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Effects of phenol on physicochemical properties and treatment performances of partial nitrifying granules in sequencing batch reactors

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ABSTRACT

This study attempts to investigate the effect of phenol on physicochemical properties and treatment performances of partial nitrifying granules (PNGs). Two sequencing batch reactors (SBRs) fed with synthetic ammonium wastewaters were operated in absence (R1) or presence (R2) of phenol. The PNGs in R1 maintained excellent partial nitrification performance and relatively stable physicochemical properties, and exhibited compact and regular shaped structure with a cocci-dominant surface. However, as phenol concentration was stepwise increased from 0 to 300 mg/L in R2, filamentous bacteria appeared and gradually dominated within granules, which in turn resulted in settleability deterioration. Most notably, granules in R2 got easier to agglomerate in the reactor walls and then been washed out with effluent, leading to significant biomass loss, frequent outflow pipe blockage, and eventual system failure. The extracellular polymeric substances (EPS) contents including proteins and polysaccharides in R2 reached 1.8 and 1.7 times of that in R1, respectively, indicating that the presence of phenol played an important role on EPS production. Removal efficiency of ammonium and phenol remained high, but dropped sharply when phenol concentration reached 300 mg/L. Moreover, the failed maintenance of partial nitrification was observed due to the revival of nitrite oxidizing bacteria (NOB) within granules after phenol exposure, which was confirmed by quantitative fluorescence in situ hybridization (FISH) analysis. Overall this study demonstrates that phenol had negative effects on PNGs, and pretreatment to eliminate phenolic substances is recommended when using PNGs for wastewater treatment.

1. Introduction

Biological nitrogen removal (BNR) based on partial nitrification for treating ammonium-rich wastewaters is drawing wide attention around the world [1,2]. Different from traditional BNR processes, partial nitrification reaction biologically converts ammonium to nitrite instead of nitrate as the intermediate for both nitrification and denitrification steps. Energy needed for aeration and organic carbon required for denitrification could be saved with partial nitrification. In addition, use of aerobic granules for partial nitrification (i.e., partial nitrifying granules, PNGs) instead of activated sludge flocs is promising because of good sludge settleability, long-term retention of slow-growing nitrifying bacteria and high specific nitrification rate [3–6].

Ammonium wastewaters containing phenolic compounds are typical from several diverse industries, such as petroleum refinement, coal tar processing and petrochemicals manufacturing, paints and resins producing industries [7]. The phenolic compounds have potential inhibitory or toxic effect over conventional biological treatment systems such as the activated sludge process [8], specifically over the nitrification process [9,10].

These substrate inhibition difficulties may be overcome by the technology of aerobic granules [11,12]. Granular biomass are considered to be resilient to toxic compounds with the compact and dense granular structure serving as a substrate diffusion barrier, and a diversified microbial aggregate within the granular structure provides a platform for simultaneous removal of ammonium and phenolic compounds [13–17]. Nevertheless, several studies have also demonstrated nitrifiers existing within the granules are sensitive and could be inhibited by the presence of phenolic compounds [18–22]. So that, the application of

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simultaneous removal of ammonium and phenolic compounds using aerobic granules needs more research attention.

Simultaneous partial nitrification and phenolic removal have been reported in continuous airlift reactors with aerobic granular biomass [23,24]. Continuous reactors can mitigate the toxic effect of the recalcitrant compound over the biomass, since the bulk liquid concentration in the continuous reactor is expected to be low [24]. But the development and maintain of aerobic granules is commonly achieved through SBRs where high hydrodynamic stress is applied. While sequencing batch reactors (SBRs) are not advisable for treatment of phenolic compounds that usually exhibit inhibition by substrate [24]. In this sense, investigation on the effect of phenolic compounds on treatment performance of PNGs in SBRs is of paramount important.

Besides, the information about the physicochemical properties and stability of PNGs in the presence of phenolic compounds is still limited. This information is very valuable for the practical application of PNGs in treating industrial wastewaters that are associated with toxicants. To the best of our knowledge, no published work has been emphasized on toxic effect of existing PNGs system influenced by phenolic based wastewater. Therefore, this work aims to study the influence of a model phenolic compound, phenol, on physicochemical properties, treatment performances and stability of PNGs in SBRs. It is expected that this study would lead to a better understanding of the behavior of PNGs in the presence of inhibitory organic compounds.

