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Biohydrogen facilitated denitrification at biocathode in bioelectrochemical system (BES)

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HIGHLIGHTS

- Enhanced denitrification at the biohydrogen facilitated biocathode was conducted.
- Activity of nitrate reductase was improved for the biohydrogen facilitated group.
- Electrochemical performances were bettered for the biohydrogen facilitated group.
- Shift of bacterial communities between the two biocathode groups was confirmed.

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ABSTRACT

Reductive removal of nitrate in bioelectrochemical system (BES) at abiotic cathode, biocathode and biohydrogen facilitated biocathode were investigated. It was found that nitrate removal efficiency reached 95% and 59% at the biohydrogen facilitated biocathode and biocathode respectively, while which was only 13% at the abiotic cathode. Meanwhile, activity of nitrate reductase reached 0.701 g-N/L h for the biohydrogen facilitated group, which was about 9.3 times of the biocathode group. Moreover, electrochemical performances as power density, ohmic resistance, and polarization resistance of the biohydrogen facilitated group reached 76.96 mW/m^3, 8.63 ohm and 383 ohm, respectively, which were better than two other groups. Finally, an obvious shift of bacterial community responsible for the enhanced nitrate reduction between the two biocathode groups was observed. Therefore, nitrate reduction in BES could be enhanced at the biocathode than that of the abiotic cathode, and then be further boosted with the combination of biohydrogen.

1. Introduction

On account of the unlimited discharge of industrial wastes, septic systems, and the massive use of both pesticides and fertilizers, accumulated nitrogenous compounds in the environment would lead to the increasing waterbody eutrophication and human health problems (Nolan et al., 2002). To date, numerous treatment methods have been put forward for the control of nitrate contamination, such as ion exchange (IE), reverse osmosis (RO), electrodialysis (ED) and biological processes (Lee et al., 2006; Samatya et al., 2006; Choi and Batchelor, 2008; Ghafari et al., 2008). However, more sustainable approaches are expected for the efficient removal of nitrate in wastewater, due to the high cost and secondary pollution of traditional treatment processes.

In terms of its environmental compatibility, versatility and low sludge yields, bioelectrochemical system (BES) is now widely considered as a competitive option for the reductive removal of recalcitrant pollutants against conventional processes (Sun et al., 2013). In addition to the nitrate reduction at abiotic cathode, biocathode inoculated with either autotrophic or heterotrophic denitrifiers could also be adopted for the reductive removal of nitrate in BES. However, nitrate removal through heterotrophic denitrification would be more costly than that of the autotrophic denitrification, as heterotrophic denitrifiers could not work nitrate without external supply of organics (Puig et al., 2008). Thus, to conduct the autotrophic denitrification at biocathode would be more attractive for the nitrate reductive removal.

Limited by the size and infrastructure of BES reactor, electron productivity and conductivity would also depend on the capacity of electron transfer between microorganisms and electrodes (Patil et al., 2012). Theoretically, the addition of external electron donor would help improve the nitrate reduction. Regardless of its

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poor solubility in catholytes, hydrogen would be more competent than any other reductive compounds for electron supply, as which would not generate any by-product but only water (Huang et al., 2013; Li et al., 2013). Comparing with the denitrification by external hydrogen supply for electrons supply, simultaneous biohydrogen at biocathode might fundamentally facilitate the autotrophic denitrification with the lowest cost, as which were cost-efficient and renewable (Lee and Rittmann, 2002; Lin et al., 2014). Moreover, to figure out the succession of microbial communities in BES through molecular technique, namely the coexisting or competing between denitrifiers and hydrogen producers at the biocathode, would be indicative to further explore the microbe–electrode–interaction based microbial BES (Patil et al., 2012). To date, few publications could be tracked about this field yet.

In this study, reductive denitrification at abiotic cathode, biocathode and the biohydrogen facilitated biocathode in BES were investigated. In addition with the activities of nitrate reductase and hydrogenase, electrochemical performances such as polarization curve and electrochemical impedance spectroscopy (EIS), the shift of bacterial community at the biocathode were determined as well, in order to further understand the biohydrogen facilitated denitrification at biocathode.

