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Insight into the microbial community and its succession of a coupling anaerobic-aerobic biofilm on semi-suspended bio-carriers

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ABSTRACT

This work aims at establishing a coupling anaerobic-aerobic biofilm within a single bioreactor and revealing its microbial community and succession. By using a semi-suspended bio-carrier fabricated with 3D printing technique, an obvious DO gradient was gradually created within the biofilm, which demonstrated that a coupling anaerobic-aerobic biofilm was successfully established on the surface of bio-carriers. The results of metagenomic analysis revealed that the microbial community on the bio-carriers experienced a continuous succession in its structure and dominant species along with the operational time. The formed coupling biofilm created suitable micro multi-habitats for the co-existence of these microorganisms, including nitrifying and denitrifying bacteria, which were beneficial to the removing of organic pollutants and converting nutrients. Along with the succession, the microbial community was gradually dominated by several functional microorganisms. Overall, the results presented an approach to improve the microbial biodiversity by constructing a new structure and floating status of bio-carriers.

1. Introduction

Biofilm is a self-aggregated microbial community, which contains lots of functional microorganisms, and is capable of degrading organic pollutants and converting nutrients. Comparing with a conventional activated sludge (CAS) process, a biofilm process has many advantages, including higher biological volumetric conversion rate, greater tolerance to shock loads and toxins, higher biomass density, and less production of excess sludge, thus, biofilm processes have been considered as a modified method for CAS in some wastewater treatment plants (WWTPs) (Luostarinen et al., 2006). In biofilm, large amounts of microorganisms are immobilized, which constitute a stable and robust microbial ecosystem with a special community structure (Lu et al., 2014). The formed biofilm can extend the sludge retention time (SRT) for promoting the growth of those microorganisms having a long generation cycle, and on the other hand, the aged and detached biofilm may be preyed on by protozoa, metazoan and oligochaeta (Derlon et al., 2013; Hendrickx et al., 2011; Li et al., 2013), which can largely limit the production of excess sludge.

In terms of a microbial ecosystem, a common viewpoint has been widely accepted, that is, not an individual microbial species, but a whole system, actually realizes the function of degrading organic pollutants and converting nutrients. Generally, higher biodiversity of an ecosystem, greater stability and more effective performance will be realized (Torresi et al., 2016), therefore, necessary measures should be taken for promoting the biodiversity of an artificial ecosystem (such as a bioreactor) according to this principle. In an actual ecosystem, these environmental conditions, including nutrients, dissolved oxygen (DO), growing space, and the availability of removing metabolites is essential and prerequisite (Tang et al., 2014). However, to each biofilm process, apart from necessary nutrients, the growing space may be the second important factor for the microorganisms to survive. In this meaning, a reasonably constructed bio-carrier is of primary importance for its providing the growing space for most suspended microorganisms to attach on to form a layer of biofilm, and to grow to a mature microbial ecosystem. Only with an integrated microbial community with lots of functional microorganisms, can a biofilm reactor achieve the environmental function of decomposing organic pollutants and converting nutrients (Mohanty et al., 2016; Tang et al., 2016). Traditional bio-carriers are generally fixed in bioreactors, and have long been used in the engineering fields for treating both domestic and industrial wastewater. Their merits have been verified by so many successfully operating engineering projects, but the drawbacks are also very obvious, including the clogging of bioreactor and low mass transfer efficiency. In recent decade, a new kind of bio-carrier, called suspended bio-carrier, was invented and widely studied (Barwal and Chaudhary, 2014). With

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this kind of bio-carrier, a moving bed biofilm reactor (MBBR) was constituted, which had attracted lots of interests in the fields of theoretical investigation and engineering applications (Nogueira et al., 2015; Young et al., 2016). Comparing with traditional fixed bio-carriers, suspended bio-carriers can move freely in a biofilm reactor, which totally overcome the shortcoming of clogging, and obviously improve the mass transferring within a biofilm reactor. However, due to the abrasion among bio-carriers caused by drastic hydrodynamic conditions, the formation of biofilm on suspended bio-carriers is very difficult, which generally prolongs the start-up of an MBBR.

In a bio-process for treating both industrial and domestic waste water, a combining anaerobic-aerobic condition is needed for totally converting N- and P- containing substances. Based on such a mechanism, an alternative aerobic and anaerobic condition in time or space has been commonly adopted in practical engineering fields, which has been used to develop many practical bio-processes, including so-called the Anoxic/Oxic (A/O) process, Anaerobic-Anoxic-Oxic (A^2/O) process, and Sequencing Batch Reactor (SBR), and they have been successfully used in numerous engineering projects. However, in a previous investigation (Tang et al., 2014), an interesting phenomenon was found, namely, a coupling aerobic-anaerobic environment could be formed in a single bioreactor by utilizing the DO gradient created by mass transferring resistance. The formed coupling conditions composed a multi-habitat environment and greatly promoted the biodiversity within the bioreactor, which enhanced the efficiency of pollutants removal.