2. Materials and methods

2.1. Partial nitrifying granules reactor

Experiments were performed in two identical 3.5 L internal-circulate sequencing batch reactors (SBRs; R1 and R2; 100 cm in height and 8 cm in diameter for down-comer; 70 cm in height and 5.4 cm in diameter for internal riser) at room temperature of $23 \pm 2\,^{\circ}C$. The two reactors were operated in 6 h cycles consisting of 12 min of influent filling, 342 min of aeration, 3 min of settling and 3 min of effluent withdraw. The aeration was supplied through fine air bubbles located at the reactors bottom with an air-flow rate of 1.2 cm/s. The minimum dissolved oxygen (DO) concentrations detected in both reactors were above 3 mg/L. The effluent of the reactor was discharged at a volumetric exchange ratio of 50% and the hydraulic retention time (HRT) was maintained at 12 h.

The PNGs used in our experiments were previously cultivated with synthetic wastewater for more than six months (Fig. S1), which had a mean diameter of 0.83 mm and sludge volume index (SVI) of 18.72 mL/g. The two reactors were inoculated respectively with 9.87 g/L PNGs. The solids retention time (SRT) was not controlled, but varies naturally with changes in the solids concentration in the effluent from the two reactors, which was a function of the sludge settleability. The walls of the reactors were cleaned every week, and the biofilm growth was discarded. R1 was fed with synthetic ammonium wastewater mainly consisted of ammonium chloride (100 mg NH_4^+-N/L), sodium bicarbonate (used to adjusted influent pH at 7.8 ± 0.1) and other necessary mineral-salts medium including $FeSO_4$, 40 mg/L; KH_2PO_4, 22 mg/L; $FeCl_3 \cdot 6H_2O$, 1.5 mg/L; EDTA, 20 mg/L. R2 was initially fed with the same wastewater as R1, and since day 7 an increasing amount of phenol (from 50 to 300 mg/L) was added to the influent (Fig. 1).

2.2. Three-dimensional excitation and emission matrix (EEM) fluorescence spectroscopy

To investigate the componential changes of extracellular polymeric substances (EPS), an F-4600 fluorescence spectrophotometer (Techcomp LTD., Japan) was applied to measure all EEM spectra. EEM spectra were collected with subsequent scanning emission spectra from 200 to 500 nm at 5.0 nm increments by varying the excitation wavelength from 200 to 400 nm at 5.0 nm increments and recorded at a scan rate of 30000 nm/min, using excitation and emission slit bandwidths of 5 nm. The voltage of the photomultiplier tube was set to 400 V for high level light detection. The blank scans were performed at an interval of 10 using deionized water.