2. Methods

2.1. Enrichment of biohydrogen and denitrification inocula

Prior to inoculating for biohydrogen production, the anaerobic granular sludge was autoclaved at 121 °C for 15 min to eliminate non-spore-harboring methanogens, with other conditions for the same as described previously (Lin et al., 2014). Ingredients of the media for biohydrogen fermentation contained (mg/L): glucose 1500, NH_4HCO_3 1048, $NaHCO_3$ 1344, $NaH_2PO_4{\cdot}2H_2O$ 2000, $K_2HPO_4{\cdot}3H_2O$ 2000, $FeSO_4{\cdot}7H_2O$ 50, $MgCl_2{\cdot}6H_2O$ 100, $MnSO_4{\cdot}H_2O$ 15, $NiCl_2{\cdot}6H_2O$ 1, $ZnCl_2$ 0.5, finally the pH were adjusted to 6.5. Enrichment of autotrophic denitrifiers was conducted in an anaerobic reactor with the working volume of 3.0 L. Except for the initial 130 mg/L of nitrate in this study, other constituents of the enrichment media for autotrophic denitrifiers consisted of M9 medium (g/L: KH_2PO_4 4.4, K_2HPO_4 3.4, $NaHCO_3$ 2.0, NaCl 0.15, $MgSO_4{\cdot}7H_2O$ 0.15, $CaCl_2$ 0.015) and trace element as reference (Rabaey et al., 2005). After 25% v/v of anaerobic sludge (Wuxi Genencor Bioproducts Plant, China) was inoculated, the reactor was kept at room temperature with intermittent stirring at 100 rpm for about 30 days. Once the effluent nitrate was reduced to about 10.0 mg/L, it indicated that the autotrophic denitrification bacteria were successfully enriched for BES inoculating.

2.2. BES reactor setup

Assembled by two polycarbonate chambers (16 cm in external diameter, 14 cm in height) and then separated by cationic exchange membrane (CEM, Yuejin CMI-7000s, China), the BES reactor adopted in this study had a working volume of approximately 0.55 L of each chamber. Anode and cathode electrode were made of graphite fiber brushes (6 cm in diameter, 12 cm in length, TOHO, Japan). Moreover, electrodes were connected via a copper wire with a 100 Ω high-precision resistor in between.

2.3. BES reactor operation

Firstly, anode chambers of three batches of BES reactors were inoculated with anaerobic sludge obtained from Wuxi Genencor Bioproducts Plant, then the cathode chambers were kept abiotically and aeratedly until the anode potential across electrodes poised at −0.55 V. After about 130 mg/L of nitrate were added into the cathode chambers, three BES reactors then were operated as abiotic group (with cathode chamber uninoculated), biocathode group (cathode chamber inoculated only with autotrophic denitrifiers), and biohydrogen facilitated group (cathode chamber inoculated with autotrophic denitrifiers and hydrogen producers at 0 and 48th hour, respectively)

As for other ingredients of the catholytes, cathode chamber was fed with only 100 mM PBS as pH buffer for abiotic group. In addition to PBS, the catholyte for the biocathode group also consisted of few minerals and trace elements as described in Zhang et al. (2009b), together with a quarter of denitrifying bacteria solution (v/v) containing about 10 g of sludge. For biohydrogen facilitated group, 15 mL of biohydrogen solution containing about 0.1 g of sludge was added into the biocathode chamber every 48 h. In addition, TS/VSS of the sludge inoculated for denitrification and hydrogen sludge were 0.081/0.039 g and 0.057/0.029 g, respectively.

2.4. Analytic methods

TS and VS were determined with the gravimetric method according to the standard protocol of State Environmental Protection Administration of China (SEPA, 2002). BES reactors were sampled every 48 h, nitrate (NO_3^--N) and nitrite (NO_2^--N) were also determined according to the protocol of SEPA (2002).

2.5. Nitrate reductase

After being centrifuged and then ultrasonicated for 5 min and 20 min, 2 mL of the crude enzyme was mixed with 1 mL of 0.1 M KNO_3 and 2 $mg{\cdot}mL^{-1}$ of fresh NADH successively. Secondly, the mixture was added with 3 mL of 1% sulfanilamide and 3 mL of 0.05% n-(1-naphthyl)-ethylenediaminedihydrochloride (NPED), and was then kept at 30 °C for 30 min. Finally, nitrite was determined spectrophotometrically at 420 nm (UV-2100, Unico, China). Activity of nitrate reductase in this study was defined as the amount of enzyme which could reduce 1 g/L of nitrate per hour (Iwamoto et al., 2001).