Comprehensively considering both the advantages and disadvantages of the traditional fixed and the suspended bio-carrier, a novel semi-suspended bio-carrier was designed in the authors' laboratory and fabricated with 3D printing technique (Tang et al., 2017). This bio-carrier was designed to be a spindle shape to reduce hydraulic resistance, whose smaller end was fixed and the larger end could move freely in a biofilm reactor. With such a configuration, the bio-carrier can be evenly installed in the bioreactor and avoid stacking in the dead zone; on the other hand, it is fixed on one end and the other end can move freely in water, which greatly improves the mass transferring and avoids of collusion to stimulate the formation of biofilm on the surface, therefore, all of the drawbacks of both the traditional fixed and suspended bio-carriers are expected to be totally overcome, and more importantly, an obvious and stable DO gradient was found to form within the biofilm on the bio-carrier (Tang et al., 2017), which might be a new approach to improve the biodiversity of a bioreactor. To the best of our knowledge, arranging bio-carriers in a semi-suspended status is a totally new idea, on which, the growing pattern of biofilm and the contained microbial communities are quite different from that on the traditional fixed or suspended bio-carriers, and may have a novel effect on the microbial community of the related bioreactor. In this regard, it is very essential to have a full understanding of the microbial community on this novel bio-carrier and further reveal its succession along with time. For this purpose, the present investigation carried out an experiment that continuously operated for 100 days in a biofilm reactor packed with this new kind of semi-suspended bio-carrier, which aimed at revealing the composition of the microbial community and its succession on this novel bio-carrier.

2. Methods and materials

2.1. Start-up of the experiment and the basic operational parameters

The used semi-suspended bio-carrier was fabricated by 3D printing technique, whose shape and structure details are shown in the Supporting Information (SI). After inoculating the original seed sludge (1500 mg/L MLSS) from the secondary sedimentation tank of a local WWTP (Lijiao municipal wastewater treatment plant, located in Haizhu district, Guangzhou, China), a rectangular bioreactor packed with these novel bio-carriers was started to carry out all experiments. Other experimental details are described in SI.

2.2. Measurement of the water quality indexes

Water samples were taken from the bioreactor and measured for the concentration of each water index in both the influent and effluent of every day. Regular water quality indexes, including COD, TN and TP, were chosen as the parameters to evaluate the performance of the bioreactor, and NH_3-N, NO_2^--N, and NO_3^--N were also measured simultaneously to evaluate their variation in the bioreactor. All of these water quality indexes were analyzed according to the standard methods (APHA et al., 2005), and they were measured in triplicate with the average value as the final result.

In the present experiment, two DO probes were installed in zone "A" and "B" separately, and the DO value in these two zones was measured simultaneously every 10 s by the connected DO meters. The DO profile within the biofilm was measured by a microelectrode system (including an oxygen probe (Unisense, OX25, Denmark) and a three-dimensional microelectrode propeller (Unisense, MM33-Z8140, Denmark)), whose stepping accuracy was 10 μm. All the measured DO data were transmitted to a connected computer and processed automatically with the average value as the final result.

2.3. Collection of biofilm samples

All the biofilm samples for analyzing the microbial community were collected at the middle line of each semi-suspended bio-carrier. To reveal the succession of the microbial community during the operational period, the biofilm samples were taken simultaneously from the same position of zone "B" and "A" after the reactor being operated for 19, 26, 36, 47, and 61 days, respectively. For making a comparison, the inoculated sludge was also sampled and labeled as "S0", which represented the original community in the bioreactor, and the other samples from different time points, representing the microbial community at different succession stages, which were marked as S19B/A, S26B/A, S36B/A, S47B/A, and S61B/A (the number represented for the day of sampling, and "A" and "B" stood for the corresponding zone that collected samples from), respectively.

2.4. DNA extraction

The biofilm samples collected from both zones at different date were cut into pieces with a sterilized cutter and totally mixed. The total DNA was extracted by using E.Z.N.A.™ Soil DNA Kit (Omega, Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. DNA samples were conducted a quantitative analysis by Qubit double-stranded DNA High Speed Assay Kit with the Qubit 2.0 fluorometer. And then, the extracted DNA was amplified using the bacterial specific primers forward primer Nobar_341F and reverse primer Miseq_805R annealing to the V3–V4 region of the 16S bacterial gene. Two polymerized chain reactions (called PCR) were performed by using Master Mix (Genebase, China). The PCR protocol was illustrated as follows: 94 °C for 5 min followed by 25 cycles (denaturing at 94 °C for 30 s, annealing at 55 °C for 20 s, extension at 72 °C for 30 s) and a final extension step at 72 °C for 8 min (Pitta et al., 2016).

Based on the requirement of DNA sequencing analysis, the quantification PCR products with balanced mixture was utilized by using a sequencing analysis again. Due to the relationship between the paired-end reads and overlap, DNA subsequences were spliced coupled reads, separated by a barcode and filtrated with quality control. Finally, 16S rRNA gene, the purified products were sent for sequencing by using the Miseq platform (Miseq, Illumina Inc, USA). The software preprocessing and information of sequences (PRINSEQ) were used for parsing and processing the acquired information after sequencing.

2.5. Sequence analysis

By citing the method described in a previous publication (Douterelo et al., 2013), the MOTHUR software (http://www.mothur.org) was used to trim the barcode and primer sequences and eliminate the sequences shorter than 200 bp for optimizing the original sequences obtained from the sequencing analysis, after that, the obtained data were clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity by using the "cluster" command of Mothur software. The sequence of each OTU was classified by matching in the corresponding taxonomic ranks using the software Ribosomal Database Project classifier (RDP) (Shu et al., 2015). Based on the obtained OTU table, both alpha and beta diversity was conducted and used as the comprehensive analysis indicator.

