Biochemical characteristic along UBAF in a one-stage autotrophic nitrogen removal reactor

Tao Liu, Dong Li and Jie Zhang

ABSTRACT

The Up-flow biological aerated filter (UBAF) based on a one-stage autotrophic nitrogen removal process has been widely investigated nowadays. In this work, the biochemical characteristic along the volcanic-filled UBAF reactor had been studied. The results indicate that short-rod, spherical and elliptical (averaged 0.2–1.0 μm) microorganisms with a specific irregular cauliflower profile existed in the system. Species identification showed Nitrosococcus- and Nitrosomonas-related aerobic ammonium-oxidizing bacteria (AerAOB) and Candidatus Kuenenia stuttgartiensis-like anaerobic ammonium-oxidizing bacteria (AnAOB) were the predominant functional bacteria that mixed with each other and showed no distinct niche in the system. However, the bioactivity of functional microorganisms displayed differently at different filter layers, with a better pollutant-removal activity in the lower parts than in the upper parts of the UBAF. In the lower parts, compact and small zoogae formed, whereas it trended to be larger and looser along the filter. Moreover, there was better biodiversity of AerAOB in the lower part, while AnAOB showed stable and low biodiversity along the filter.

Key words | biochemical characteristic, CANON, community structure, filter height, UBAF

INTRODUCTION

The one-stage autotrophic nitrogen removal process, also known as the completely autotrophic nitrogen-removal over nitrite (CANON) process, is regarded as a novel and effective nitrogen removal technology nowadays. This process has been widely investigated both in laboratorial and industrial activities. In this, ammonium is autotrophically oxidized to dinitrogen gas with nitrite as the electron acceptor, with the harmonious cooperation of aerobic ammonium-oxidizing bacteria (AerAOB) and anaerobic ammonium-oxidizing bacteria (AnAOB) in oxygen-limited conditions (Third et al. 2001). Compared with traditional nitrogen removal systems, the CANON process consumes 63% less oxygen and nearly 100% less reducing agent (Sliekers et al. 2003), making it one of the most efficient and economical processes for nitrogen removal from wastewater. For CANON operation, the following three aspects are vital and essential: (i) strict dissolved oxygen (DO) concentration to support the metabolism of both AnAOB and AerAOB; (ii) long sludge retention time; and (iii) strong sludge intercept ability to maintain efficient biomass. Consequently, up-flow biological aerated filters (UBAF) are usually preferred for CANON to meet these requirements for its advantages, like high oxygen transfer efficiency, high biomass and organic loading, strong impulsion load resistance, etc.

However, as an up-flow process, the sewage and air are introduced from the bottom of the reactor and pushed up along the filter, leading to variations of substrate concentrations and hydraulic conditions along the filter. A previous study indicated filter height was one of the most important factors to effect treatment activity in a UBAF, in particular with those heterotrophic and autotrophic bacteria that survive simultaneously to remove chemical oxygen demand (COD) and ammonia in one tank (Yan et al. 2010). Consequently, a function division is expected to form concerning heterotrophic and autotrophic microorganisms, resulting in different microbial distribution and pollutant-removal efficiency along the filter. Therefore, it is important and necessary to investigate the appropriate filter height for pollutant removal in UBAF so as to establish guidelines for process monitoring (Wang & Dou 2016).

Although few heterotrophic microorganisms inhabit the UBAF-based CANON process, it may lead to different NH₄⁺-N loading and hydraulic conditions at different layers.

doi: 10.2166/wst.2016.443
It is known that the biological characteristics have a close relationship with the substrate and hydraulic conditions, and the bioactivity and community structure of functional microorganisms at different filter layers may display differently accordingly. Therefore, the nitrogen removal capacity along the filter may be differently (Zhang et al. 2006). Up to now, little attention has been paid to the relationship between biochemical characteristics and filter height in the UBAF-based CANON process, which results in relatively aimless control and instable operation of the process.