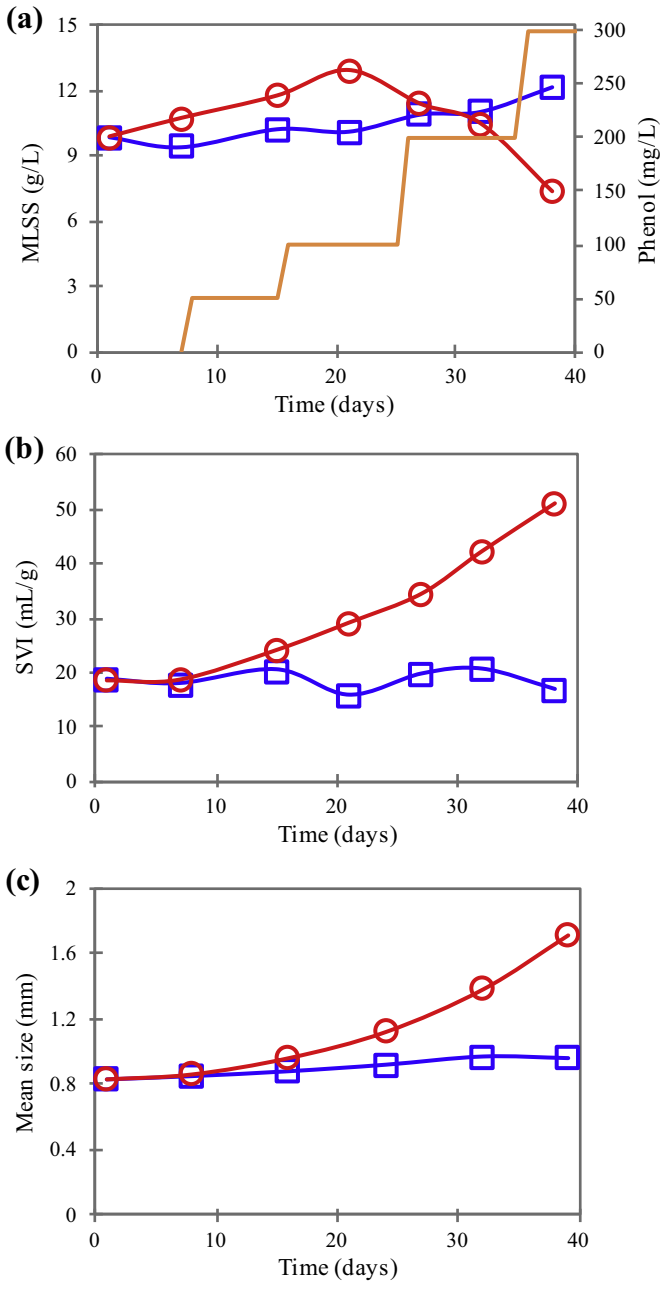

Fig. 1. Time profiles of MLSS (a), SVI (b) and mean granule size (c). Symbols: R1 (□), R2 (○), and phenol concentration (—).

2.3. Fluorescence in situ hybridization (FISH) analysis

FISH analysis was carried out to research the microbial communities of PNGs under the influence of phenol. Pretreatment and hybridization processes were according to previous report [25,26]. The 16S rRNA-targeted oligonucleotide probes used were

as follows: EUB338 (labeled with fluorescein isothiocyanate (FITC)) for all active bacteria, Nso190 (labeled with hydrophilic sulfoindocyanine dye (Cy3)) for AOB, Ntspa662 and Nit3 (labeled with Cy3) for nitrite oxidizing bacteria (NOB). Hybridized samples were viewed with a confocal laser scanning microscope (LEICA TCS SP2, Germany). For quantitative analysis of FISH images, digital images were analyzed with a program Image Pro Plus (version 6.0). The percentage of AOB or NOB was calculated from the ratio of the specific target biovolume to the total bacterial biovolume. For each condition, three samples were measured for average and at least 10 different fields were examined for each sample.

2.4. Analysis

The measurements of mixed liquor suspended solids (MLSS), sludge volume index (SVI), pH, phenol and N concentrations were conducted according to standard methods [27]. DO values were measured with a digital, portable DO meter (YSI Model 85, USA). The size distributions of the granules were measured by a laser particle size analysis system (Mastersizer 2000, Malvern Instruments, UK). The granule structure and surface morphology were viewed via a scanning electron microscope (SEM, HITACHI S-570, Japan). The granule samples were fixed with 3.0% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2. The samples were then dehydrated with ethanol, silver-coated by a sputter and observed in the SEM. The extraction of EPS was based on a cation exchange resin (Dowex-Na form) method [28]. EPS was analyzed as the sum of polysaccharides (PS) and proteins (PN), which were analyzed using phenol-sulfuric acid method [29] and coomassic brilliant blue method [30], respectively.

3. Results and discussion

3.1. Granule properties

The PNGs which had MLSS of 9.87 g/L, mean granule size of 0.83 mm, and SVI of 18.72 mL/g, were seeded into R1 and R2 after taken from a parent reactor. During the entire operation period, the granule properties in terms of MLSS, SVI and granule size in the control reactor R1 with no phenol feeding were relatively stable. The MLSS and granule size kept rising slightly and the SVI fluctuated without obvious directional changes (Fig. 1). The seed granules had a spherical or elliptical shape and were orange in color (Fig. S1). Detailed micro-structure examined by SEM showed that the seed granules had compact and regular shaped structure with a cocci-dominant outer surface (Fig. 2a). Both the macro- and micro-structure of granules was stable and no detectable changes in granule morphology were observed for PNGs in R1.