3. Results and discussion

3.1. DO profile in the bioreactor and the biofilm

DO value is a limiting factor that determines the performance of a bioreactor and the distribution of microbial species. As shown in SI, the removal of organic pollutants and nutrients keeps a relatively high level except two declines during the operation period. The simultaneous removal of organic pollutants and nutrients implies a coupling anaerobic-aerobic biofilm has gradually formed, which is actually determined by the DO profile within the bioreactor, therefore, the DO profile is necessary to be detected systematically. The DO profile in the bioreactor was obtained by averaging the daily value of the measured DO data, and within the biofilm, it was plotted according to the results measured by a microelectrode system on the representative date. All the results are shown in Fig. 1.

In Fig. 1(a), with a lower aeration rate in zone "A", the DO concentration kept a lower value during the whole experimental period, and it decreased almost linearly from the beginning to the end of the experiment. While, in zone "B", a relative higher aeration rate was provided, which obviously increased the DO value and kept it stable in this zone before changing the operating mode. However, the changing of operational mode influenced the DO value in zone "B", after that, the DO value gradually recovered to its original level.

Unlike in the bulk solution, diffusion is a main pattern for the mass transferring of DO in the biofilm, but it is heavily influenced by the formed biofilm. However, due to the driving force of mass transferring is mainly caused by the gradient of DO value, the DO concentration in the bulk solution also influences the mass transfer within the biofilm. As shown in Fig. 1(b) and (c), the DO value declined very quickly within the biofilm no matter in zone "A" or "B". It should be noted that the DO profile within the biofilm varied with the operational time, and it exhibited an obvious difference in the DO profile in different zones. In zone "A", after operating for 20 days, the DO value in the bulk solution was about 1.8 mg/L, and it nearly decreased linearly within the biofilm with the maximum penetration distance of 470 μm. Continuously operated for 60 days, the DO concentration in the bulk solution declined to a very low value (0.2 mg/L), and thus, it penetrated a very short distance inside the biofilm (only 250 μm). While in zone "B", for its relatively higher aeration rate, the DO value almost kept a stable level during the operational period (the DO values on day 20 and day 60 were very close to each other in the bulk solution), which caused a relatively small difference in the DO profile within the biofilm on different days. In Fig. 1(c), the penetration distances of DO on day 20 and day 60 were almost the same (about 550 μm), which was very close to the maximum distance of DO penetrating in a biofilm. The difference in DO profile is a prerequisite to form a multi-habitat environment (Tang et al., 2014), which is also a necessary condition to promote the biodiversity of a microbial community (Li et al., 2013). The obtained results of this section demonstrated that a combining aerobic-anaerobic biofilm had formed on the used semi-suspended bio-carriers, which actually provided a suitable condition to achieve richer biodiversity.

3.2. Similarity and difference of the microbial communities

The microbial communities contained in the biofilm are the main factor responsible for degrading organic pollutants and converting nutrients. However, many factors heavily influence the formation of microbial community, which may cause its changing along with the operational time, and further influence the performance of the bioreactor. For illustrating the similarity and difference of the microbial

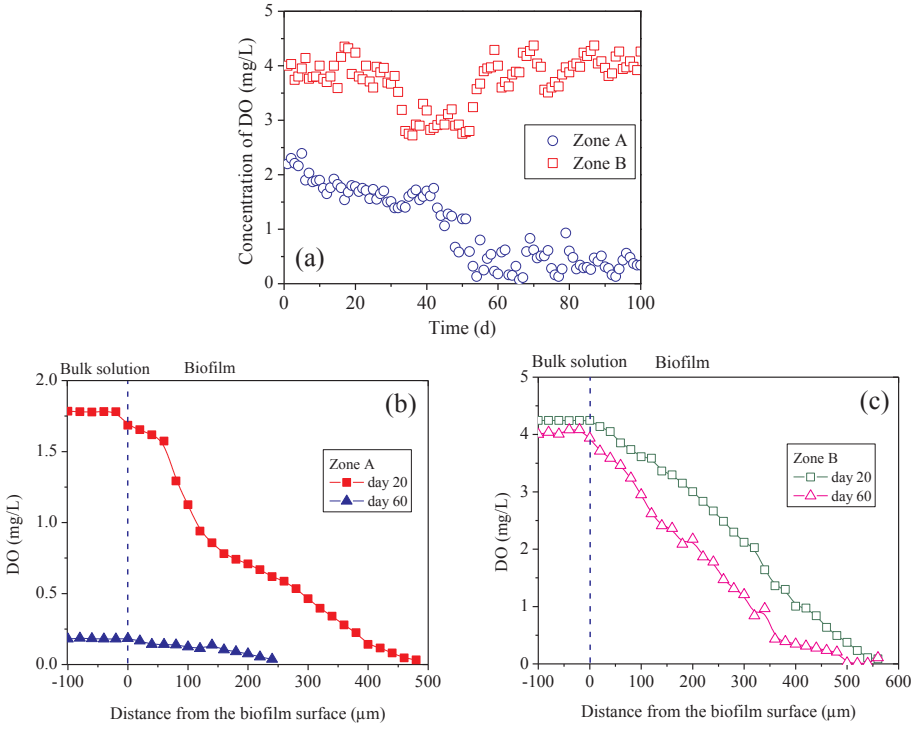


Fig. 1. Profile of DO in the bulk solution and the biofilm: (a) DO profile in the bulk solution; (b) DO profile in the biofilm of zone "A"; (c) DO profile in the biofilm of zone "B".