Consequently, the overall goal of this study is to attempt to evaluate the biochemical characteristics along a volcanic-filled UBAF-CANON reactor. Here it is worth mentioning that fixed/moving carriers were known to be ideal biomass retention materials in CANON systems and it was reported that volcanic rocks showed more distinct biomass retention capacity than sponge or modified polyethylene carriers (Fu 2010). Consequently, we choose volcanic rocks as the supporting material in this study. The concentrations of N-compounds were detected to evaluate pollutant-removal efficiency along the filter. The community structure of the functional bacteria was investigated by using polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) techniques based on the diversity of amoA and 16S rDNA fragments, while the identification and phylogeny were performed by molecular clone methods. Besides, the biomass morphology and spatial distribution were observed using a scanning electron microscope (SEM) and fluorescent in situ hybridization (FISH) techniques in this study. The biochemical experimental results are expected to guide the process, improve removal efficiency, and further provide microbiological theoretical guidance for the popularization and application of the UBAF-based CANON process.

METHODS

Reactor and sample collection

A laboratory-scale UBAF-based CANON reactor made of polymethyl methacrylate that was filled with volcanic rock (4–6 mm in diameter and 80% porosity) was used in this study (Figure 1). The inner diameter, total volume and working volume were 150 mm, 8.15 L and 1.80 L, respectively. The reactor was operated in a high total nitrogen (TN) concentration (300 mg L\(^{-1}\)) at ambient temperature (16–20°C) with a DO and hydraulic retention time of around 0.5 mg L\(^{-1}\) and 1.0 h, respectively. The reactor was fed with synthetic wastewater, containing NaHCO\(_3\) (C source and buffer, approximately 1,200 mg L\(^{-1}\) of inorganic C), (NH\(_4\))\(_2\)SO\(_4\) (N source, 300 mg L\(^{-1}\) of NH\(_4\)\(^+\)-N) and KH\(_2\)PO\(_4\) (P source and buffer, 40 mg L\(^{-1}\) of PO\(_4\)\(^{3-}\)) together with a small amount of trace element solution. The wastewater and air was pumped continuously from the bottom of the reactor and output from the upper outlet (Figure 1).

Concentrations of NH\(_4\)\(^+\)-N, NO\(_2\)-N and NO\(_3\)-N in influent and effluent were measured according to standard methods (APHA 1995). The pH value, temperature and DO were all detected by the online multi-function instruments (WTW inoLabStirrOx). In this study, the reactor had been operated stably for a couple of months with steady nitrogen removal capacity: an ammonia removal rate of 83%, ammonia removal loading of 1.4 kg·(m\(^3\) d)\(^{-1}\), TN removal rate (NRR) of 75%, and TN removal loading (NRL) of 1.1 kg·(m\(^3\) d)\(^{-1}\). Except for those in the influent and effluent, N-compound concentrations were also measured at the four effluent points, with different heights of 100, 300, 500 and 700 mm, respectively, from bottom to top. Each measurement was repeated in triplicate. For molecular analysis, some pieces of volcanic carriers were collected at the four sampling points and stored at –20°C.

SEM

Some pieces of volcanic filler were collected randomly at 100, 300, 500 and 700 mm from bottom to top and fixed with 2.5% (V/V) glutaraldehyde for 1 h followed by dehydration in 50%, 70%, 90% and 95% (V/V) ethanol for 10 min per each step. Afterwards, the samples were steeped in hexamethyl disilazane (HMDS) twice for 10 min, air dried, and coated with...
gold. Morphology characterization was conducted on an SEM device (S-4300, Hitachi High-Tech., Co.).

**DNA extraction, PCR and DGGE**

Biomass was removed from the carriers and genomic DNA was extracted on the basis of the methods described previously (Liu et al. 2012). The biodiversity of the two types of functional bacteria, AerAOB and AnAOB, was evaluated by the PCR-DGGE technique. The primers and the relevant annealing temperatures for AerAOB and AnAOB are shown in Table 1. Primer pair amoA-1F and amoA-2R was used to amplify the amoA fragments of AerAOB. To amplify 16S rDNA of AnAOB, a nested PCR approach was adopted. Primer set 27F/1492R was used for the first round, followed by a second round using primer set Amx368f/Amx820r. Thermocycling was performed in a Takara PCR Thermal Cycler Dice, and PCR products were detected by 1.5% (w/V) agarose gel electrophoresis to confirm the product size. Afterwards, the products were purified with the TIANgel midi purification kit (Tiangen) according to the manufacturer's instructions.