2.6. Hydrogenase

Hydrogenase was determined according to methylviologen (MV) method (Riberio et al., 2011). Firstly, 20 mL of the sample with 10 mL of 0.1 M PBS including 10 mM dithiothreitol were disrupted by sonifier (JY99-II DN, Scientz, China) for 20 min. Then the liquid was cultured using MV substrate to render the biohydrogen for 5 min at room temperature. Finally, the hydrogen gas was determined using gas chromatography (GC9790, Fuli, China) as described before (Lin et al., 2014). Activity of hydrogenase in this study was defined as the amount of enzyme that catalyzes the production of 1 L of H_2 per hour (Riberio et al., 2011).

2.7. Electrochemical performances

Polarization curves were obtained by measuring the stable voltage generated at various external resistances ranging from 33,000 Ω to 100 Ω, and the voltage readings of each point on the polarization curves were conducted with a multimeter when the voltage stabilized (Logan et al., 2006). Current (I) and power (P) were calculated as previously described (Zhang et al., 2009a). Next, volumetric current density and power density were normalized with the 0.55 L of net cathode compartment (NCC). Coulombic efficiency (CE) for nitrate reduction was then calculated as the ratio of current flowing across the BES reactors and theoretical current estimated according to the oxidized nitrogen compounds removed at the cathodes (Virdis et al., 2008).

Meanwhile, electrochemical impedance spectroscopy (EIS) analysis was measured by the electrochemical workstation (CHI660D, Chenhua, China) with a two electrode mode. That is, the cathode was set as a working electrode, and the anode electrode was set as counter and reference electrode. The frequency ranged from 10^5 to 10^{-2} Hz with the amplitude of 5 mV. To characterize the specific resistance, the EIS curves were fitted and resolved with ZSimpWin 3.20d (Princeton Applied Research, USA).

2.8. PCR-DGGE based microbial communities analysis

DNA was collected and extracted following the instruction of Powersoil DNA isolation kit (Mobio, USA). Subsequently, about 230 bp of fragments from the V-3 region of 16S rDNA were amplified using the primer set GC-357f (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3′) and 518r (5′-ATT ACC GCG GCT GCT GG-3′) (Muyzer et al., 1993), respectively. After that, the amplification, PCR-DGGE and the sequencing of bands of interest were the same as reported previously (Lin et al., 2014).

3. Results and discussion

3.1. Enhanced nitrate reduction at biohydrogen facilitated biocathode in BES

In this study, simultaneous biohydrogen was firstly adopted to improve the denitrification efficiency at biocathode. As indicated in Fig. 1A, initial 130 mg/L of nitrate was reduced to 120.1, 54.1 and 6.4 mg/L after 16 days of operating, resulted in the removal efficiency of 13%, 59% and 95% for the abiotic, biocathode and biohydrogen facilitated groups, respectively. Denoted by slopes of the three fitted lines, it was discovered that the constants of nitrate degradation reached 1.29, 4.70 and 7.67 for the abiotic, biocathode and biohydrogen facilitated groups, respectively (Fig. 1A). Moreover, as the direct product from nitrate reduction, nitrite concentration increased sharply to the maximal 10.75 mg/L at the 4th day in the case of biohydrogen facilitated group, while which reached only 8.74 and 0.45 mg/L for the biocathode and abiotic groups at the end of the reductive process, respectively. Therefore, nitrate removal could be enhanced after the introduction of autotrophic denitrifiers at biocathode, and then be further bettered with the combination of hydrogen producers.

Responsible for the hydrogen accumulation and nitrate reduction, activities of both hydrogenase and nitrate reductase were found to be increased gradually to 0.045 L-H_2/L h and 0.701 g-N/L h for the biohydrogen facilitated group at the 8th day during the nitrate removal process, respectively (Fig. 1B). Remarkably, activity of the nitrate reductase of the biohydrogen facilitated group was 9.3 times higher than that of the biocathode group, as which reached only 0.075 g-N/L h. As a kind of key enzyme participated in the reversible oxidation of molecular hydrogen, hydrogenase could be involved in the electron transmission (ET) as redox mediator for nitrate reduction with accumulated H_2 as electron donor (Vignais et al., 2001). Therefore, enhanced nitrate removal could be achieved at the biohydrogen facilitated biocathode.

