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The response of anaerobic ammonium oxidation process to bisphenol-A: Linking reactor performance to microbial community and functional gene

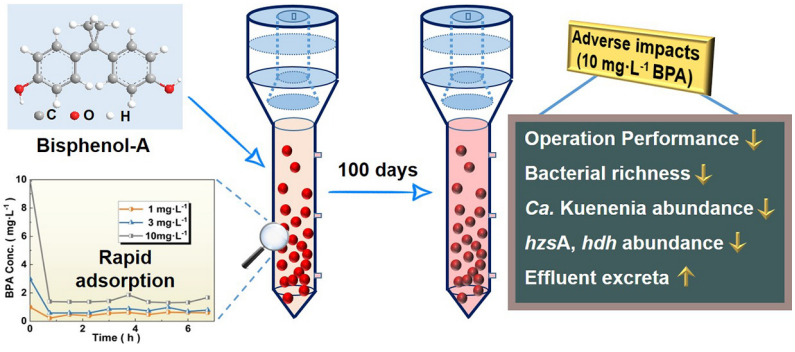
Jing-Peng Li [1], Qi Liu [1], Ye-Nan Gu [1], Shi-Xu Wang, Gui-Feng Li, Nian-Si Fan, Bao-Cheng Huang *, Ren-Cun Jin

School of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 311121, China

HIGHLIGHTS

- 10 mg L^{-1} bisphenol-A (BPA) would cause 60% decline of specific anammox activity.
- BPA would affect sludge metabolism and lead to effluent dissolved organic matter increase.
- BPA would reduce *Ca. Kuenenia* abundance but increase SBR1031 richness.
- 10 mg·L^{-1} BPA would induce reduction of *hzs*A and *hdh* abundances.

GRAPHICAL ABSTRACT



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ABSTRACT

As a typical endocrine disruptor, bisphenol A (BPA) has been widely detected in various water bodies. Although the influence of BPA on traditional biological treatment system has been investigated, it is not clear whether it has potential impact on anaerobic ammonium oxidation (anammox) process. The short- and long-term influences of BPA on reactor operational performance, sludge characteristics and microbial community were investigated in this study. Results revealed that 1 and 3 mg L^{-1} BPA exhibited a limited adverse impact on granular sludge reactor performance. However, exposure of sludge under 10 mg L^{-1} BPA would cause an obvious inhibition on nitrogen removal rate from 10.3 ± 0.2 to 7.6 ± 0.4 kg N m^{-3} d^{-1}. BPA would affect granular sludge metabolic substance excretion and lead to effluent dissolved organic content increase. Both the microbial community and redundancy analysis showed that BPA exhibited a negative influence on *Ca. Kuenenia* but a positive correlation with *SBR1031*. Low BPA concentration appeared a limited impact on functional genes while 10 mg L^{-1} BPA would cause decline of *hzs*A and *hdh* abundances. The results of this work might be valuable for in-depth understanding the potential influence of endocrine disruptor on anammox sludge.

1. Introduction

Emerging environmental pollutants have potential adverse effects on humans and wildlife. Among which, pharmaceuticals and personal care products have attracted wide attention in the past decades. Both of the European Union and the United States of Environmental Protection Agency have listed them as priority pollutants (Ebele et al., 2017). As a typical pharmaceuticals and personal care products, bisphenol-A (BPA) has been widely used as an important organic chemical raw material in the production of polymer materials (Liu et al., 2009) and fine chemical products (Chen et al., 2016). During the production and daily use process, BPA would inevitably release into the environment (Huang et al., 2017). Up to now, it has

* Corresponding author.
E-mail address: huangbc@hznu.edu.cn (B.-C. Huang).
[1] These authors contributed equally to this work.

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been widely detected in a variety of places including industrial wastewater, urban sludge, rivers and river bottom mud, at varied degrees (Fürhacker et al., 2000). BPA released into environment is possible to induce potential toxicity risks and deteriorate endocrine system stabilization. Herein, comprehensively evaluating the fate and ecotoxicity of BPA is of great significance for its pollution dispersal control. Bioreactor will be of high possibility to become a sink for micropollutants when treating this contaminant-contained wastewater. From this perspective, clarifying the potential influence of BPA on treatment performance of biological treatment system is essential.