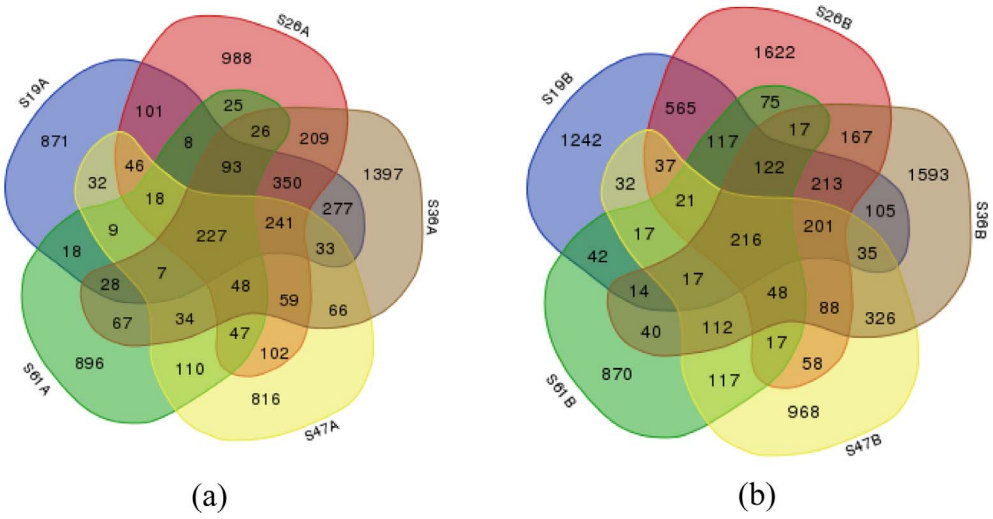


Fig. 2. Similarity and difference among the samples: (a) samples from zone "A"; (b) samples from zone "B".

composition at different time points and locations, the biofilm samples at zone "A" and "B" were collected at different time points to detect the composition of their microbial community with an HTS method, and with which, the similarity and difference of the microbial communities contained in the biofilm samples were evaluated. The results are shown in Fig. 2.

In Fig. 2, Venn diagrams are used to show the similarity and difference of OTU numbers of the samples collected at different time point. The samples in Fig. 2(a) are all from zone "A", they have 227 shared OTU. However, their difference is also quite obvious, which can be illustrated by the unique OTU among the five samples, namely, the unique OTU in S19A is 871, in S26A is 988, in S36A is 1397, in S47A is 816, in S61A is 896. The number of unique OTU is far more than that of the shared OTU. In the samples from zone "B", a similar phenomenon was also observed. In Fig. 2(b), the shared OTU among the five samples is 216, but the number of unique OTU in S19B, S26B, S36B, S47B and S61B, is 1242, 1622, 1593, 968 and 870, respectively. The numbers of the shared OTU between these two zones are very close to each other, but the number of unique OTU in zone "B" is higher than that of in zone "A". For further comparing the difference among the samples and revealing their relationship, the method of Principal Co-ordinates Analysis (PCoA) based on the weighted uni-frac beta diversity was used to evaluate the difference in the microbial structure of the samples, and the results are shown in Fig. 3.

Weighted uni-frac is a useful tool, which can be used to intuitively compare the difference of two samples in their characteristic by the distance between them. S0 stands for the original microbial community in the inoculated sludge, as shown in Fig. 3, at the initial stage (from day 1 to 19), there was a very short distance between S19A and S19B, and also, they were all close to S0, which indicated that the microbial communities contained in these three samples were very similar. At this stage, the DO value in zone "A" and "B" was different to some extent, but the operational time was not so long, which caused a similar composition in their microbial community. However, as the extension of operational time, the environmental conditions gradually changed, which led to an obvious variation in the microbial community among the biofilm at different time point. As shown in Fig. 3, the distances between the two samples of both zones on the same day gradually enlarged, which demonstrated the difference of the two zones in their microbial community was becoming more and more obvious (day 36–61).

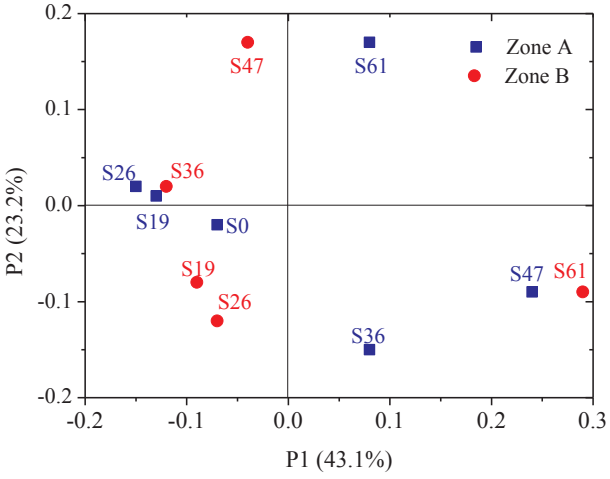


Fig. 3. Weighted uni-frac beta diversity measured at different dates.