3.2. Electrochemical performances during the denitrification process in BES

To investigate the effect of biohydrogen on power generation in BES, polarization data of the three groups were measured with

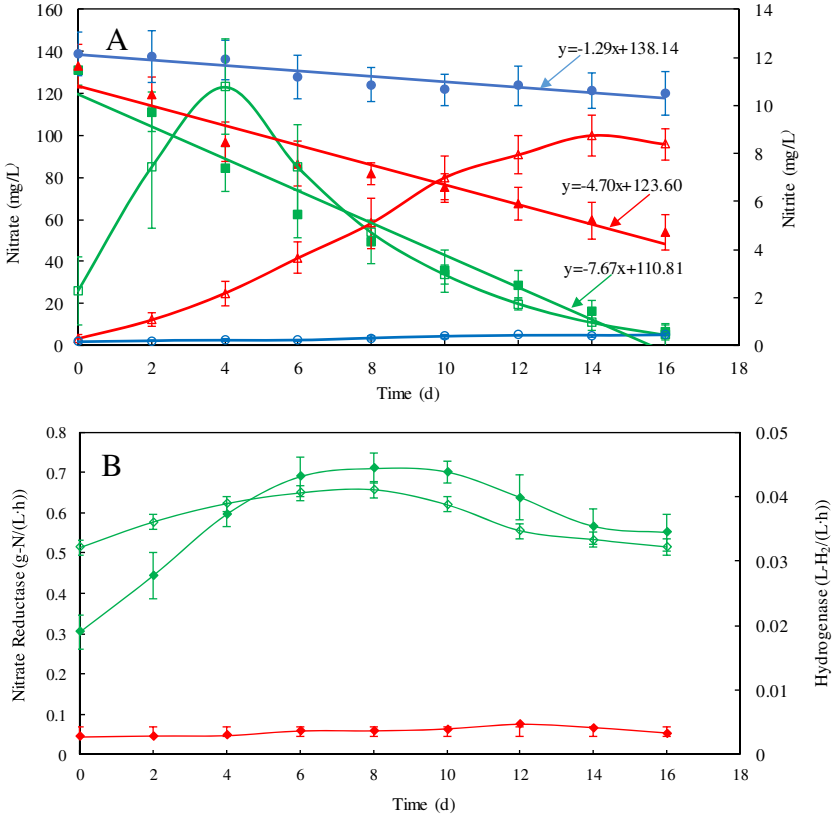

Fig. 1. Nitrate reductive removal at the abiotic, biocathode and biohydrogen facilitated biocathode in BES: (A) nitrate (■) and nitrite (□) concentration for biohydrogen facilitated group; nitrate (▲) and nitrite (△) concentration for biocathode group; nitrate (●) and nitrite (○) concentration for abiotic group. (B) Activities of nitrate reductase (◆) and hydrogenase (◇) for biohydrogen facilitated group; activities of nitrate reductase for biocathode group (◆).

various external resistances (Fig. 2A). It was found that power density of biohydrogen facilitated group reached the maximum of 76.96 mW/m^3, while which were only 66.40 mW/m^3 and 49.56 mW/m^3 for the biocathode and abiotic groups, respectively. Also, the biohydrogen facilitated group could achieve higher cell voltage than the other two groups at the same condition (Fig. 2A). Furthermore, CE reached 81.5 ± 0.4% with 100 Ω resistor throughout the operation at the biohydrogen facilitated cathode, which was higher than those of the biocathode (69.2 ± 0.6%) and abiotic cathode (18.4 ± 0.3%). As current density could be significantly improved when biocathode was adopted for the reductive removal of refractory pollutants in BES (Zhang et al., 2010), power generation in this study was apparently further increased with the combination of hydrogen producers at biocathode.

According to the EIS analysis, it was observed that both ohmic resistance (*R*s) and polarization resistance (*R*p) of the abiotic group were evidently higher than those of the two biocathode groups (Fig. 2B). Especially, *R*s of the abiotic group reached 104.9 ohm, while which were only 17.97 ohm and 8.63 ohm for the biocathode and biohydrogen facilitated groups, respectively. Noticeably, the decrease of ohmic resistance at biocathode was in accordance with the increasing of power density as shown in Fig. 2A. Other than that, *R*p of the abiotic cathode group reached 1501 ohm, while which were only 507 ohm and 383 ohm for the biocathode and biohydrogen facilitated group, respectively. Consequently, mass transfer resistance from the electrodes to catholytes within the biocathode chambers could be sharply decreased with the development of biofilm onto the biocathode (Sun et al., 2013), then the electron flux between electron acceptors and donors was fastened, and finally facilitated the nitrate reduction at biocathode (Ter et al., 2011).

