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Metagenomic insight into the bioaugmentation mechanism of *Phanerochaete chrysosporium* in an activated sludge system treating coking wastewater

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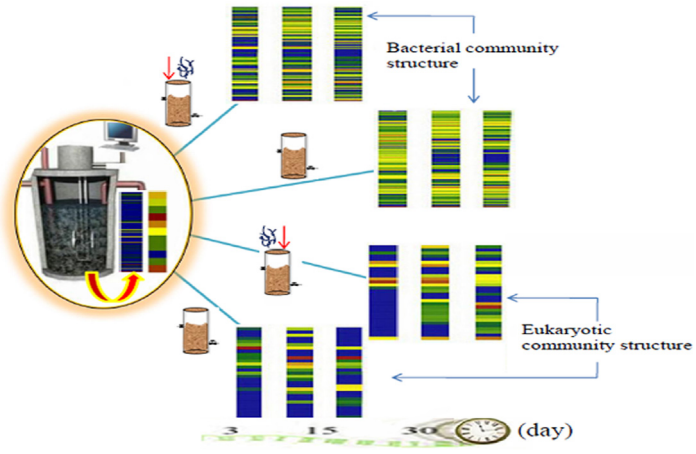
HIGHLIGHTS

- Bioaugmentation with *P. chrysosporium* is a good alternative for phenol wastewater treatment.
- Bioaugmentation increases bacterial and eukaryotic richness, but reduces their diversity.
- The fungus improves reactor performance by influencing microbial community structure.
- This work impacts the conventional criteria for selection of bioaugmentation microbe.

GRAPHICAL ABSTRACT



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ABSTRACT

Phanerochaete chrysosporium was seeded to a sequencing batch reactor treating phenol wastewater. Compared to the contrast reactor (R1), the bioaugmented reactor (R2) exhibits better performance in sludge settling ability, as well as biomass and phenol removal, even though the added fungus is not persistently surviving in the reactor. Bioaugmentation improved bacterial population, growing up to 10,000 times higher than that of eukaryotes. Metagenomic sequencing results show the bioaugmentation finally increases bacterial and eukaryotic richness, but reduces their community diversity. In contrast to R1, bacterial distribution in R2 is more concentrated in *Proteobacteria*. The relative abundances of filamentous fungi, yeast and microalgae in R2 are all higher than those in R1 at different treatment phases, and two reactors are finally dominated by different protozoan and metazoan. In conclusion, *P. chrysosporium* improves reactor performances by influencing microbial community structure, and this phenomenon might be attributed to the ecological competition in sludge and toxicity reduction of phenol wastewater. The novelty of this study emphasizes why a species which is not persistently active in bioreactor still plays a crucial role in enhancing reactor performance. Results obtained here impact the conventional criteria for selection of bioaugmentation microbes used in activated sludge systems.

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1. Introduction

In industrial fields, one of the most common environmental pollutants is phenol, which can be detected in effluents from pharmaceutical firms, plastic synthesis industries, coking plants and so on [1,2]. Phenol poses hazardous effects to human health and ecology, and should thus be completely removed before wastewaters are discharged [3]. Thus, several chemical, physical, and biological methods for phenol removal have been developed [4–6]. Biological treatment, specifically the conventional activated sludge (AS) process, is the most widely used among these techniques for phenol wastewater treatment [7,8]. However, the high toxicity of phenol in wastewaters inhibits microbial growth in AS, leading to the breakdown of AS systems [9]. Bioaugmentation is considered a possible solution for treating wastewaters with high concentrations of phenol. The performance of a wastewater treatment system can be improved by amending AS with specialized microorganisms that can produce versatile enzymes to enhance phenol biodegradability [10,11]. Nonetheless, even though a bioaugmented AS system is regarded as a powerful tool for improving the efficiency of wastewater treatment [12,13], only a few microorganisms are suitable for bioaugmentation, because they have to meet at least three conventional criteria for strain selection, namely, active, persistent, and compatible [14,15]. Therefore, the majority of previous works have focused mainly on the usage of bacterium and yeast with rapid reproduction capability, high phenol tolerance, and degradation capability for bioaugmentation in phenol wastewater treatment [16–18]. Depending on the criteria, filamentous fungi may be unsuitable for bioaugmentation because they reproduce at a slower rate than bacteria and yeasts, and a slow reproduction rate is disadvantageous to the ecological competition that occurs in AS. Nevertheless, the effective treatment of wastewaters by AS bioaugmented with filamentous fungi has also been reported, although the survival of the fungi in AS is uncertain, and limited information is available in the literature as regards the fungal bioaugmentation mechanism [19,20].

Phanerochaete chrysosporium is a white-rot fungus that is extensively used in environmental engineering fields, and the fungus has promising potential use in environmental pollution control because it can degrade a wide variety of nonphenolic and phenolic compounds by producing Mn-dependent peroxidase (MnP) and lignin peroxidase [21,22]. In this study, this fungus was seeded to a sequencing batch reactor (SBR) that received a high concentration of phenol wastewater; its effects on the performance and microbial composition of AS were revealed by metagenomic sequencing, which is widely used to investigate the microbial communities associated with various samples, such as sludge and freshwater [23–25]. The objectives of this work were to (i) evaluate the feasibility of treating phenol wastewater with AS bioaugmented with *P. chrysosporium* and (ii) clarify the fungal bioaugmentation mechanism from the aspect of microbial community change. To the author's knowledge, limited information is available in the literature as regards the bioaugmentation mechanism of filamentous fungi in an AS reactor. This study provides insight into the function of *P. chrysosporium* and the ecological succession of microbial communities under high phenol levels.

2. Materials and methods

2.1. Chemicals, AS, and microorganism

All of the chemicals used were of analytical grade unless otherwise stated. Seed sludge was obtained from the Xinxiang Municipal Wastewater Treatment Plant, China. *P. chrysosporium* (ATCC24725) was obtained from the Henan Province Key Laboratory for Microbial Resource and Functional Molecules, China.