DGGE was conducted at a constant temperature and a voltage of 60 °C and 120 V, respectively, for 5 h on a Dcode Universal Mutation Detection System (Bio-Rad). Polyacrylamide gel (8%) was used with a 30–60% gradient of urea-formamide denaturant. The loading amount in each lane was approximate 500 ng. After electrophoresis, the gel was stained using the silver-staining method followed by visualizing on a Gel Doc XR system (Bio-Rad). Specific gel bands were excised and stored in 1× TE buffer for the following cloning and sequencing.

**Cloning, sequencing and taxonomic identifications**

The dissolved DNA fragments were re-amplified using the primes without GC-clamp, purified according to the instructions of the TIANgel midi purification kit (Tiangen). The purified DNA was cloned by pMD19-T plasmid vector system (TaKaRa) and transferred into competent Escherichia coli DH5α. The positive colonies were chosen randomly and sequenced on an ABI 3730 DNA sequencer by a commercial service (Sangon). The sequences were assembled and trimmed prior to alignment by using ClustalW with 99% dereplication (MEGA software, version 5.05). All dereplicated DNA sequences obtained were deposited to the GenBank database with accession numbers of JN367453-JN367457 and JQ753318 related to AerAOB and AnAOB, respectively. Homology analyses were carried out by a basic local alignment search tool and phylogenetic trees were constructed based on the neighbor-joining (NJ) algorithm using the Kimura two-parameter model with a bootstrap value of 1,000 replicates.

**FISH analysis**

The spatial distribution of AerAOB and AnAOB was examined by FISH. Biofilm samples were dispersed to small clusters by ultrasonication for 10 min before fixing in a 4% (V/V) paraformaldehyde solution for 4 h and then suspended in a mixture of phosphate buffered saline and 100% ethanol (1:1, V/V). Hybridization was carried out in accordance with the previous report on an Olympus BX51 microscope (Amann et al. 1990). The oligonucleotide probe used in this experiment targeting AerAOB was NSO190 (5-CGATCCCCTGCTTTTCTCC-3) labeled with Cy3 dye while the probe targeting AnAOB was Amx368 (5-CCTTTCGGGCATTGCGAA-3) labeled with AMCA dye (Liu et al. 2016). Semi-quantitative analysis of the microorganisms was conducted by calculating the ratio of the colored area to the total biofilm area using CellSens Dimension software (version 1.6).

**Table 1** | Primers and annealing temperatures used for PCR-DGGE in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Annealing temperature</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>amoA-2R</td>
<td>CCCCTCGGAAAGGCTTCTTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27F</td>
<td>AGAGTTTGATCMTGGCTCAG</td>
<td>58</td>
<td>Total bacteria</td>
<td>Fan et al. (2008)</td>
</tr>
<tr>
<td>1492R</td>
<td>TACGGYTACCTTGTTACGACTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amx368f (GC-clamp)</td>
<td>CTTTTCCGGGATTTGCCGAA</td>
<td>52</td>
<td>AnAOB</td>
<td>Liu et al. (2012)</td>
</tr>
<tr>
<td>Amx820r</td>
<td>AAAACCCCTCTACTTAGTGC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*GC-clamp: CGCCCCCGCGCGCCGCCGGCCCAGGGCCGCGCGCGCCGGC.
RESULTS AND DISCUSSION

N-compounds concentrations along bio-filter

Concentrations of NH$_4^+$-N, NO$_2^-$-N, NO$_3^-$-N and TN along the bio-filter were measured and shown in Figure 2. It showed a relatively high nitrogen removal capacity with an NRR and NRL of 75% and 1.1 kg·(m$^3$·d$^{-1}$), respectively (Figure 2(a)), indicating a kind of balance between the two types of functional bacteria, AerAOB and AnAOB. However, the slope variation of the NRR line and NRL line in Figure 2(a) reveals a trend of decrease in the TN removal rate along the filter. At a filter height below 300 mm, there was a faster TN removal rate, while in the upper parts of the bio-filter it slowed down. This phenomenon suggested the N-removal bacteria in the lower part had better bioactivity.