3.3. Shift of bacterial communities between two biocathode groups

According to the band migration and intensity (Fig. 3), it was investigated that the PCR-DGGE band pattern of the biocathode group was different from that of the biohydrogen facilitated group, which indicated an obvious community shift after the introduction of hydrogen producers. Comparing to those of the biocathode group, intensity of bands numbered 1, 3, 4 kept almost unchanged, while bands numbered 2, 5, 7 became stronger, and the bands numbered 6, 8, 9 faded for the biohydrogen facilitated group. Due to the BLAST (Basic Local Alignment Search Tool) results from GenBank database, it was found that the nine retrieved sequences could be attributed to four different bacterial phyla as *Bacterioidetes*, *Proteobacteria*. *Firmicutes*, and *Actinobacteria* (Table 1). Significantly, *Firmicutes*, *Proteobacteria* and *Bacteroidetes* harbored all the four nitrate reductive genes as *narG*, *nir*, *nosZ*, and *nor* responsible for nitrate, nitrite, NO, and N_2O reduction (Zumft, 1997), respectively. However, *Actinobacteria* harbored only nitrate reductase encoding gene *narG*, which was involved in a severely truncated denitrification pathway (Shapleigh, 2013).

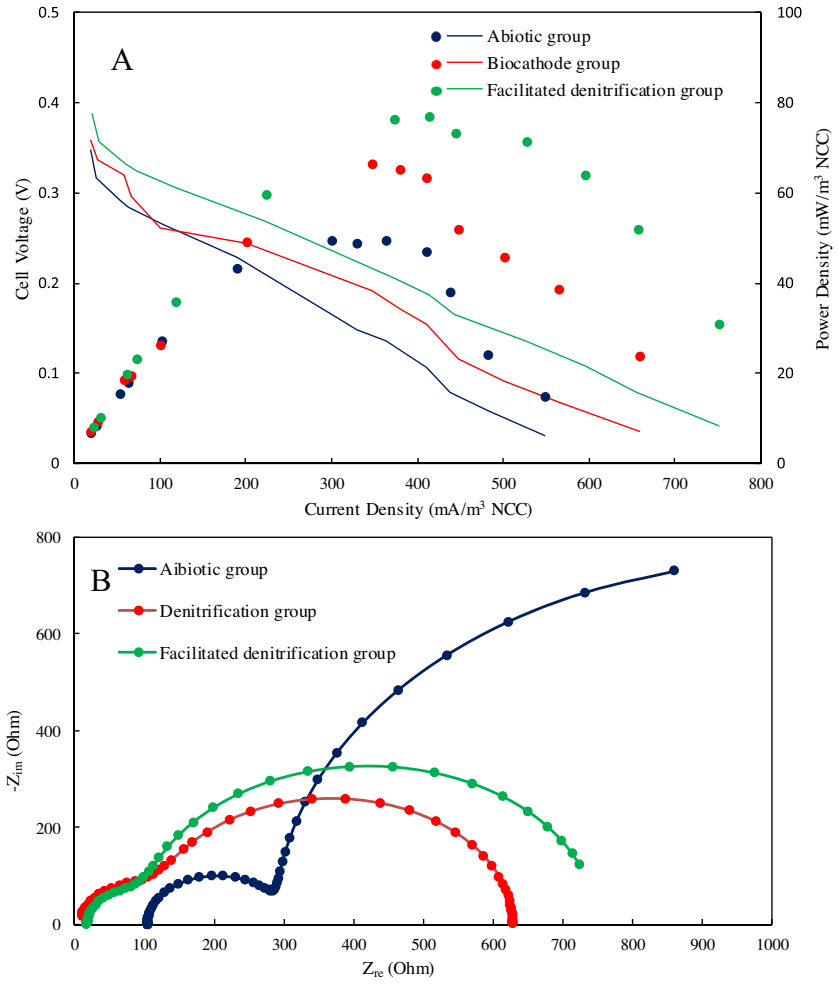

Fig. 2. Electrochemical performances of the three nitrate removal groups. (A) Polarization curves (circles for the power density and lines for the cell voltage); (B) electrochemical impedance spectroscopy (EIS).