2.2. Cultivation of P. chrysosporium

P. chrysosporium was incubated on a potato dextrose agar (PDA) plate and sub-cultured for 3 days at 35 °C. Subsequently, conidium suspension (5×10^7 spores/ml) was prepared in sterile water. The suspension (5.0 ml/flask) was inoculated to six 500 ml flasks containing 200 ml of liquid medium (Table 1). The flasks were placed in a shaking incubator at 100 rpm and 35 °C. After a 4-day submerged cultivation, the mycelial pellets in the fermentation broth (5.8 g/l of biomass in dry weight) were smashed in a homogenizer for 60 s at 10000 rpm and 25 °C. The shattered mycelia were centrifuged at 5000 rpm and used as inoculum.

2.3. Operation of SBRs

Two groups of SBRs (R1 and R2), which consist of six reactors (work volume of each reactor: 6.0 l) controlled by a microcomputer controlled system, were equipped with a heater, a pH meter, an aerator and an air diffuser [26]. The sludge inoculation process was performed as follows: at 30 °C, the contrast reactors (R1), which included three parallel reactors, were inoculated with 2.0 l of seed sludge [mixed liquor suspended solids (MLSS), 12.8 g/l]; the bioaugmented reactors (R2) were inoculated with 2.0 l of seed sludge and 3.0 g of inoculum (dry weight). During the start-up phase (days 1–3), the reactors were fed with 2.0 l synthetic wastewater (Table 1). After day 3, the synthetic wastewater was replaced with the coking wastewater (Table 1) collected from a wastewater treatment plant of the Anyang Iron and Steel Group (China), and the influent volume was increased from 2.0 l to 4.0 l. The phenol concentration of the coking wastewater was gradually adjusted to a final concentration of 1800 mg/l by adding solid phenol to the wastewater depending on the requirements of the test. The influent pH and the dissolved oxygen were controlled at the levels of 6.5 and 4.6 mg/l, respectively. The operational phases of SBR comprised the feeding phase (10 min), the aerobic phase (4.5 h), the settling phase (30 min) and the discharge and idle phases (50 min).

2.4. Kinetic analysis of phenol degradation

Separate batch tests were conducted on the AS obtained from R1 (AS_{R1}) and R2 (AS_{R2}) on day 30 with the use of synthetic wastewater with different initial phenol concentrations (400–2000 mg/l). Kinetic analysis of phenol degradation was performed based on Haldane's equation for an inhibitory substrate, which is expressed as:

$$V = \frac{V \max S}{Ks + S + S^2/Ki} \quad (1)$$

where V and V_{max} [g phenol g^{-1} volatile SS (VSS) day^{-1}] are the specific phenol degradation rate and the maximum specific phenol degradation rate, respectively; S (mg/l) is the phenol concentration; K_s (mg/l) is the half-saturation constant; and K_i (mg/l) is the inhibition constant.

2.5. Quantification of bacterial and eukaryotic populations

The microbial population in the AS samples was quantified by quantitative real-time polymerase chain reaction (qRT-PCR) method. The total DNA of the samples was extracted by cetyltrimethylammonium bromide method [27]. The yield and fragmentation of DNA were determined by agarose gel electrophoresis and ultraviolet visualization after ethidium bromide (EB) staining. The primer pairs of 27F (5′-AGAGTTTGA-

Table 1
The media and wastewaters used in this work.

Media and wastewaters	Component or characteristic
Liquid medium	10.0 g/l glucose, 2.5 g/l $(NH_4)_2SO_4$, 0.8 g/l $MnSO_4$, 1.0 g/l KH_2PO_4, 0.8 g/l $MgSO_4$, 0.5 g/l $CaCl_2$, and 0.7% trace element solution containing 0.5 g/l glycine, 0.1 g/l $FeSO_4{\cdot}7H_2O$, 0.1 g/l $ZnSO_4$, 10 mg/l $CuSO_4{\cdot}5H_2O$, 10 mg/l H_2BO_3 and 10 mg/l $Na_2MoO_2{\cdot}2H_2O$
Synthetic wastewater	2.0 g/l glucose, 0.5 g/l phenol, 2.0 g/l NH_4Cl, 2.0 g/l KH_2PO_4, 0.8 g/l $MgSO_4{\cdot}7\,H_2O$, 0.5 mg/l $MnSO_4$ and 0.1 g/l $CaCl_2$
Coking wastewater	COD: 2031 mg/l; phenol: 700 mg/l; suspended solids: 660 mg/l; pH: 7.5–8.2
Meat-peptone agar medium	3.0 g/l beef extract, 10 g/l peptone, 5.0 g/l NaCl and 20 g/l agar
Martin agar medium	10 g/l glucose, 5.0 g/l peptone, 1.0 g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$ and 20 g/l agar

TCCTGGCTCAG-3′) and 533R (5′-TTACCGCGGCTGCTGGCAC-3′) for bacteria and 3NDF (5′-GGCAAGTCTGGTGCCAG-3′) and V4euk_R2 (5′-ACGGTATCT(AG)ATC(AG)TCTTCG-3′) for eukaryotic cells were used for PCR quantification [28,29]. The PCR was run at 95 °C for 30 s, 40 cycles of denaturing (5 s at 94 °C), annealing (34 s at 63 °C for bacteria; 60 °C for fungi), and a melting curve analysis from 60 °C to 95 °C, with the plate being read every 0.5 °C. The qRT-PCR assay was conducted in a volume of 25 μl on an ABI 7500Q-PCR machine (USA) using an SYBR green detection system and an SYBR premix EX Taq™ kit (Takara, China). Both agarose gel electrophoresis and melting curve analysis were conducted to confirm the specificity of the amplified products. Three independent PCR assays were performed on each of the three replicate DNA samples.