Biodegradation and adsorption are the leading micropollutant removal mechanisms in biological system. In a conventional activated sludge bioreactor, BPA was reported to be biologically transformed (Jewell et al., 2014). However, anaerobic system was found to exhibit a limited BPA removal performance (Abargues et al., 2018; Chen et al., 2018). Although the fate and removal efficiency of endocrine disrupter in traditional wastewater treatment system has been previously surveyed and investigated (Hu et al., 2019; Wang et al., 2019), its impact on novel autotrophic nitrogen removal process, *i.e.*, anaerobic ammonium oxidation (anammox), has not been fully evaluated. Anammox bacteria not only distribute at a variety of habitats, but also demonstrate a promising engineering application prospect (Harry et al., 2012; Ma et al., 2020). Considering its promising application for wastewater treatment, comprehensively evaluating the influence of BPA on anammox process is then meaningful.

Both of the Alvarino et al. (2015) and Kassotaki et al. (2018) observed a high removal efficiency of pharmaceuticals and personal care products in one stage nitritation/anammox process. However, in their studies, the exact role of aerobic nitritation and anoxic anammox process on micropollutants removal was unclear. Comparing to the aerobic or heterotrophic biological process, the response of anoxic-autotrophic anammox process to the BPA might be different. To date, the treatment capacity of such micropollutants in a single anoxic anammox process is still unclear. More importantly, the microbial community response to BPA stress is also not fully studied yet.

Therefore, the main objective of this study was to evaluate the potential impact of BPA on the anammox process. Firstly, the short-term influence of BPA on the activity of anammox sludge was explored. Then, studies were carried out in a continuous bioreactor by long-term dosing BPA at 1, 3, and 10 mg L^{-1}. The changes in sludge properties including extracellular metabolic substance excretion, kurtosis of functional genes, and specific anammox activity (SAA), were studied. Furthermore, the evolution of bacterial communities under BPA stress was evealed. The results of this work might help us better understanding the fate of BPA in anammox bioreactor and its interference mechanism to the sludge.

2. Materials and methods

2.1. Seeding sludge and anammox reactor operation

The granular sludge used in this experiment was collected from our bench-scale up-flow anaerobic sludge blanket reactor (60 L), which has been operated for about one year under the nitrogen loading rate (NLR) of 6.0 g $N{\cdot}L^{-1}{\cdot}d^{-1}$. The dominated anammox bacteria were the genus *Candidatus Kuenenia stuttgartiensis* (Zhang et al., 2016). Continuous-flow experiments were carried out in two identical up-flow anaerobic sludge blanket reactors (1.0 L effective volume) at 35 ± 1 °C. The initial suspended solids, hydraulic retention time, NLR and pH was maintained at 27 g L^{-1}, 0.88 h, 11.5 kg-N m^{-3} d^{-1} and 7.5, respectively. In this study, synthetic wastewater consisted of substrate, trace elements and minerals was used, which has been reported in our previous work (Zhang et al., 2021). $(NH_4)_2SO_4$ and $NaNO_2$ were used as sources of ammonium and nitrite, respectively.

The above two reactors were initially operated in parallel. After their treatment performances were stabilized (days 1–24, phase I), 1, 3, and 10 mg L^{-1} BPA was introduced into reactor B (R_B) at phase II (days 25–48), phase III (days 49–73), phase IV (days 74–98), respectively. In this work, reactors were assumed to be stable once nitrogen removal efficiency stabilized at ≥85% for more than seven days. In comparison, reactor A (R_A) without BPA addition was served as control.