For further perceptions of N-compounds’ variations along the filter, concentrations of NH$_4^+$-N, and NO$_2^-$-N, were tested (Figure 2(b) and 2(c)). Similarly to the TN concentration distinction, the NH$_4^+$-N concentration decreased sharply from 300 mg L$^{-1}$ to 100 mg L$^{-1}$ at filter heights below 300 mm, whereas it reduced smoothly from 100 mg L$^{-1}$ to 46 mg L$^{-1}$ in the upper parts. NO$_2^-$-N and NO$_3^-$-N were maintained at low levels in the CANON reactor, whereas the NO$_2^-$-N concentration tended to decrease while NO$_3^-$-N increased with filter height. According to the stoichiometric equation of the CANON process, the ratio of TN removal to nitrate production ($\Delta$TN/$\Delta$ NO$_3^-$-N) was around 8.0 (Sliekers et al. 2005). In this study, this ratio was 8.4, indicating relatively stable and complete nitrogen removal by the CANON process. It meant that NO$_3^-$-N generated in the reactor was attributed to the CANON process itself rather than the oxidation of NO$_2^-$-N by nitrite oxidizing bacteria (NOB), indicating the effective elimination of NOB. Consequently, little NO$_2^-$-N could be oxidized by NOB in this system. Nevertheless, the negligible NO$_2^-$-N accumulation along the filter demonstrated a relatively stable anaerobic ammonia oxidation (anammox) process in the reactor.

As a whole, the bioactivity of functional microorganisms at different filter layers displayed differently, and there was better pollutant removal activity in the lower parts of the UBAF. The most probable reason was that the sufficient substrate and air were introduced from the bottom of the filter.
and would be utilized first by the bacteria, which might be high concentration-favored microorganisms located subja-
cently. While in the upper parts, the substrate concentration decreased and the bacterial bioactivity reduced accordingly. Therefore, for better UBAF-based CANON operation, pumping substrate and air from the top of the filter at appropriate intervals might be an effective strategy. Moreover, some biomass in the upper parts might be run off with the effluent to some extent depending on the hydraulic force, which would result in an adverse impact for nitrogen removal. Consequently, it is suggested that a sludge retention device be set up at the outlet to recycle the effluent CANON biomass in a timely way.

Morphology and bacterial distribution along the filter

The biomass morphology along the filter was visualized by SEM (Figure 3). As was known, Nitrosomonas were considered to be the predominant AerAOB in sewage treatment plants with a short rod-shaped morphology, while AnAOB was reported as regular or irregular spherical and ellipsoidal, dispersed or in clusters (Liu et al. 2015). SEM images showed various morphologies of microorganisms in the system, of which short rod, spherical and ellipsoidal were predominant at a diameter of 0.2–1.0 μm, which might indicate the presence of AerAOB and AnAOB. Nevertheless, the biomass morphology released differently along the filter. Specifically, it showed compact and small zoogla with CANON-specific irregular cauliflower profiles mainly consisting of spherical shaped and short rod-shaped bacteria in the lower part (Figure 3(a)) while the zooglae trended to be larger and/or looser in the middle part (Figure 3(b) and 3(c)). At a height of 700 mm, there was a relatively smaller amount of microorganisms and few zooglae could be detected (Figure 3(d)). It was considered that since sufficient substrate and DO were first utilized by bacteria in the lower part of the UBAF, CANON-specific zoogla formed and ensured sufficient microorganisms for nitrogen removal. With the filter height increase, the concentration of TN and \( \text{NH}_4^+ - \text{N} \) dropped sharply (Figure 2), which might affect the biomass morphology. It could be predicted that the bigger and looser zooglae with smaller spacing in the middle part might decrease the zooglae specific surface area and further be adverse for substrate transferring between and/or inside zoogla, which might be disadvantageous for nitrogen removal. While in the upper part, fewer biomass might lead to worse pollutant-removal activity in the UBAF (Figure 2).

The spatial distribution along the filter was visualized by FISH (Figure 4). The previous study reported the acceptable bacterial distribution in a CANON system was that AerAOB were distributed abundantly in the outer layer while AnAOB existed in the inner part of biofilm (Nielsen et al. 2005).