3.3. Biodiversity analysis

After a suitable pretreatment, all the biofilm samples were analyzed by using an HTS method, with which, species richness and diversity indices (i.e., the observed OTUs, Chao1 estimator, Shannon index, Simpson index and abundance-based coverage estimator (ACE)) were calculated by using the MOTHUR software at a 3% dissimilarity cutoff. The results are shown in Table 1.

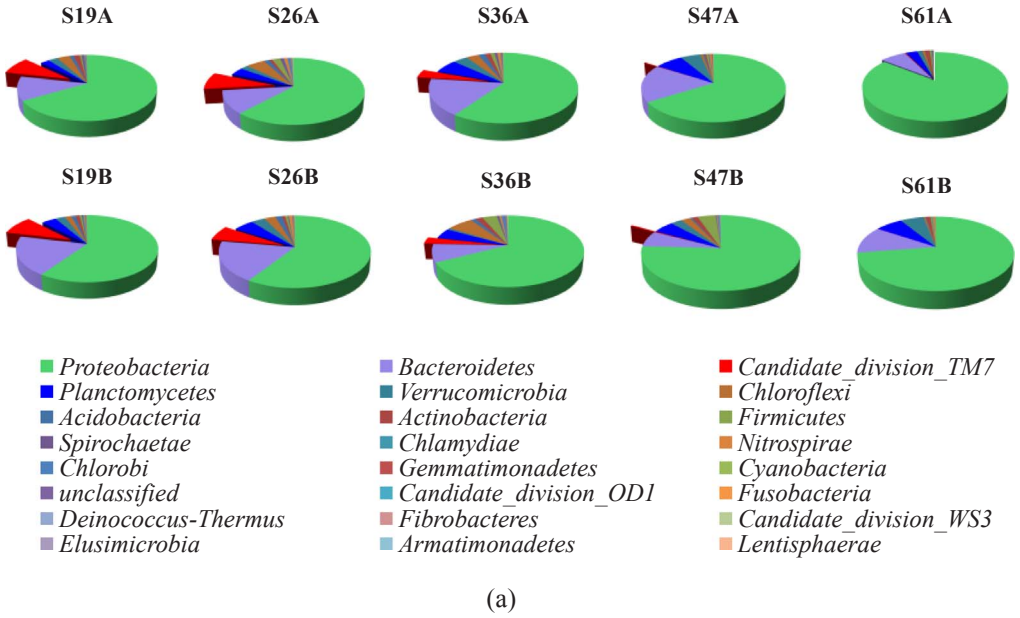
The biodiversity in an ecosystem is generally expressed by the community abundance and diversity of bacterial colony. Shannon index is used to express both the richness and evenness of species, Chao1 and ACE index is very useful to estimate the community abundance of the samples, Simpson index is used for comparing species diversity and evenness, and Coverage index is the coverage of each clone library and a measure of captured diversity, which reflects the reliability of the sequencing result. The results in Table 1 indicate that the biodiversity of both zones increases first, and then decreases during the observing period, and in zone "B", the relative abundance is shown to be higher than that in zone "A". Higher biodiversity implies a more stable microbial ecosystem, which is a guarantee for the performance of bioreactor. Compared with the performance results of the bioreactor (SI), the varying trend of biodiversity seems has some extents of relationship with the pollutants removal and nutrients conversion, and will be further illustrated by the HTS results.

3.4. Microbial composition and its succession

Results of Sections 3.2 and 3.3 indicated there were obvious differences among the biofilm samples in their microbial communities. For

Table 1
Biodiversity evaluation.

Sample	Sequence number	OUT number	Shannon index	ACE index	Chao1 index	Coverage	Simpson
S0	21107	4260	6.925911	19084.79	11356.46	0.872886	0.003848
S19B	22517	3242	6.094575	12220.4	8025.53	0.913754	0.012646
S19A	15926	2582	5.647374	9623.457	6320.253	0.901042	0.044513
S26B	31035	3894	6.165437	15255.56	9626.266	0.924633	0.015342
S26A	23405	3377	6.439092	9003.324	7015.735	0.92335	0.007987
S36B	18355	3501	6.246846	14453.37	9051.35	0.879978	0.018335
S36A	16715	2720	6.421145	9419.064	6655.032	0.906072	0.006464
S47B	18854	2465	5.413376	8896.632	5768.458	0.921184	0.023773
S47A	12237	2077	5.85516	7541.964	4753.585	0.898668	0.012822
S61B	18499	1994	5.192166	6697.595	4445.968	0.93697	0.023415
S61A	19241	1944	4.867652	7040.477	4608.827	0.939504	0.040559