2.2. Batch test

To evaluate the acute toxicity of BPA on anammox sludge, batch tests were also conducted. After washing three times, sludge collected from R_B was inoculated into serum flasks (100 mL) and the biomass concentration in each flask was about 1.7 g volatile suspended solids (VSS) L^{-1}. Synthetic wastewater was supplied to bring the substrate concentration to 200 mg N L^{-1} in each flask. HCl (0.1 M) or NaOH (1 M) was used to adjust the initial pH to 7.5 ± 0.1 in each serum flask. All flasks were pre-purged with high purity (99.99%) argon to remove dissolved oxygen and then cultivated in a shaker (180 rpm, dark, 35 ± 1 °C). At a certain time interval, collecting water samples to determine ammonium, nitrite, nitrate and BPA concentration (APHA, 2005).

2.3. High-throughput sequencing and functional genes quantification

Sludge samples at the days 48, 73, 98 were collected from reactor. DNA extraction was performed by using Power Soil DNA kit (Mo Bio Laboratories, USA). The DNA quality was determined by 1% agarose gel, and the quantity was determined by a spectrophotometer (Nano Drop ND-1000, Thermo Scientific, USA). The universal primer 341F/805R targeting V3-V4 region was used for 16S rRNA gene amplification. Microbial community analysis was performed by high-throughput sequencing, through the Illumina Mi Sep system by Majorbio Bio-pharm Technology (Shanghai, China). The functional genes including *hdh*, *hzs*A, and *nir*S were quantified by quantitative polymerase chain reaction (qPCR), according to the previously reported operation procedures (Zhang et al., 2020). In this study, relative gene abundance, which was obtained *via* dividing absolute gene abundance with total sludge amount used, was reported.

2.4. Other analysis

The standard method (APHA, 2005) was applied to measure NH_4^+-N, NO_3^--N, NO_2^--N, VSS, pH and suspended solids. BPA was detected by a high performance liquid chromatograph (Essentia LC-16, Shimadzu, Japan), with acetonitrile-water (6:4) as mobile phase. A fluorescence spectrophotometer (F-4600, Hitachi Co., Japan) was used to collect fluorescent spectra of effluent samples. Parallel factor (PARAFAC) analysis was performed to extract the overlapped fluorescent component, according to the previous work (Li et al., 2020).

3. Results and discussion

3.1. The acute toxicity effect of BPA on anammox sludge

The short-term impacts of 1, 3, and 10 mg L^{-1} BPA on anammox sludge were evaluated under 200 mg L^{-1} fixed substrate concentration condition. The SAA of the sludge treated with 1 mg L^{-1} BPA was 525 mg N g^{-1} VSS d^{-1}, close to its initial value (Fig. 1a). At 3 mg L^{-1} dosage, SAA exhibited a slight decrease while still maintained at a high value. However, after exposure of sludge to 10 mg L^{-1} BPA, SAA significantly declined to 217 mg N g^{-1} VSS d^{-1}. These results indicated that 1–3 mg L^{-1} BPA had a low acute toxicity to anammox sludge while 10 mg L^{-1} BPA would significantly inhibit sludge activity.

In order to explore the sludge adsorption capacity, the variation of BPA content in supernatant was detected. Results showed that when BPA was added to the serum flask, it was quickly adsorbed by the anammox sludge within 0.75 h, and its concentration in supernatant remained stable thereafter (Fig. 1b). In particular, more than 85% of BPA was adsorbed onto the sludge surface when the concentration of BPA was 10 mg L^{-1}, which might be the possible reason inducing inhibition effect.