![Figure 3](https://iwaponline.com/wst/article-pdf/74/11/2656/457343/wst074112656.pdf)
Nevertheless, the two types of functional bacteria (AerAOB and AnAOB) along the filter mixed with each other and showed no distinct niche. The explanations for this type of distribution in a one-stage autotrophic nitrogen removal systems have been given elsewhere (Schmid et al. 2000; Pynaert et al. 2005; Liu et al. 2016). Semi-quantitative analysis of AerAOB and AnAOB was conducted by calculating the ratio of colored area to total biofilm area. At the height of 100 mm, the abundance of AerAOB and AnAOB was (32.5 ± 3.2) %. While at the height of 300, 500 and 700 mm, this proportion decreased to (24.8 ± 2.8) %, (15.7 ± 2.4) % and (12.2 ± 1.5) %, respectively. Similar with SEM results, the population of AerAOB (red) and AnAOB (blue) was bigger in the lower part while it trended to drop along the filter height (the full color version of this figure is available in the online version of this paper, at http://dx.doi.org/10.2166/wst.2016.443). In the lower part, zoogae formed and little single cells existed; however, these two types of functional bacteria tended to cluster more loosely with the filter height increase. While at the height of 700 mm, single cells emerged and few zoogae could be detected. Considering the uneven distribution of microorganisms along the bio-filter, it was suggested to rearrange the filter at appropriate intervals during operation. However, this strategy should be used with reasonable caution because it might break the established air/substrate path in the system.

**Microbial community structure along the filter**

To evaluate the functional microbial community structure, DGGE profiles of AerAOB and AnAOB were released (Figure 5). Results indicated a relatively abundant...
biodiversity of AerAOB, whereas the fingerprints of AnAOB were simple (only two visible bands). The phylogenetic composition of AerAOB released an obvious shift along the filter, which was probably due to the varied N-compound concentrations along the filter. To be specific, the bands in lane 1 and lane 2 distributed similarly, while the profiles in lane 3 and lane 4 were homologous, indicating that the AerAOB structure was different from the lower part to the upper part. Lane 1 and lane 2 had more bands with darker color than those in lane 3 and lane 4, suggesting a more abundant biodiversity and bigger population of AerAOB in the lower part. This result was in accordance with the SEM and FISH results, which supported an earlier report where a decreasing NH4+-N concentration was regarded as a cause for the decrease in AerAOB biodiversity (Liu et al. 2012). The abundant biodiversity of AerAOB in the lower part might promise the reactor’s anti-shock capacity to some extent because different types of AerAOB usually have different metabolism habits (Purkhold et al. 2000). That means although the bioactivity of certain AerAOB might be depressed as the environment changes, other types of AerAOB that had got used to the new condition might enhance the bioactivity accordingly. Nevertheless, in the upper part, the lower AerAOB biodiversity might lead to a relatively weak anti-shock capacity. Therefore, if some appropriate strategies are adopted to enhance the biodiversity of AerAOB in the upper parts of a UBAF, the anti-shock capacity would improve correspondingly.

To identify the species of AerAOB, 5 visible AerAOB bands were excised and sequenced (GenBank No. JN367453-JN367457) with the taxonomic identification listed in Table 2. The phylogenetic tree of AerAOB was built based on the DNA sequences of 5 bands (Figure 6). *Nitrosomonas*, *Nitrospira*, *Nitrosococcus* and *Nitrosolobus*, known as four familiar AerAOB, were selected as exogenous ones. Results indicated that most bands had a close relationship with *Nitrosomonas*-like bacteria, indicating *Nitrosomonas* was the predominant type of AerAOB in this UBAF-based CANON system. This phenomenon was consistent with previous reports that *Nitrosomonas* was predominantly AerAOB in one-stage autotrophic nitrogen removal systems (Liu et al. 2008; Liu et al. 2016). However, band 3 and *Nitrosococcus* were in the same branch (with a similarity of 97%). It has been reported that *Nitrosococcus* can survive in high ammonia conditions (Duan et al. 2007). While in this study, the influent ammonia was around 500 mg L−1, providing suitable condition for *Nitrosococcus* survival. Notably, *Nitrosococcus* was found at filter heights of 500 and 700 mm, band 1 and band 2 related *Nitrosomonas* existed at heights of 100 and 300 mm, while band 4 and band 5 related *Nitrosomonas* inhabited along the whole bio-filter (Figure 6). The different distribution of the AerAOB phylotype might be mainly attributed to variations in N-components and hydraulic conditions along the layers (Figure 2). In other words, sufficient substrate and air were introduced from the bottom of the filter and would be utilized rapidly by the high concentration-favored *Nitrosomonas* (like band 1, 2, 4, 5 related AerAOB) located subjacentely. While in the upper parts, NH4+-N oxidation depended mainly on *Nitrosococcus* (band 3 related AerAOB), and the removal rate decreased sharply (Figure 2). This phenomenon illustrated that *Nitrosomonas*, rather than *Nitrosococcus*, was the valued contributor in this UBAF-based CANON system.