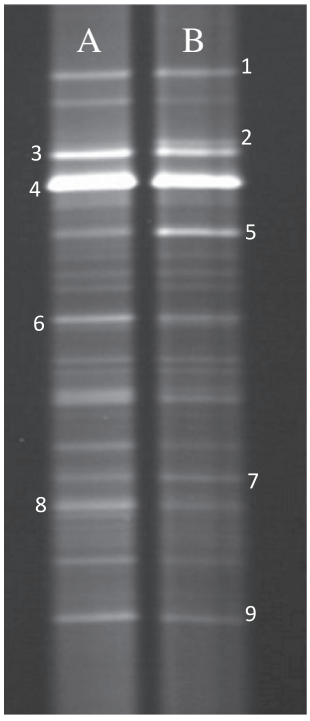

Fig. 3. Difference of bacterial DGGE pattern between biocathode and biohydrogen facilitated group.

Of the three enriched species during the biohydrogen facilitated group, it could be seen that only band 2 appeared with the addition of biohydrogen solution, while the other two were originally presented within the bacterial communities for denitrification. Especially, *Thauera terpenica*, the closest relative to band 5 with the similarity of 100%, has already been reported as a strong denitrifier, as which could reduce about 60–70 mg/L of nitrate to dinitrogen (Foss and Harder, 1998). Besides, the most close relative to band 2 and 7 were identified as hydrogen producer (*Clostridium amylolyticum*) and hydrogen consumer (*Simplicispira psychrophila*), respectively (Song and Dong, 2008; Wen et al., 1999). Belonged to the genus *Hydrogenophaga*, *S. psychrophila* could be capable of the chemolithotrophic growth with hydrogen for nitrate reduction (Wen et al., 1999). For the biohydrogen facilitated group, nitrate reduction would be enhanced with the growth strength of denitrifiers and electron shuttles, after the addition of biohydrogen solution.

Of the three faded bands in the biohydrogen facilitated group, band 9 was identified most close to *Saccharothrix espanaensis* ascribed to *Actinobacteria* phylum. Encoding only nitrate reductase responsible for an incomplete denitrification pathway (Shapleigh, 2013), *Actinobacteria* might not compete with three other predominant bacteria phyla within the biohydrogen facilitated group, as which could achieve higher nitrate removal through entire nitrate reduction pathway (Shapleigh, 2013; Zumft, 1997). However, more electrochemical and genetic evidences should be further discovered, in order to better understand the enhanced denitrification at biocathode through biohydrogen.

4. Conclusions

Nitrate reductive removal in BES could be enhanced at the biocathode with enriched denitrifiers, and then be further promoted with the combination of hydrogen producers. Other than the activity of nitrate reductase, electrochemical performances as both polarization curve and EIS profiles of the biohydrogen facilitated group could all be bettered than two other groups. Noticeably, an obvious shift of bacterial community for the biohydrogen facilitated group was investigated. Next, detailed mechanism for electron transfer between donors, shuttles and acceptors should be further demonstrated, in order to better understand the biohydrogen enhanced nitrate removal at biocathode in BES.

Acknowledgements

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Table 1
Sequencing affiliation and identifications of excised bands.

Band number	Band shift*	Closest relatives (sequence similarity%)	Accession number	Phylogenetic affiliation (phylum)
1	U	*Sediminibacterium salmoneum* (95%)	NR_044197.1	*Bacteroidetes*
2	S	*Clostridium amylolyticum* (100%)	NR_044386.1	*Firmicutes*
3	U	*Nonlabens agnitus* (89%)	NR_117858.1	*Bacteroidetes*
4	U	*Bacteroides stercorirosoris* (92%)	NR_043154.1	*Bacteroidetes*
5	S	*Thauera terpenica* (100%)	NR_025284.1	*Proteobacteria*
6	F	*Nonlabens agnitus* (90%)	NR_117858.1	*Bacteroidetes*
7	S	*Simplicispira psychrophila* (99%)	NR_028712.1	*Proteobacteria*
8	F	*Arenimonas donghaensis* (95%)	NR_043790.1	*Proteobacteria*
9	F	*Saccharothrix espanaensis* (97%)	NR_102474.1	*Actinobacteria*

* U-Unchanged, S-Stronger, F-Faded.

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