2.6. Metagenomic sequencing and bioinformatic analysis

Metagenomic DNA of the AS samples obtained from R1 and R2 was extracted by using an E.Z.N.A soil DNA kit (Omega Bio-Tek, USA). The DNA samples were spectrophotometrically analyzed by the Nanovue assay (GE Healthcare, USA) to measure DNA purity and concentration, and they were visualized on a 1.0% of agarose gel containing EB prior to amplification. PCR amplifications of the highly variable V1–V3 regions of the bacterial 16S rRNA gene and the V4 region of the eukaryotic 18S rRNA gene were conducted based on the universal bacterial primer pair (27F and 533R) and the eukaryotic primer pair (3NDF and V4-euk-R2), respectively. The thermo-cycling procedure consisted of an initial denaturation step at 95 °C for 2 min, followed by 25 cycles (16S rRNA) or 30 cycles (18S rRNA), where each cycle consisted of 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing), and 72 °C for 30 s (extension), and a final extension at 72 °C for 5 min. Each reaction was conducted in 20 μl of reaction mixtures containing 10 ng of template DNA, 5 μM of each of the primers, 2.5 mM of the deoxynucleoside triphosphate mix, and 1 unit of FastPfu Polymerase (TransGen Biotech, China). The PCR cycling reactions were performed in a GeneAmp®9700DNA thermocycler (ABI, USA), and the amplified products were visualized on agarose gels containing EB and purified with a DNA gel extraction kit (Axygen Inc., USA). Prior to sequencing, the DNA concentration of each PCR product was determined, and the amplicons from each PCR reaction were pooled together in equimolar ratios to reduce the biases of each individual reaction and subjected to emulsion PCR to generate amplicon libraries. Deep sequencing was performed on a Roche Genome GS FLX system at the Majorbio Company (Shanghai, China). The metagenomic data were deposited in the National Center for Biotechnology Information sequence read archive under the accession number of SRP064766.

Any sequence with more than two base mismatches was discarded by Seqcln software analysis. The low-quality sequences and the redundant reads were further trimmed by the Mothur software. The “dist.seqs” command was performed to identify operational taxonomic unit (OTU) by 97% similarity. The obtained sequences were subjected to Megablast and searched against SILVA aligned 16S/18S small subunit rRNA sequence database (version111) to acquire a high taxonomic resolution [30]. Rarefaction curves, Chao1 richness, and Shannon diversity index were performed by Mothur analysis [31].

2.7. Analytical methods

Chemical oxygen demand (COD), total SS, VSS, MLSS, effluent SS (ESS) and sludge volume index (SVI) were analyzed by standard methods for wastewater examination, and phenol was measured by 4-aminoantipyrine colorimetric method [32]. MnP was spectrophotometrically assayed by the method of Asgher [33]. Sludge floc diameter was measured by using a stage micrometer. Quantification of *P. chrysosporium* was determined by the colony-forming unit method [34]. After coating of the diluted sludge samples on PDA plates, the plates were incubated at 35 °C for 2–3 days, and the specific powdery white colonies were selected and enumerated. Suspected colonies were further identified according to a fungi identification manual [35]. The prevalent bacteria and fungi in AS_{R1} and AS_{R2} were isolated by meat-peptone agar and Martin agar plates, respectively (Table 1), and their identification was conducted according to morphological, physiological, and biochemical characteristics[36] and phylogenetic analysis [37,38]. The partial sequences of 16 or 18S ribosomal RNA gene of the isolates were deposited in the GenBank database under accession numbers KT353080–KT353087. Their phenol degradation capabilities were evaluated in a flask at 150 rpm and 30 °C for 3 days.

Statistical analyses of difference (*t*-test) and correlation (Pearson) significance were performed by the software SPSS, and the difference or correlation coefficient (Coe.) was considered significant when p-value was less than 0.05. Dissimilarity test of sludge samples was performed in R based on the Bray–Curtis dissimilarity index using analysis of similarities[39,40].

3. Results and discussion

3.1. Sludge and reactor performance

Give, its low yield, conidia of *P. chrysosporium* produced by solid cultivation are unsuitable as inoculum at industrial scale. Thus, its mycelia were prepared for bioaugmentation in this work. During the start-up phase, the microbes in sludge rapidly propagated because the reactors were fed with synthetic wastewater with 2.0 g/l of glucose. The color of sludge in both reactors gradually changed from black to yellow or white, and sludge settling performance became better with time. In R1, small flocs with diameters of 0.2–1.1 mm began to shape on day 5, and the sludge was incompact during the entire cultivation. In R2, a number of floc sludges appeared on day 4; after day 5, besides the floc sludges, inconsiderable rodlike sludge also appeared.

The SVI of sludge decreased with time during the operation (Fig. 1a). On day 9, the SVIs in R1 and R2 were 98 and 76 ml/g, respectively. Subsequently, the sludge in both reactors had a stable settling performance, although phenol concentration gradually

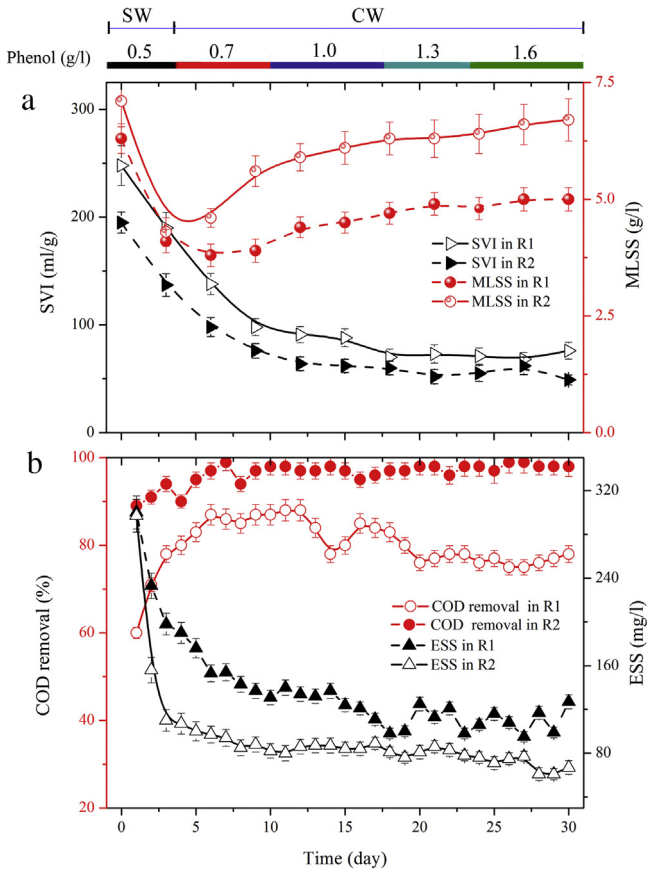

Fig. 1. Variations of the SVI, MLSS, COD removal, and ESS in R1 and R2. (a) Variations of SVI and MLSS with time; (b) Variations of COD removal and ESS with time.

increased. The SVI in R2 was less than that in R1, which indicated that the bioaugmentation can improve sludge settling performance.