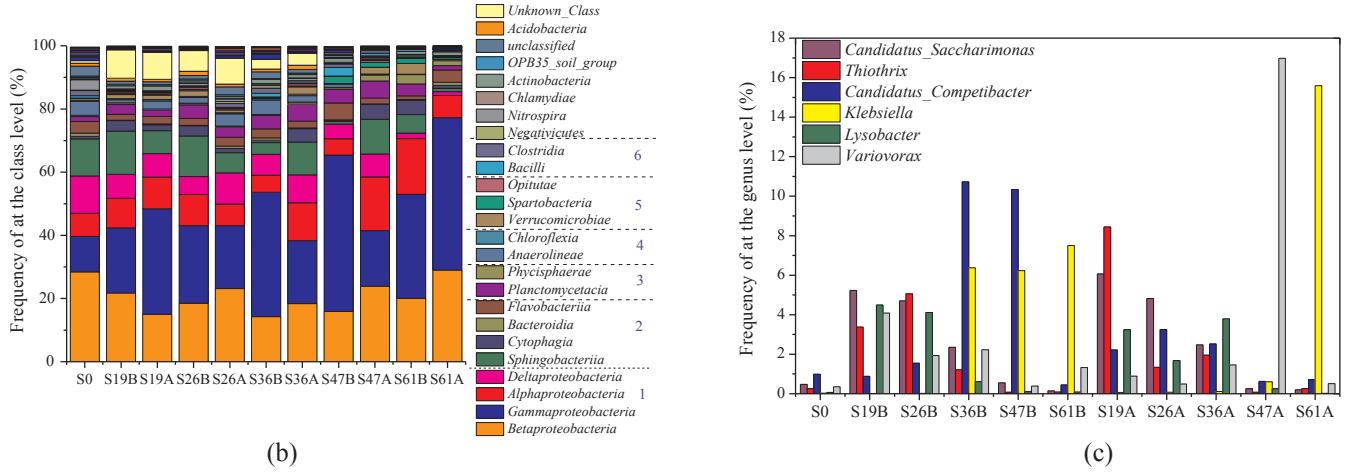


Fig. 4. Microbial composition: (a) Relative abundance at the phylum level; (b) Relative abundance at the class level (Numbers in the parentheses denote their belonging phylum, as follows: (1) *Proteobacteria*; (2) *Bacteroidetes*; (3) *Planctomycetes*; (4) *Chloroflexi*; (5) *Verrucomicrobia*; (6) *Firmicutes*); (c) Relative abundances of the maximum genus contained in each sample.

further illustrating the microbial composition of the biofilm samples at different level of taxonomic classification, the microbial compositions of the samples at the phylum, class and genus level are shown in Fig. 4.

Fig. 4(a) indicates that *Proteobacteria* are the dominant phylum contained in all samples, whose ratio exhibits a continuously increasing tendency in both zones with a percentage more than 50%. In zone "A", they increased from 61.08% to 82.51% (S19A: 61.08%, S26A: 59.66%, S36A: 58.32%, S46A: 67.75%, S61A: 82.51%); while in zone "B", they increased from 60.81% to 67.93% (S19B: 60.81%, S26B: 57.6%, S36B: 62.37%, S47B: 70.19%, S61B: 67.93%). The bacteria occupying the position of the second and the third dominant phylum are *Bacteroidetes* (average ratio 11.84%) and uncultured *Candidate_division_TM7* (average

ratio 8.95%), respectively. In zone "A", *Bacteroidetes* increased continuously (S19A: 3.53%, S26A: 11.36%, S36A: 15.6%) before day 47, then decreased to 7.28% after that day. In zone "B", an opposite trend was observed, namely, *Bacteroidetes* decreased continuously (S19B: 17.37%, S26B: 16.41%, S36B: 8.42%, S47B: 6.07%) before day 61, then suddenly increased to 12.04%. In both zones, uncultured *Candidate_division_TM7* decreased simultaneously and disappeared at the final stage (the ratios in zone "A" were: S19A: 6.06%, S26A: 4.82%, S36A: 2.47%, S47A: 0.26%, S61A: 0.02%; the ratios in zone "B" were: S19B: 5.23%, S26B: 4.7%, S36B: 2.35%, S46B: 0.05, S61B: 0.14%). *Proteobacteria* and *Bacteroidetes* are very common in an ordinary process for wastewater treatment (Xiao et al., 2016), and on the outer surface of *Proteobacteria*, most components are lipopolysaccharide, which make them are very easier to attach on the surface of bio-carrier (Tang et al., 2016). Therefore, with a higher adhesion force, *Proteobacteria* have more obvious advantages to grow on the surface, which greatly improves their percentage in the biofilm, especially encountering the predation of *Metazoa*.

Bacteroidetes include *Bacilli*, *Flavobacteria* and *Sphingobacteria*, whose occurrence and distribution is determined by the environmental factors, such as COD, temperature and pH value as well as nitrite, and in turn, the existence of these microorganisms guarantees the removal of organic pollutants and nutrients. Due to a large difference in the aeration rate between zone "A" and "B", there exist quite different environmental conditions between them, which cause obvious differences in the microbial composition and heavily influence the distribution of *Bacteroidetes*. *Candidate_division_TM7*, as a member of *Candidatus Microthrix Parvicella* (Mielczarek et al., 2012), is a kind of keystone microorganism, which generally plays a supporting role for other bacteria. As the growing of other microorganisms (such as non-filamentous bacteria), the strength of biofilm increases continuously and gradually inhibits the growth of *Candidate_division_TM7*, and as a result, it is replaced by *Proteobacteria* with an obvious decreasing ratio at the final stage.