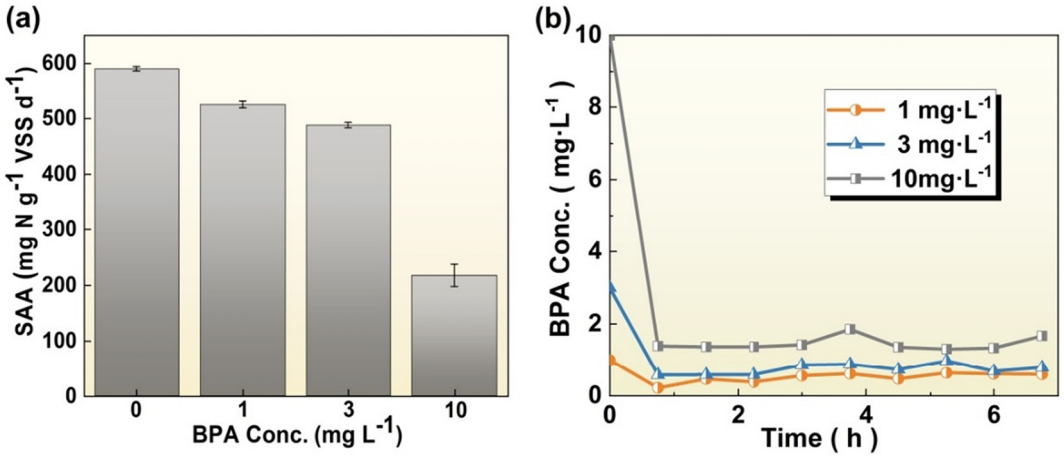

Fig. 1. Short-term effect of BPA on anammox granules: (a) variation in specific anammox activity and (b) concentrations of BPA in supernatant.

3.2. The long-term effect of BPA on nitrogen removal performance

At phase I, two bioreactors operated under the same conditions during days 1–24, where no BPA was dosed. During this phase, although there is fluctuations in influent nitrogen content, the effluent quality of two reactors remained as stable (Fig. 2a and b). Under the NLR of 11.8 ± 1.2 kg N m^3 d^{-1}, the nitrogen removal rate (NRR) maintained stable at 10.2 ± 0.4 kg N m^3 d^{-1} (R_A) and 10.3 ± 0.2 kg N m^3 d^{-1} (R_B), respectively (Fig. 2c). Nitrogen removal efficiency (NRE) was up to 90%, implying a good treatment performance. The stoichiometric ratio of reactants and products in a balanced anammox reaction provides evidence for the enrichment of anammox bacteria (Ni et al., 2011; Sun et al., 2011). The calculated R_S