The MLSS curves of R1 and R2 indicated a sharp decline during the start-up phase (Fig. 1a) because substantial dispersed sludge with poor settling performance was washed out with the effluent. After day 4, the biomass in both reactors increased with microbial propagation, and MLSS curves had an ascending trend. The MLSS in R2 was higher than that in R1 throughout the test because *P. chrysosporium* was added to R2 besides the domestic sludge.

Compared with R1, the COD removal efficiency of R2 was significantly improved (Fig. 1b). On day 5, 83.3% and 95.1% of COD removals were obtained in R1 and R2, respectively. After day 6, the removal in R2 was kept at the levels of 95.2%–99.0%, whereas that in R1 was below 88.0%. Hence, the reactor without bioaugmentation had a lower treatment capability to phenol wastewater.

Both ESS curves of the two groups of reactors demonstrated a sharply descending trend before day 5. On day 6, the ESSs of R1 and R2 were 153 and 94 mg/l, respectively (Fig. 1b). After day 7, the ESS of R2 stabilized, and all values were less than 85 mg/l. By contrast, the ESS of R1 was considerably higher. In particular, when phenol exceeded 1.0 g/l (on day 15), the ESS curve of R1 fluctuated in the range of 98–143 mg/l, thus validating limited capability of the reactor to withstand an extreme phenol load.

3.2. Kinetic analysis of phenol degradation

The maximum specific degradation rate (SDR) of AS_{R2} reached 1.45 g phenol g^{-1} VSS day^{-1} at 600 mg/l of phenol, and sludge performance remained stable at a phenol concentration as high as 1800 mg/l. A good fit between the degradation data and Haldane's

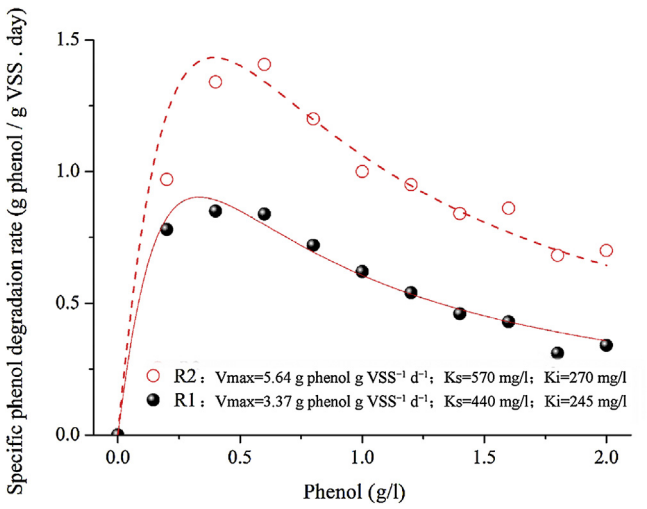

Fig. 2. Batch phenol degradation test with AS from R1 and R2.

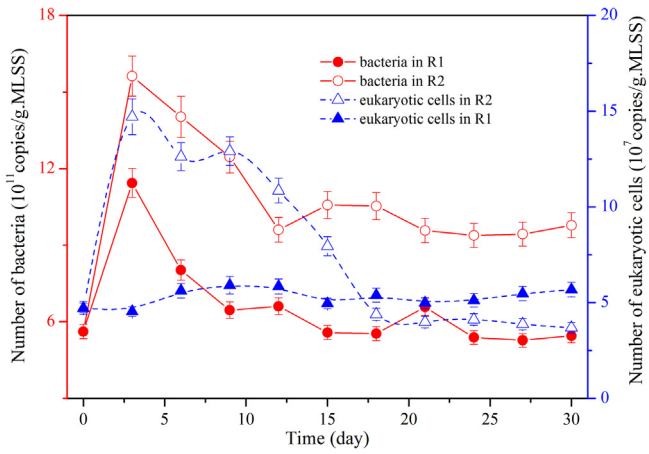

Fig. 3. Enumeration results of bacteria and eukaryotic cells in R1 and R2 during the wastewater treatment.

equation is shown in Fig. 2. High kinetic parameters indicated that AS_{R2} developed an uptake system to counteract the adverse effects of phenol toxicity. AS_{R1} could degrade phenol at a maximum SDR of 0.84 g phenol g^{-1} VSS day^{-1}. After a 12 h treatment, 78.9% of removal was achieved only when phenol was less than 1200 mg/l. Clearly, AS_{R2} exhibited a better removal performance than AS_{R1}. Bacteria, fungi, and other eukaryotes including microalgae, protozoa and metazoa, are the main components in AS, and they are also the crucial forces for pollutant removal during wastewater treatment [41]. Thus, the microorganisms in AS_{R2} were more suitable for phenol degradation.

3.3. Quantification of microbial population

The microbial population in reactors was determined by a qRT-PCR method because some microorganisms are uncultivable [42], and archaea were not considered in view of their low concentration in an aerobic reactor [43,44]. The AS in both reactors harbored an amount of bacteria and eukaryotes, but bacteria were dominant because their quantity is approximately 10,000 times higher than that of eukaryotic cells (Fig. 3). During the start-up phase, bacterial quantity increased in both reactors owing to their rapid propagation. The reactors were supplied with glucose and a low concentration of phenol (0.5 g/l). After day 3, as the phenol con-

centration in the wastewater was gradually increased, bacterial concentration declined in both R1 and R2 until day 12, indicating that bacterial growth was inhibited by phenol toxicity [45]. The bacterial quantity in R2 was greater than that in R1. The quantity of eukaryotic cells in R1 remained relatively stable during the test. In R2, the enhancement of eukaryotic quantity resulting from the addition of *P. chrysosporium* only occurred before day 18. After day 18, the eukaryotic population in R2 was less than that in R1. The addition of *P. chrysosporium* thus changed the microbial quantity in AS during wastewater treatment, and bioaugmentation resulted in an increased bacterial quantity.

3.4. Metagenomic analysis on the bacterial community

3.4.1. General analysis

A total of 78,125 trimmed sequences with an average sequence length of 476 bp were obtained. The OTUs identified for seven samples are shown in Table S1. Good's coverage implicated that 94.09%–99.81% of species in samples were recovered at a cutoff of 97% sequence similarity. The slope of taxon rarefaction curves of samples remained incomplete, except for seed sludge, thereby suggesting that the ture bacterial diversity was likely to exceed the current perceptions (Fig. S1).