As influent phenol concentration was stepwisely increased from 0 to 300 mg/L in R2, both physical properties and micro-structure of PNGs changed obviously. The MLSS increased initially to plateau at 12.94 g/L and then decreased since day 21 to 7.33 g/L at the end of operation (Fig. 1). Both the SVI and mean granule size were gradually increased throughout the experiment, from 18.72 to 51.20 mL/g and 0.83 to 1.71 mm, respectively (Fig. 1). The PNGs in R2 still showed similar macro-structure as seed granules with intact appearance and orange color during the whole operation. However, by using SEM, Fig. 2 shows that the micro-structure of granules in R2 gradually modified in a loose and irregular structure covered with filamentous bacteria. Filament development on surface of phenol-fed aerobic granules has been reported previously [31–33]. For PNGs exposed to other toxic compounds, such as *p*-nitrophenol and *o*-cresol, the development of the filamentous structures was also found [23,24]. Possible explanation for these phenomena include the ability of filamentous bacteria to compete in a toxic environment [33], and the growth of bacteria related to biodegradation of toxic compounds [23]. Therefore, the deterioration of settleability and modification of microstructure in our study was thought to be linked to the development of heterotrophic biomass able to degrade phenol in the form of filamentous structures.

Most notably, after influent phenol concentration increased to 200 mg/L since day 26, some granules in R2 were found to agglomerate together in the walls of the reactor R2 and this phenomenon was getting more serious with operation time (Fig. S2). While no such phenomenon occurred in the control reactor R1. The formed agglomerates, which can be considered as special biofilm, were easy to be washed out with effluent and in turn caused obvious decrease in MLSS as shown in Fig. 1. Biofilm wall growth was generally a significant problem during initial stages of aerobic granules formation (transition from flocculating to granular sludge) [34,35]. However, similar to our study, it was also reported that the biofilm growth on the walls was especially prolific after granule formation when granules became filamentous and reactor performance worsened [36]. Thus, the granules agglomeration was speculated to be ascribed to filamentous

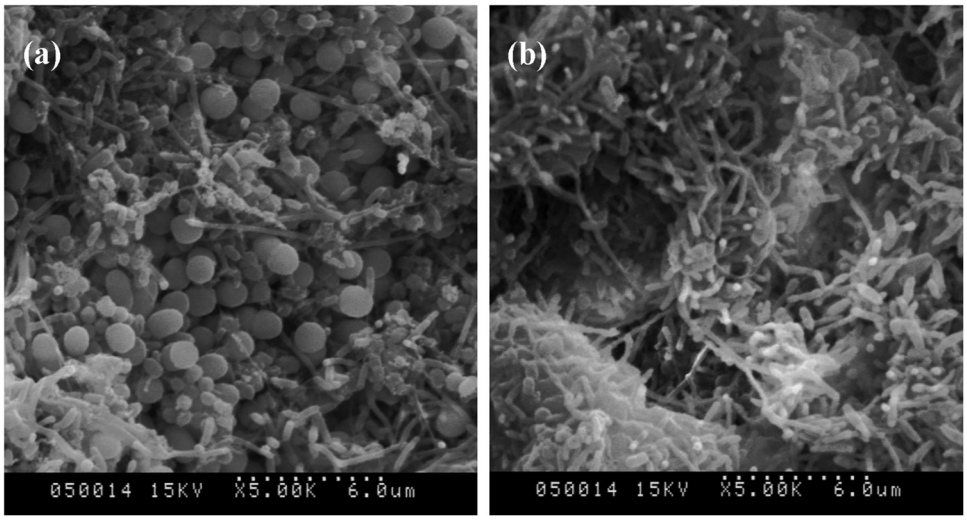

Fig. 2. SEM images of (a) seed granule in both reactors and (b) granule in R2 on 30th day.

overgrowth. Biofilm wall growth and biomass washout yielded biomass overflow, and more seriously, blocked the outflow pipelines and led to SBR crash. As frequent crashes occurred, the operation of R2 was forced to stop on day 40.

3.2. EPS characterization

EPS are metabolic products accumulating on the surface of bacterial cell within activated sludge flocs, biofilms and microbial granules. Their layer forms a protective shield for aerobic granule cells against harsh external environments. Intensive researches have shown that the compositions of EPS could contribute primarily to the structural stability of aerobic granules, which determines the long-term operation effect of aerobic granule system. To have a better understanding of the variations of EPS production during the exposure of phenol, the main components of EPS, including PN and PS, were measured and compared for R1 and R2 (Fig. 3).