Fig. 4(b) indicates the content and distribution of microorganisms at the class level (all the selected microorganisms have the ratio more than 1%), which shows that the dominant microorganisms are *Gamma-Proteobacteria*, *Beta-Proteobacteria*, *Alpha-Proteobacteria*, and *Detla-Proteobacteria.* Among them, *Gamma-Proteobacteria* have the highest ratio in all *Proteobacteria*, and their variation is also the most obvious during the operational period (e.g. in S61A and S47B, the ratios of *Gamma-Proteobacteria* are 40.08% and 40.20%, respectively, and the other three classes just fluctuate in a small range without any obvious variation). In general, *Alpha-Proteobacteria* are capable of decomposing complex organic pollutants (such as polycyclic aromatic hydrocarbon and organic sulfur compound) (Rani et al., 2008), while in *Beta-Proteobacteria*, the contained *Nitrosococcus oceanus* and *Rhodocyclus* have a close relationship with the removal of nitrogen and phosphorus from wastewater (Wang et al., 2013; Zhu et al., 2015), and *Delta-Proteobacteria* are a kind of microorganisms that commonly play an important role in sulphur cycle. In addition, *Proteobacteria*, *Sphingobacteriia*, the second dominant microorganisms, belong to *Bacteroidetes* and they occupy a large ratio, especially in zone "A", which are the major kind of microorganisms in sulfur metabolism, removing COD and nitrogen. The above findings further verify that the contained dominant microbial species within the coupling biofilm play an important role in keeping the stable operation of the bioreactor, and the used semi-suspended biocarriers provide suitable environmental conditions for removing nitrogen, phosphorus, sulfur, and organic pollutants in the biofilm reactor.

Due to the contained numerous microorganisms in the biofilm samples at the genus level, it is really difficult to give an accurate description about the variation of each microorganism at this level, thus, the microorganisms at the genus level with the highest abundance in each sample were listed and compared to evaluate their variation during the succession. The results shown in Fig. 4(c) indicate that *Candidatus_Saccharimonas*, *Thiothrix*, *Candidatus_Competibacter*, *Klebsiella*, *Lysobacter*, *Variovorax* have the highest abundance among all the samples. At the initial stage (day 19 and 29), the dominant genuses were *Candidatus_Saccharimonas*, *Thiothrix*, and *Lysobacter*, but they gradually decreased in the subsequent period. *Candidatus_Saccharimonas* belongs to *Candidate_division_TM7*, it is capable of promoting the formation of biofilm at the initial stage. *Thiothrix* has a similar variation tendency with that of *Candidatus_Saccharimonas*, they all occur in the biofilm with a filamentous structure, which verifies that the bacteria with a filamentous structure have an important influence on the formation of biofilm at the initial stage. *Lysobacter* is a kind of genus with a function of myceliolysis, which decreases as the reduction of hyphae (decreasing from 3.24% to 0.02% in zone "A", and decreasing from 4.08% to 0.09% in zone "B"). However, in the samples collected during the mid-term of operation (S36B, S47B and S26A, S36A), *Candidatus_Competibacter* was observed to be a dominant genus, which was a kind of glycogen accumulating organisms (GAOs) and generally competed resources with phosphorus accumulating organisms (PAOs). Its ratio in zone "A" was higher than that in zone "B", which indicated PAOs in zone "B" was not dominant. At the final stage (samples of day 47 and 61), the ratio of the two kinds of bacterial genus suddenly increased (*Klebsiella*: S61A 15.59% and S61B 7.5%; *Variovorax*: S61B 1.33% and S47A 16.98%). *Klebsiella*, as a kind of microorganisms with strong flocculability (Liu et al., 2013), may play a role of flocculation when they co-exist with *Metazoa* at the final stage, and are generally used to extract biological flocculants, while, *Variovorax*, one of *Proteobacteria,* may obtain competitive superiority for their high viscosity at the same stage.

On the surface of the semi-suspended bio-carrier, it was also found the bacteria with the function of removing nitrogen and phosphorus. *Nitrospira*, a kind of nitrifier (nitrite oxidizing bacteria, NOB), were detected to be 0.36% in zone "A" and 0.26% in zone "B". Other aerobic ammonia oxidation bacteria (AOB) were also commonly found in zone "A" and "B", e. g. in zone "A", the average percentage of *Pseudomonas*, *Bacillus*, *Comamonas*, *Paracoccus*, *Klebsiella*, *Rhodocyclus*, *Nitrosomonas*, and *Nitrosococcus* was 0.14%, 0.22%, 1.89%, 0.01%, 3.29%, 0.74%, 1.58%, and 0.15%, respectively, while in zone "B", the corresponding average percentage was 0.178%, 0.79%, 0.79%, 0.03%, 4.03%, 0.55%, 1.79%, and 0.16%. The co-existence of AOB and NOB in the biofilm is the primary reason to achieve total ammonia oxidation. Though, in zone "A" and "B", different rate of aeration was provided, a kind of anaerobic microorganism (*Anaerolineae*) was detected to have an average percentage of 1.76% in zone "A" and 1.70% in zone "B", which verified that an anaerobic micro-environment still co-existed in both zones. In addition, *Flavobacterium*, a kind of denitrifying bacteria (Huang et al., 2015), was found in the biofilm samples, whose percentage in zone "A" increased from 2.26% to 3.78%, but in zone "B", it increased first from 1.83% to 5.33%, then decreased to 1.16%. *Planctomycetacia*, a kind of anaerobic AOB (Connan et al., 2016), can oxidize ammonia with nitrite to nitrogen gas under an anoxic environment, which is also an important factor for the nitrogen removal from the bioreactor. In the used biofilm reactor, *Planctomycetacia* was commonly detected in both zones, in zone "A" and "B", its average ratio was 3.98% and 4.846%, respectively. However, the ratio in each sample was S19A: 2.75%, S26A: 3.53%, S36A: 5.74%, S47A: 5.78%, S61A: 2.09% in zone "A"; S19B: 3.64%, S26B: 4.83%, S36B: 5.14%, S47B: 5.93%, S61B: 5.19% in zone "B". The above findings demonstrated that nitrifying bacteria, denitrifying bacteria actually co-existed in the biofilm, which verified that a multi-habitat was formed on the semi-suspended biocarriers. Additionally, *Acinetobacter* was so far found to be a kind of genus to accumulate phosphorus with high efficiency, which was detected to have an average percentage of 1.04% in zone "A" and 1.01% in zone "B", and was higher than that of in the inoculated sludge (S0). In the bioreactor, *Acidobacteria* was also detected in both zones (S36A: 1.36%; S26B: 1.35%), though it could not remove phosphorus directly, the aliphatic acid it produced was beneficial to the phosphorus removal