Chao1 was used to estimate the bacterial richness in sludge [46], and seed sludge had the lowest Chao1 value. On day 3, the Chao1 estimator of AS_{R2} (1642) was considerably lower than that of AS_{R1} (2666). This phenomenon can be explained by the addition of *P. chrysosporium*, and bacterial richness was reduced because of the ecological competition caused by the fungal growth. On day 15, the estimator of AS_{R2} increased to 2633; to day 30, it surpassed the estimator of AS_{R1}. Therefore, the bioaugmentation finally increased bacterial richness. This fact was in accordance with the PCR analysis of the bacterial population. Shannon diversity index represents the bacterial diversity in sludge [47]. AS_{R2} had a lower Shannon index than AS_{R1}, and the bioaugmentation reduced the bacterial community diversity.

3.4.2. Bacterial community structure

The bacterial OTUs of the sludge samples were grouped into 23 phyla (Fig.S2), and a more than 97.9% of the total relative abundance (RA) of *Actinobacteria*, *Bacteroidetes*, *Candidate*, *Firmicutes* and *Proteobacteria* suggested that they were the main bacteria in the reactors. The RA of *Actinobacteria* exhibited different changes in R1 and R2. By contrast, the RAs of *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Candidate* displayed the same change trend in the reactors. On day 30, both reactors were dominant with *Proteobacteria* (71.60% in R1; 50.43% in R2), followed by *Bacteroidetes* (11.86% in R1; 21.63% in R2) and *Actinobacteria* (9.00% in R1; 9.95% in R2). In many mature wastewater treatment systems, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* as main bacterial groups co-exist for pollution disposal with a few other groups [48–51].

At the genus level, OTUs were divided into 342 genera. The top 100 genera in seed sludge (Fig. S3) were significantly different from those in the sludge sample obtained during wastewater treatment (Fig. 4), which showed the ecological succession of the bacterial community caused by phenol and bioaugmentation. The top 100 genera during wastewater treatment occupied 96.33%–99.29% of RA. These prevalent bacteria, including *Alicycliphilus*, *Ferruginibacter* and so on, were mainly distributed in *Proteobacteria* and *Bacteroidete*. On day 30, 50.69% of the bacteria in R2 were distributed in *Proteobacteria*. By contrast, the distribution in R1 was not such concentrated, and the RAs of *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* were 40.09%, 14.69%, and 8.45%, respectively. Dissimilarity analyses on the AS from the two reactors also showed that their bacterial community structures were significantly different ($F = 0.63$, $p < 0.01$ on day 3; $F = 0.48$, $p < 0.01$ on day 30), thus indicating that the bacterial community structure in R2 was significantly changed by bioaugmentation.

3.5. Metagenomic analysis on the eukaryotic community

3.5.1. General analysis

The 43,739 eukaryotic sequences with an average sequence length of 434 bp were identified as 1414 OTUs by phylogenic analysis (Table S1). Moreover, 94.79%–98.66% of Good's coverages at the 97% similarity level indicated that the sequencing results were sufficient to represent the natural eukaryotic community [52]. Rarefaction curve analysis also revealed that the overall eukaryotic diversity was well exhibited (Fig. S1).

During the treatment, the eukaryotic Chao1 estimators in both reactors increased with time. On day 3, the estimator of AS_{R2} was higher than that of AS_{R1}. This phenomenon was caused by the addition of *P. chrysosporium*. However, the estimator of AS_{R2} was lower than that of AS_{R1} on day 15, and a probable reason was that the rapid growth of *P. chrysosporium* inhibited the propagation of other eukaryotes. On day 30, the richness of AS_{R2} was considerably increased considering that its Chao1 estimator was 1.8 times of that of AS_{R1}. Nonetheless, the bioaugmentation reduced the eukaryotic community diversity, and AS_{R2} had a lower Shannon diversity index than AS_{R1}. The enhancement of community richness and the reduction in diversity meant that some specific eukaryotes were enriched in R2.

3.5.2. Eukaryotic community structure

The eukaryotic community structure was investigated at the class level because of the limited accuracy of molecular identification based on 18S rDNA analysis at the genus level [53]. The eukaryotic OTUs were grouped into 28 classes (Fig. 5) containing fungi (7 classes), microalgae (6 classes), protozoa (11 classes), micrometazoa (4 classes), and uncultured and unclassified eukaryotes. Yeast and filamentous fungi were the main eukaryotes in seed sludge (Fig. S3). On day 3, the RAs of protozoa and metazoa were minimal in AS_{R2} (Fig. 6). However, it reached 97.42% in AS_{R1}, and *Craniata* almost dominated the reactor. On day 15, the RAs of yeast and microalgae decreased in AS_{R2}, whereas the RAs of protozoa, metazoa, and filamentous fungi were enhanced. In AS_{R1}, except that of protozoa and metazoa, the RAs of all other eukaryotes increased. On day 30, the two reactors were dominated by protozoa and metazoa. The prevalent eukaryotes in R2 were protozoa, including *Conosa* (53.05%) and *Lobosa* (8.57%). By contrast, *Rotifera* (67.72%) that was affiliated with micro-metazoa was dominant in R1.

The occurrence of eukaryotes, such as protozoa and metazoa, in wastewater treatment was well documented [54], and they were considered good indicators of the treatment quality of wastewater in aerobic AS systems [55]. Thus, the appearance of different protozoa and metazoa in R1 and R2 suggested their significant difference in reactor performance. The findings of this study also reveal that the total RA of protozoa and metazoan cannot be considered a standard for evaluating reactor performance. The RAs of protozoa and metazoan in R2 were lower than that in R1 during the entire treatment process; nonetheless, R1 did not exhibit a better reactor performance than R2 (Fig. 1b). Except those of protozoa and metazoa, the RAs of yeast, filamentous fungi, and microalgae in AS_{R2} were higher than those in AS_{R1} at all phases. Yeast, filamentous fungi, and microalgae were generally believed as the main eukaryotic groups responsible for pollutant removal [56–58]. Therefore, the bioaugmentation made R2 harbor more eukaryotic cells for phenol removal.