The PN and PS concentrations in R1 were slightly increased from 16.3 ± 1.3 and 9.3 ± 0.6 mg/gVSS for seed granules to 24.1 ± 1.4 and 13.9 ± 0.7 mg/g VSS, respectively. Compared with R1, both the PN and PS concentrations increased greatly to 42.7 ± 1.0 and 23.8 ± 0.9 mg/g VSS after the stepwise addition of phenol in R2, respectively. In other words, the contents of PN and PS in R2 reached 1.8 and 1.7 times of R1, implying that the presence of phenol played an important role on the EPS production. Previous studies have also demonstrated that EPS production was stimulated in the presence of phenol [37,38]. Bacteria could survive the inhibitory effect of toxic chemicals through the metabolic pathway by producing more EPS, which was considered to act as an important role to protect microorganism [11]. Increased COD concentration due to the addition of phenol may also stimulate EPS production. It has been reported that an increased substrate COD/N stimulated the production of EPS, because enriched heterotrophic bacteria can produce larger amounts of EPS than autotrophic bacteria [39].

EEM spectroscopy has been widely applied to investigate the interaction of EPS and toxic chemicals, as fluorescence characteristics are greatly related to their structure and functional groups in molecules. Fig. S2 shows the 3D-EEM results of EPS in both reactors on day 30. Both 3D-EEM spectra provides information about two major substances of EPS: aromatic protein (peak A, Ex/Em = 200–250/280–380) and tryptophan protein like (peak B, Ex/Em = 250–310/320–380) [40]. The locations of peaks have not changed in R2, indicating no distinct changes of the structure and functional groups of EPS. The respective intensity of peak A and peak B was 33.74 and 107.50 in R1, while they were obviously increased to 98.62 and 221.10 with the addition of phenol in R2. As fluorescence intensities correlated with the EPS concentration, the result was consistent with the increased EPS contents in Fig. 3, which further proved that phenol stimulated the secretion of EPS.

The stimulated EPS production would be helpful for maintaining the structural integrity for aerobic granules [41,42]. However, excessive EPS production also had adverse influence on sludge-water separation [43], which might be another reason for granules agglomeration. Wu et al. [39] has found that excessive EPS secreted resulted in a lower cell surface hydrophobicity and failure of granule formation. High EPS quantity was also reported to result in high SVI of granular sludge [44], which is in good agreement with this study. The SVI of granular sludge in R2 increased with the increase of influent phenol concentration (Fig. 1b), and showed

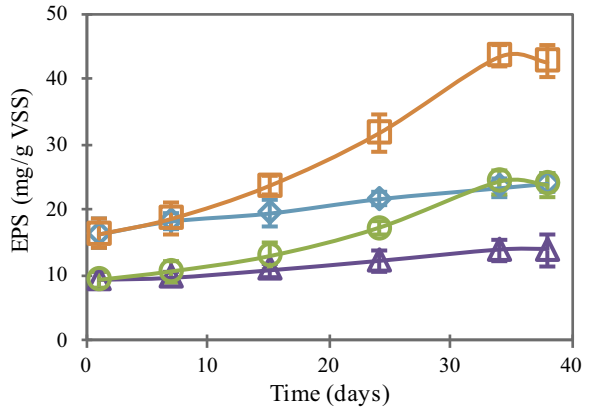

Fig. 3. Changes in EPS contents during the operation. Symbols: PN in R1 (◇), PN in R2 (□), PS in R1 (△), and PS in R2 (○).