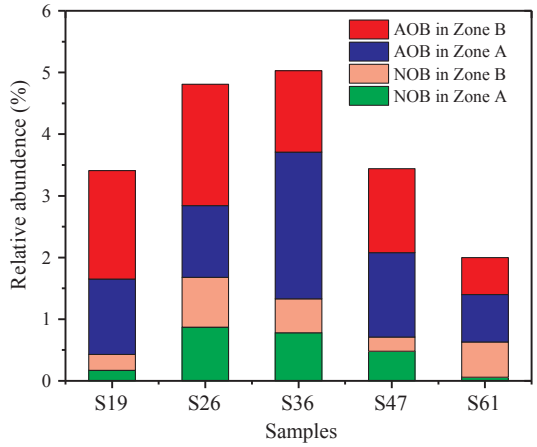


Fig. 5. Relative abundance of AOB and NOB within the bioreactor.

from the bioreactor (Xiao et al., 2016).

The above results revealed that the biodiversity was obviously enriched due to the formed micro multi-habitat within the biofilm, and the contained microbial community experienced a serious succession, during which, the microbial species were gradually dominated by those functional genus, including *Nitrospira*, *Pseudomonas*, *Bacillus*, *Comamonas*, *Paracoccus*, *Klebsiella*, *Rhodocyclus*, *Nitrosomonas*, *Nitrosococcus*, *Anaerolineae*, *Flavobacterium*, *Planctomycetacia*, and *Acidobacteria*.

The microbial community in a bioreactor greatly influences its performance (Neoh et al., 2017). However, the microbial species in the biofilm were found to be not evenly distributed at different stages, the dominant species occupied a large ratio in the microbial community and also acted as a keystone in maintaining its stability.

3.5. Variation of AOB and NOB within the biofilm

Total removal of nitrogen from wastewater involves several processes, including ammonification, aerobic ammonium oxidation, nitrite oxidation, anaerobic nitrification, heterotrophic nitrification, aerobic denitrification, and anaerobic ammonium oxidation (Ji et al., 2013). However, in an ordinary WWTP, AOB and NOB are commonly found to be the major microorganisms to achieve biological nitrification. In the collected samples, AOB and NOB were also detected with their relative abundance shown in Fig. 5.

AOB and NOB are two distinct groups of bacteria that play important role in the process of nitrification. Previous studies based on amoA-gene-based cloning analysis indicated that the genus *Nitrosomonas*, *Nitrosococcus* and *Nitrosospira* belonging to AOB, and were responsible for ammonia oxidation (Shangguan et al., 2015; Tait et al., 2015), while, *Nitrobacter* and *Nitrospira*, two major members of NOB, actually acted as the role to change the chemical valence of the nitrogen for achieving nitrite oxidation. Fig. 5 is a column plot about the distribution of NOB and AOB in the relative abundance, which shows, as a whole, the amounts of AOB and NOB increased during day 0–30, and decreased after day 47, which were accordance with the results of alpha diversity indices, and could be used to explain the tendency of TN removal. In zone “A”, AOB gradually predominated due to the low DO value (Gong et al., 2008), but after day 40, total AOB and NOB obviously decreased, and the amount of AOB in zone “A” was far less than that of in zone “B”. The optimal conditions for the growth of AOB are DO 2–3 mg/L at 30 °C (Zhang, 2009), thus, during the operational period, when the environmental factors satisfied the optimal conditions (around day 40), AOB in zone “A” and “B” attained the highest percentage.

4. Conclusions

A coupling anaerobic-aerobic biofilm was successfully established on the semi-suspended bio-carriers fabricated by a 3D technique, which promoted the performance of removing organic pollutants and nutrients. Lots of functional microorganism, including AOB, NOB, and denitrifier co-existed in the formed coupling biofilm to form a complete microbial community, which verified that a multi-habitat was formed on the semi-suspended bio-carriers, and it further promoted the biodiversity within the bioreactor. During the continuous succession of the coupling biofilm, the microbial community was gradually dominated by some functional genus, and showed an increasing difference in the dominant species and the structure of microbial community.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.09.147.

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