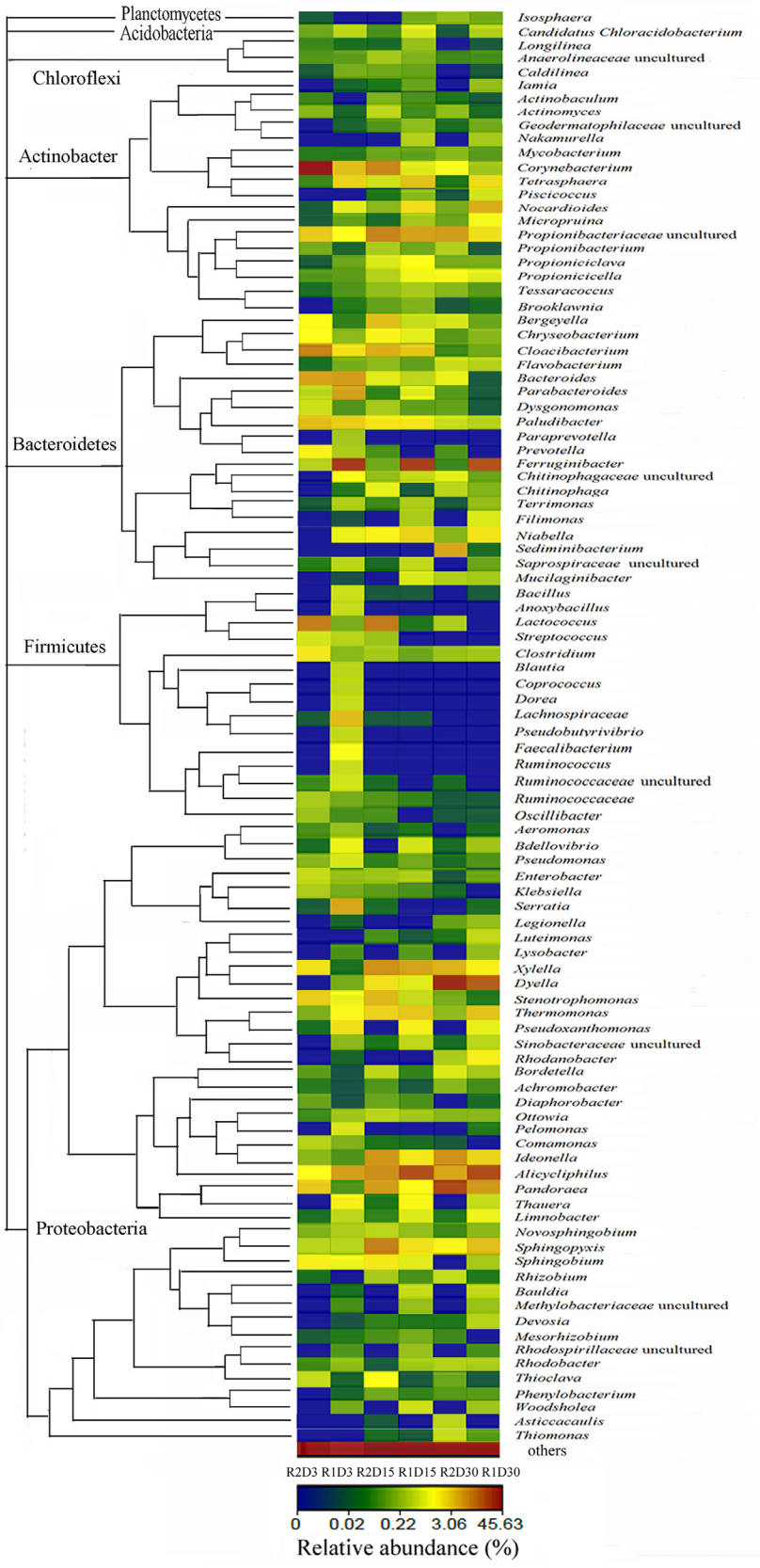

Fig. 4. Heat map illustrating the RA change of the top 100 genera in sludge samples. The color scale indicates the magnitude of RA.

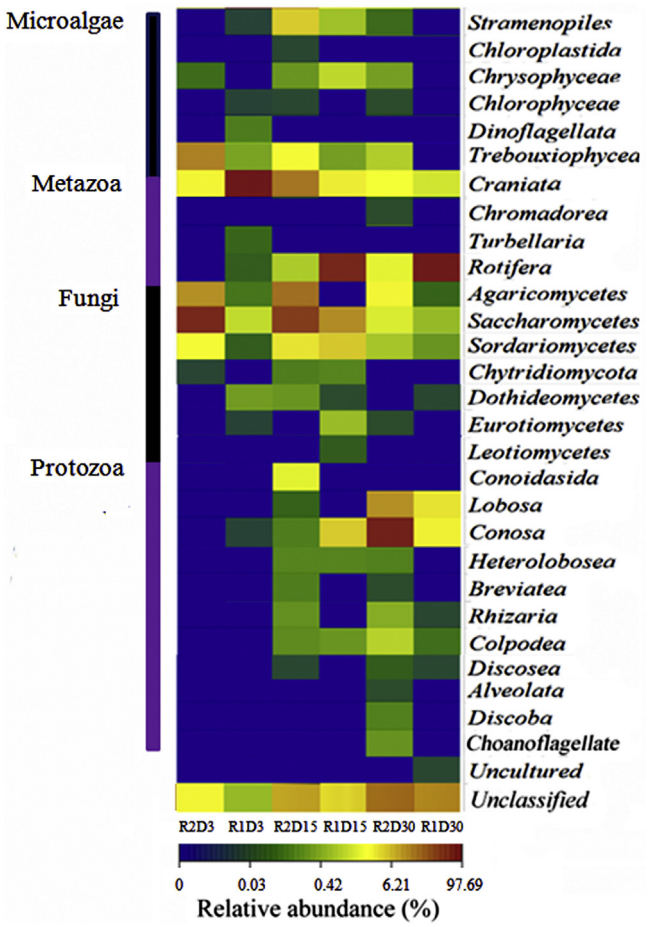

Fig. 5. Heat map illustrating the RA change of the eukaryotes in sludge samples. The color scale indicates the magnitude of RA.