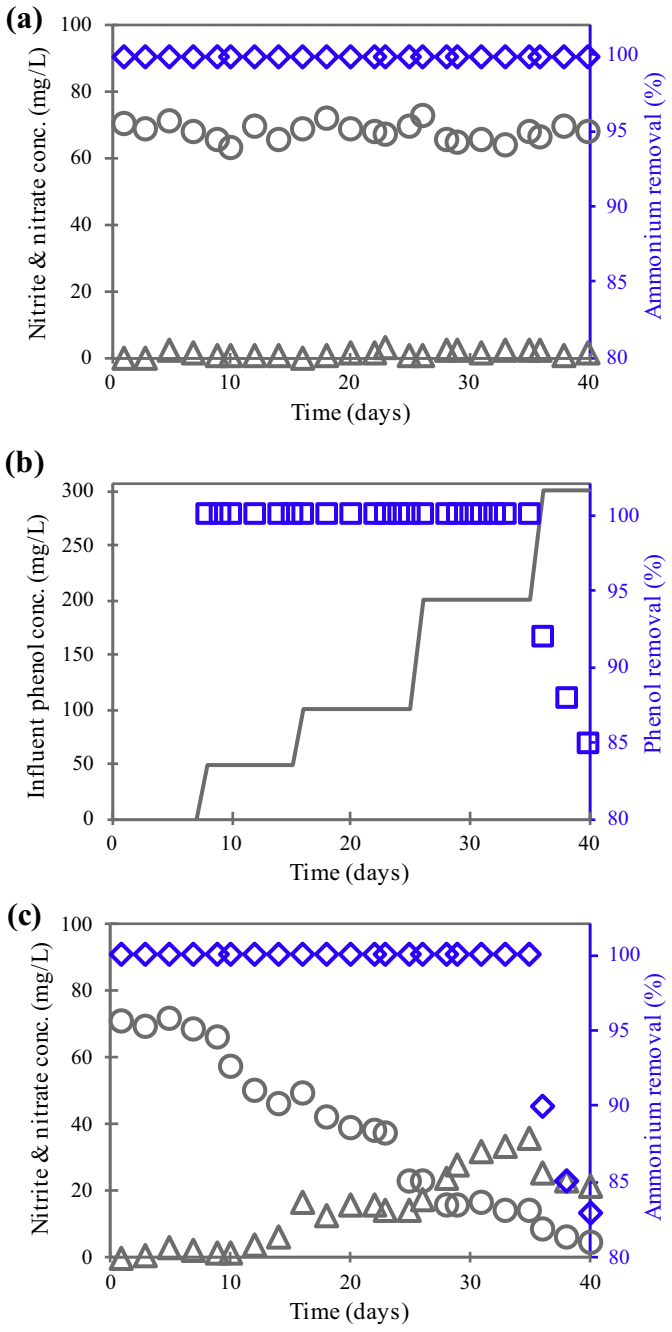

Fig. 4. Profiles of long-term performances of R1 (a) and R2 (b & c). Symbols: ammonium removal (◇), nitrite (○), nitrate (△), phenol concentration (—), and phenol removal (□).

positive liner correlations with PN ($R^2 = 0.9215$) and PS ($R^2 = 0.9294$).

3.3. Treatment performances

The overall performances of R1 and R2 in nitrogen and phenol removal are shown in Fig. 4. The inoculated PNGs obtained from the parent SBR had an excellent partial nitrification performance. In the control reactor R1 without phenol addition, ammonium was almost completely removed throughout the experiment and above 96% of effluent was composed of nitrite, indicated that a stable partial nitrification was maintained (Fig. 4a). Stable BNR via nitrite with granular biomass has been reported in other studies treating both high- and low-strength ammonium wastewaters [45–47]. This could be due to the higher size of aerobic granules than activated sludge. The higher the granule size, the lower the specific surface; consequently, a reduced oxygen flux towards the granule is obtained for a given biomass amount, favoring partial nitrification due to oxygen limitation [48]. Although organic carbon source was absent in the influent, about 30% of total nitrogen (TN) removal was achieved in R1 (Fig. 4a). Assimilation by nitrifiers and heterotrophs presumably accounts for some of the observed nitrogen loss, but this should be very small [49]. Such a nitrogen loss phenomenon might be attributed to denitrification by heterotrophs with soluble microbial products (SMP) released by nitrifying bacteria as a carbon source [50].