As a whole, these observable changes to DGGE patterns of AerAOB represented the impact of the filter height on the microbial structure. However, from the point of view of engineering, the extent to which the performance and stability of the UBAF-based CANON reactor could be unequivocally inferred from the results of this study is still not clear. Consequently, further study is still needed to improve the nitrogen removal efficiency based on the bacterial community variations along the filter.

<table>
<thead>
<tr>
<th>Band no.</th>
<th>Accession number</th>
<th>Closest phylotype</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>JN367453</td>
<td>Uncultured AerAOB (HQ142897)</td>
<td>99</td>
</tr>
<tr>
<td>2</td>
<td>JN367454</td>
<td>Uncultured AerAOB (HQ123432)</td>
<td>99</td>
</tr>
<tr>
<td>3</td>
<td>JN367455</td>
<td><em>Nitrosococcus</em> (AF047705)</td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>JN367456</td>
<td><em>Nitrosomonas europaea</em> (L08050)</td>
<td>97</td>
</tr>
<tr>
<td>5</td>
<td>JN367457</td>
<td><em>Nitrosomonas sp.</em> (AY958703)</td>
<td>96</td>
</tr>
<tr>
<td>6, 7</td>
<td>JQ753318</td>
<td><em>Candidatus Kuenenia stuttgartiensis</em> (AF375995)</td>
<td>98</td>
</tr>
</tbody>
</table>

As a whole, these observable changes to DGGE patterns of AerAOB represented the impact of the filter height on the microbial structure. However, from the point of view of engineering, the extent to which the performance and stability of the UBAF-based CANON reactor could be unequivocally inferred from the results of this study is still not clear. Consequently, further study is still needed to improve the nitrogen removal efficiency based on the bacterial community variations along the filter.
with spherical or ellipsoidal appearance (Egli et al. 2001), which was consistent with SEM results in Figure 3. Previous study showed only one genus of AnAOB was dominant in a certain habitat (Hu et al. 2010), and this viewpoint was in accordance with the result here. Candidatus Kuenenia stuttgartiensis had been reported as a kind of AnAOB that can tolerate relatively high salt concentrations (Hu et al. 2010), therefore, it could survive in this UBAF with an influent ammonia concentration of around 300 mg L$^{-1}$. 
CONCLUSION

In the UBAF-based CANON reactor, *Nitrosococcus* and *Nitrosomonas*-related AerAOB and *Candidatus Kuenenia* stuttgartiensis-like AnAOB were the predominant functional bacteria, which mixed with each other and showed no distinct niche in the system. However, the bioactivity of functional microorganisms in different filter layers displayed differently. There was better TN and NH$_4^+$-N removal in the lower parts than the upper parts of the UBAF. Filter height was also regarded as an important factor affecting the biomass morphology, spatial distribution and biodiversity. The biomass in the lower parts formed compact and small zoogla, whereas it tended to become larger and looser along the filter. Moreover, there was a better biodiversity of AerAOB in the lower part, while AnAOB showed stable and low biodiversity along the filter. The biochemical experimental results are expected to guide the process and further provide theoretical microbiological guidance for the application of the UBAF-based CANON process.

ACKNOWLEDGEMENTS

This research was financially supported by National Natural Science Foundation of China (Grant No. 51408095), China Post-doctoral Science Foundation (Grant No. 2014M561236, Grant No. 2015T80257), Foundation of Liaoning Educational Committee (Grant No. L2014021) and the Fundamental Research Funds for the Central Universities (Grant No. DUT16RC(4)13).

REFERENCES

Aman, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. & Stahl, D. A. 1990 Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology* 56 (6), 1919–1925.


First received 6 May 2016; accepted in revised form 2 September 2016. Available online 19 September 2016