3.6. *Fate of* P. chrysosporium

P. chrysosporium is affiliated with the family *Phanerochaetaceae* in *Polyporales*, which is an order of fungi in the phylum *Basidiomycota*, subclass *Agaricomycetidae* [59]. The addition of *P. chrysosporium* results in the higher RA of *Agaricomycetes* in R2 (Fig. 5). The sequences in *Agaricomycetes* were subjected to Megablast against Genebank, and AS_{R1} did not contain any sequence affiliated with *Phanerochaetaceae*. Hence, *P. chrysosporium* was not native in AS. In R2, the sequences of *Phanerochaetaceae* appeared on days 3 and 15, but they disappeared on day 30. Considering that a molecular method cannot accurately identify eukaryotes at the species level, the quantity of *P. chrysosporium* was counted by colony enumeration. The fungal quantity in R2 initially decreased with time and finally disappeared on day 30 (Fig. 7a). Therefore, the better reactor performance of R2 was not attributed to the survival of *P. chrysosporium*, but it was caused by the change in microbial community structure resulting from bioaugmentation. This phenomenon also occurred in the bioreactor augmented by bacteria [12,60]. The rapid nicotine degradation strain performs a vital function in bioaugmented SBRs, that is, influencing the microbial community structure and the AS activity.

The survival and activity of individual bioaugmentation had not been monitored in several previous applications of the fungal bioaugmentation for enhanced degradation of environmental pollutants [61–63]. We deduce that the slow growth of *P. chrysosporium* is the reason why it did not persist in the AS system. The AS system was dominated by bacteria (Fig. 3), and *P. chrysosporium* has few advantages over the majority of bacteria with a higher reproduction rate in the ecological competition occurring in AS, which might result in its gradual disappearance.

3.7. *Suggested bioaugmentation mechanisms*

During wastewater treatment, phenol toxicity was one selective pressure for microorganisms in AS. Compared with seed sludge, the

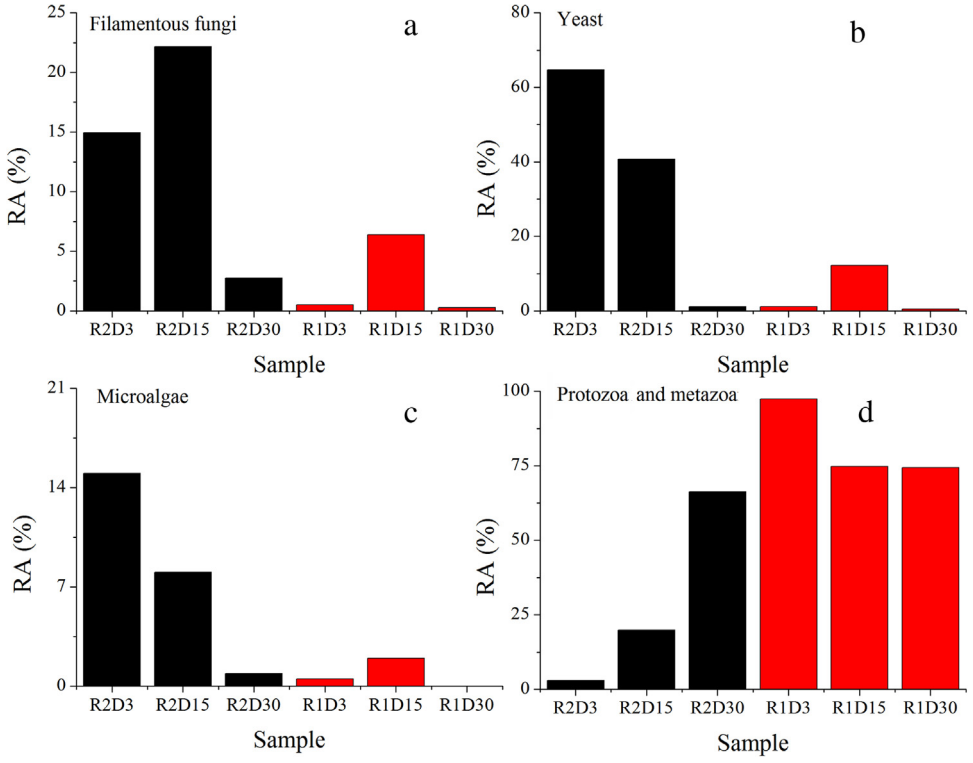

Fig. 6. RAs of filamentous fungi, yeast, microalgae, protozoa, and metazoa in sludge samples. (a) filamentous fungi; (b) yeast; (c) microalgae; (d) protozoa and metazoa.

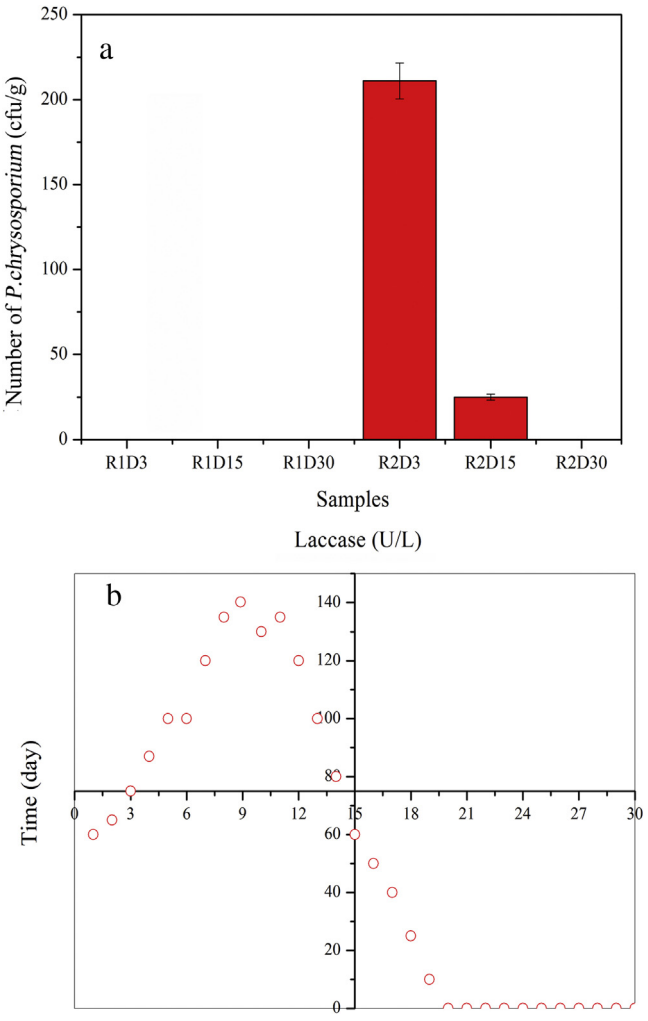

Fig. 7. Number of *P. chrysosporium* and MnP activity in AS system. (a) Number of *P. chrysosporium* in sludge samples; (b) Variation of MnP activity with time.