The effect of phenol with stepwise increased concentration (0–300 mg/L) on the performance of PNGs was evaluated in R2. As shown in Fig. 4b, once the phenol was fed to the reactor, its complete removal was observed with no lag phase. Aerobic granules contains a high diversity of microorganisms and has been demonstrated to possess enough physiological traits and functional redundancies to make it ideal candidate for use as a starting seed to rapidly produce phenol-degrading bacteria [33]. In a previous 16S rRNA gene clone library analysis for the PNGs, *Pseudomonadales* bacteria (40%) was detected [51]. Because *Pseudomonas* genus are well-known most commonly utilized phenol-degrading bacteria [52], several microbes in the PNGs may help to eliminate phenol immediately. The effect of phenol at concentration of 0–200 mg/L was observed to be insignificant on the performance of R2 in both phenol and ammonium removal. This could be due to the protection provided by the compact granule structure for microorganisms against phenol toxicity [11,12,33,53], and also the buildup of a critical population of phenol-degrading bacteria in the PNGs, such as the filamentous bacteria observed in Fig. 2b.

However, severe biomass washout would weaken the phenol biodegradation ability, and the loose granule structure might be unfavorable for protecting microorganisms. Meanwhile, long-term phenol exposure could enhance the cell membrane permeability and damage the cell membrane [54]. Because of these, further increasing the phenol concentration to 300 mg/L resulted sudden and continuous decreases in removal efficiency for both phenol and ammonium in R2 (Fig. 4b). The microbial activity may be recovered if the biomass was efficiently retained within the reactor. A previous study has demonstrated that acetate-fed aerobic granules exerted by phenol were neither permanent nor irreversible, and that the granules were able to make adaptive changes to survive and thrive under the high phenol level [53]. Another study found that after long-term acclimation, aerobic granules was able to degrade phenol at a concentration of up to 5000 mg/L without severe inhibitory effect [55]. Thus, it seems that the PNGs is more susceptible to the toxic effect of phenol.

Another worthy of attention is that nitrite concentration began to decrease after phenol addition on the 8th day and nitrate concentration gradually increased from then on in the effluent of R2 (Fig. 4b). The above phenomenon indicated a transfer from partial nitrification to full nitrification in R2. Bioreactor performance would be influenced by the changes of microbial communities, which were therefore identified by quantitative FISH analyses. Fig. 5 shows that the proportions of AOB, NOB, and other bacteria were 71.18%, 5.43%, and 23.39% on the 1 st day and 33.17%, 26.65%, and 40.18% on the 30th day in R2, respectively. The increase of other bacteria was thought to be linked to the growth of heterotrophic bacteria responsible for phenol biodegradation. NOB prevailed in the competition with AOB after long-term phenol exposure, probably due to the alteration of DO diffusion in granules, as the granule has been modified into loose structure and deeper DO penetration would favor NOB growth. Thus, the results from this study suggest that phenol had adverse effect on PNGs, and pretreatment to decrease or eliminate phenolic substances concentration would be necessary when treating ammonium wastewaters containing phenolic compounds by PNGs.

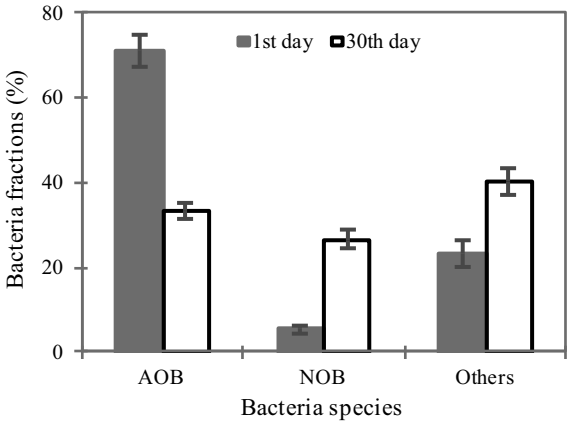

Fig. 5. Bacterial fractions of PNGs in R2 on 1st day and 30th day.

4. Conclusions

Phenol showed negative effects on the physicochemical properties and partial nitrification performance of PNGs. Overgrowth of filamentous bacteria and stimulated EPS production under the influence of phenol were considered to be the main reasons for deterioration of physicochemical characteristics of PNGs. Phenol induced changes of microbial community especially promoted NOB growth, which further resulted in the failure of partial nitrification. Therefore, phenolic substances should be paid special attention when treating ammonium wastewater containing these pollutants by PNGs.

Acknowledgments

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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.btre.2016.12.002.

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