2) DNA may leak out from cells that die during transportation and storage into the bulking water and then be washed away. For sludge samples, fixation in 50 % ethanol (final concentration) was recommended, which is the same as sample fixation for fluorescence in situ hybridization (FISH) (Xia et al. 2007). Another advantage is that 50 % ethanol would not be frozen at −20 °C. The results in this study showed that the fixation could improve DNA yield, although the reason is unclear. In addition, most of the slight variation of the bacterial community between the fixed and nonfixed treatments could not be attributed to the fixation. It could arise from the biases of PCR or sequencing.

The total OTU number and the diversity indexes based on the total OTUs could not be the key criteria for the evaluation of the efficiencies of DNA extract kits, especially under the conditions that not all species were detected by sequencing at insufficient depth, considering the extremely high diverse bacteria in activated sludge. In fact, under 46,734 sequencing depth, the ~4,000 OTUs in each treatment usually had about 50–70 % singletons and >90 % OTUs containing <10 tags (data not shown), which were obviously rare groups with little significance, having abundances of 0.002–0.02 %. The more OTUs and higher diversity indexes may represent more bacterial species at the price of biases on the abundances of the dominant or subdominant groups if the kits could not extract DNA effectively from certain such groups. On the contrary, the top 500 and 50 OTUs usually accounted for more than 80 and 50 % of total tags, respectively. Thus, they are more suitable to evaluate the efficiency of the kits. Under these conditions, the five kits with the bead-beating step are significantly better than the two kits that only used lysis buffer. However, the differences among the five kits need to be determined by taxonomic analysis.

The Gram-positive bacteria are resistant to both detergents and mechanical resistance because of their thick cell wall (Bollet et al. 1991) or because some of them can form spores (Kuske et al. 1998). Therefore, it could be simply considered that the more Gram-positive bacteria are detected, the more efficient the DNA extraction kits are. In terms of this, the most efficient two kits were FA-SS and MB-US because more Gram-positive Actinobacteria were detected in the two treatments at both the phylum and genus levels than the others. Actinobacteria is an ubiquitously dominant phylum in AS and plays key roles in polymer degradation, glycogen accumulation, and polyphosphate

Fig. 4 The abundances of the top 5 Gram-positive genera in different treatments in AS from the Stanley (a) and Shatin (b) STPs. Seven of the nine genera belong to high G+C phylum, Actinobacteria, and the other two, Oscilliospira and Streptococcus, are Firmicutes
accumulation (Seviour and Nielsen 2010). A study that used untreated sludge to perform PCR and cloning could not detect Actinobacteria, although about 13% of the cells belonged to this phylum, as determined by FISH (Snaidr et al. 1997). Another study that adopted denaturing gradient gel electrophoresis as the DNA extraction evaluation method treated this high G+C phylum as a key indicator for DNA extraction methods (Niemi et al. 2001). All these suggested the abundance of Actinobacteria could be a key sign for efficiency of DNA extraction, especially for cell lysis. Recently, a high-throughput sequencing, metagenomic study of AS found biases when comparing the sequencing data with the results from the FISH method (Albertsen et al. 2011). Very interestingly, the study also found that the data with the results from the FISH method (Albertsen et al. 2011) overlaid the operational time for bead-beating was performed only for 3×5 s. This observation indicates that the operational time for bead-beating should also be concerned. By contrast, the detection of the other Gram-positive phylum, Firmicutes, was minimally affected by different kits, even for the two inefficient kits. Also interestingly, a genus that belongs to the Firmicutes, Oscillospira, was more abundant in treatments of the two inefficient kits. This observation indicates the different efficiencies of the kits in detecting various subgroups of Gram-positive bacteria. The other underestimated phylum, Nitrospirae, has a special wide periplasmic space, which is near twice that in other Gram-negative bacteria (Watson et al. 1986). This structure may hinder the release of DNA following inefficient cell lysis treatment. Similarly, DNA from Chloroflexi was hard to extract, possibly also because of the layered cell envelopes (Sutcliffe 2011). However, the reason for the underestimation of Alphaproteobacteria is unclear.

Moreover, the results from the inefficient kits are also valuable because they imply which groups tend to be overestimated. In this study, the Gammaproteobacteria, Deltaproteobacteria, Bacteroidetes, and many rare phyla were overestimated in the two kits with low efficiency. This implies that bacteria within these groups are more likely to be destroyed and, thus, overestimated if inefficient methods are adopted to disrupt all the bacterial cells. Since the usual high richness of Gammaproteobacteria and Bacteroidetes in many environmental samples was found, it is noteworthy whether they were overestimated by the inefficient DNA extraction to some extent.

In summary, in the light of our results, the FastDNA® SPIN kit for Soil is recommended for DNA isolation of activated sludge samples because of its high yield, purity, and excellent cell-breaking capability. Although the number of total OTUs from this kit was not high, the major groups and Gram-positive bacteria that were identified indicated its reliability compared to other kits. The three MoBio kits and ZR-SM kit were also fair, but insufficient in yield and/or purity, which are essential for current metagenomic studies. In addition, the results also proved that the bead-beating step is necessary for activated sludge samples because some phyla, such as Actinobacteria and Nitrospirae, are significantly resistant to the simply chemical cell lysis treatment. Careful selection of extraction kits or methods should be considered if these phyla would exist dominantly in certain environmental samples.

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