lower bacterial and eukaryotic diversity index in R1 indicated the toxicity of phenol. However, in R2, another special selective pressure, i.e., the ecological competition caused by *P. chrysosporium*, occurred, and it significantly affected the microbial community in sludge and led to the elimination of microorganisms probably with a lower growth rate than *P. chrysosporium*. This conclusion was supported by the metagenomic data. On day 3, the number of bacterial genus in R2 was 97, which was less than half of that in R1 (208); the number of eukaryotic class in R2 was 8, but it reached 15 in R1. Clearly, the selective pressure changed the microbial community structure and made R2 have low community diversity in the wastewater treatment.

Moreover, MnP activiy was detected (<140 U/l) in R2 from days 1–20 but not in R1 (Fig. 7b). The enzyme secreted by *P. chrysosporium* can minimize wastewater toxicity by degrading phenol [64,65] and protect microbes in AS, thereby enhancing the community richness in R2.

The preceding analyses revealed the functions of *P. chrysosporium* and explained the reduction in community diversity and the enhancement of richness in R2. Nevertheless, how the change in microbial community structure improves reactor performance remains unclear. To date, AS containing a tremendous amount of microbes is a mystery, and clarifying the characteristics of every species in sludge and the intricate relationship among them is difficult. Therefore, in this work, the phenol degradation capabilities of some prevalent bacteria and fungi in sludge were evaluated by degradation test and literature survey (Table 2). For bacteria, no evidence showed that *Xylella*, *Tetrasphaera*, *Ferruginibacter*, and *Thermomonas* can assimilate phenol, although the *latter* two genera that were high in R1 always appeared in the coking wastewater treatment plants in the steel industry [66]. *Alicycliphilus*, *Corynebacterium*, *Ideonella*, and the uncultured *Propionibacterium* were phenol degraders [67–69,51]. The isolated *Nocardioides* and *Sphingopyxis* can degrade phenol wastewater with a low concentration, whereas the *Sediminibacterium*, *Pandoraea*, and *Dyella* that were enriched in R2 all exhibited high phenol degradation capabilities. In particular, the phenol degradation of an isolated *Rhizobium* strain reached 95% when the initial phenol in wastewater was 1000 mg/l. Spearman's correlation analysis showed that its RA had a significant correlation with the maximum SDR of R1 (Coe. = 1.00) and R2 (Coe. = 0.98) at the 0.01 level at different phases. For eukaryotes, only yeasts, including *Candida* and *Pichia*, were obtained. Both demonstrated a high capability in phenol degradation. According to the RAs of these prevalent microorganisms, R2 enriched more prevalent microbes with phenol degradation capability, and this might be used to explain the better reactor performance of R2.

The aforementioned facts only partly explain the relationship between microbial community structure and reactor performance because the number of prevalent microbes investigated is limited. The phenol degradation capability of the isolated strains also

Table 2
Phenol degradation capabilities of the prevalent bacteria and fungi in R1 and R2.

	Microorganisms (genus)	Number of strain	Phenol (g/l)	Maximum degradation ability (%)	Access number[a]	Literature
Bacteria	*Alicycliphilus*	N		Phenol degrader		[68]
	Ferruginibacter	N		Unknown		
	Dyella	2	400	60–89	KT353082	
	Pandoraea	6	200	90–97	KT353083	
	Sphingopyxis	5	100	76–80	KT353084	
	Sediminibacterium	1	200	65	KT353085	
	Ideonella	N		Phenol degrader		[51]
	Corynebacterium	N		Phenol degrader		[67]
	Propionibacteriaceae	N		Phenol degrader		[69]
	Xylella	N		Unknown		
	Rhizobium	1	1000	95	KT353086	
	Thermomonas	N		Unknown		
	Tetrasphaera	N		Unknown		
	Nocardioides	3	50	60	KT353087	
Fungi	*Candida*	36	800	90–98	KT353080	
	Pichia	10	400	50–87	KT353081	

N, the species in this genus is not obtained

[a] The access number of strain having the maximum phenol degradation capability.

does not represent that all of the species belonging to this genus in AS have the same capability. Further study on the microbial characteristics in AS is helpful to understand the bioaugmentation mechanism. The results presented here generally prove that filamentous fungus can also be used for bioaugmentation, and it improves reactor performances by influencing the microbial community structure in the reactor. Moreover, the conclusion obtained herein might affect the conventional criteria for selecting the strain used for bioaugmentation in an AS system considering that *P. chrysosporium* does not survive in a bioaugmented reactor.

4. Conclusions

This study demonstrates the promising potential of bioaugmentation with a filamentous fungus in improving reactor performances during phenol wastewater treatment. The results reveal that bioaugmentation with *P.chrysosporium* is a good alternative for phenol removal even though it is not persistently active in the bioreactor. The fungus improves reactor performances by influencing microbial community structure. Meanwhile, bioaugmentation results in increased microbial richness and reduced community diversity. *P. chrysosporium* altered the microbial distribution in the reactor. By contrast, bacterial distribution in the bioaugmented reactor becomes more concentrated in *Proteobacteria*. Comparable abundances of filamentous fungi, yeast and microalgae are higher at different treatment phases. In addition, the two reactors are dominated by different protozoan and metazoan. These phenomena are attributed to the ecological competition among microorganisms in sludge and to the reduced toxicity of phenol wastewater. Future studies should focus on the characteristics of specific prevalent microbes. Gaining insight into their functions would better elucidate the bioaugmentation mechanism.

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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.jhazmat.2016.09.072.

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