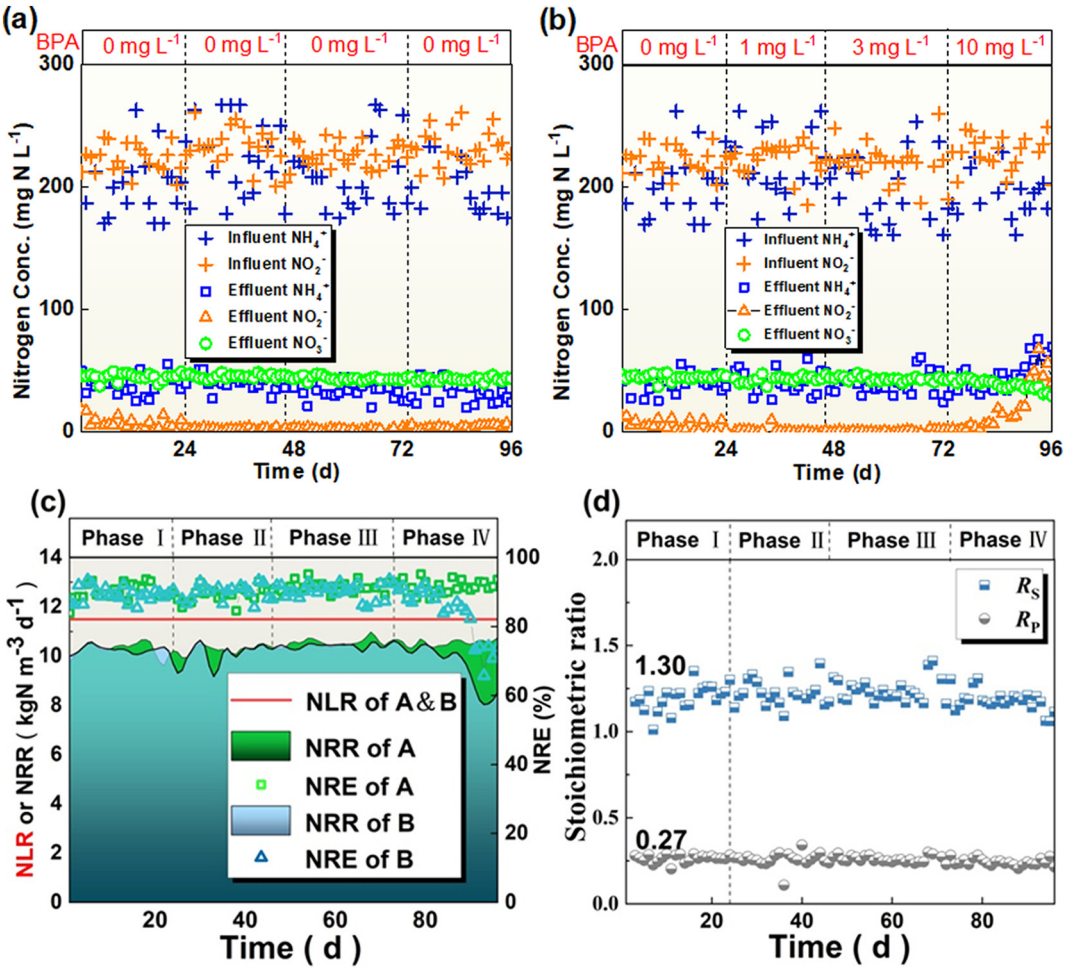

Fig. 2. Changes in nitrogen removal performance of R_A (a) and R_B (b) under different concentrations of BPA during the periods of phase I to phase IV. (c) The nitrogen-loading rate and removal rate (NLR and NRR). (d) The stoichiometric ratios (R_S = NO_2^--N conversion/NH_4^+-N depletion and R_P = NO_3^--N production/NH_4^+-N depletion).

(the ratio of NO_2^--N conversion to NH_4^+-N depletion) and R_P (the ratio of NO_3^--N production to NH_4^+-N depletion) at phase I were 1.28 ± 0.11 and 0.28 ± 0.06, respectively (Fig. 2d). The values were similar to the reference values (R_S = 1.32 and R_P = 0.26) of the anammox reaction, indicating that anammox sludge was responsible for the TN removal in bioreactors.

When 1 mg L^{-1} BPA was dosed to R_B (phase II), the treatment performance remained as negligibly changed. The effluent NH_4^+-N, NO_2^--N, and NO_3^--N concentration of R_B was consistent with R_A and remained at 46.8, 6.3, and 41.9 mg L^{-1}, respectively (Fig. 2b). At the end of phase II, the SAA of granules in R_A and R_B was 585.5 and 688.5 mg N g^{-1} VSS d^{-1} approximately, close to their initial level. Further increase into 3 mg L^{-1} BPA dosage (phase III) also shows negligible impact on bioreactor performance. The concentration of NH_4^+-N, NO_2^--N, and NO_3^--N in R_B effluent was 39.6, 1.0, 43.8 mg L^{-1} and the corresponding NRR and NRE reached 10.4 kg N m^{-3} d^{-1} and 90%, respectively. The above results indicated that 1 and 3 mg L^{-1} BPA can hardly affect the treatment performance of anammox bioreactor.

However, 10 mg L^{-1} BPA (days 74–98, phase IV) was found to cause an obvious inhibition on sludge activity. During the initial 10 days (days 74–83), the NRR of R_B declined from 10.3 ± 0.2 kg N m^{-3} d^{-1} to 9.6 ± 0.3 kg N m^{-3} d^{-1}, and finally dropped to 7.6 ± 0.4 kg N m^{-3} d^{-1}. Correspondingly, the NRE of R_B decreased from 90% to 83% (Fig. 2c). The above results indicated that the effects of BPA on sludge experienced a latent period. Effluent NH_4^+-N and NO_2^--N contents increased to 48.3 and 19.4 mg N L^{-1}, respectively, and the accumulation of NO_2^--N in the effluent was observed. NRE and NRR declined to 71% and 7.6 kg N m^{-3} d^{-1} at the end of this period. Also, the average values of R_S and R_P during days 74–98 were 1.20 ± 0.13 and 0.21 ± 0.03, respectively (Fig. 2d). The dramatic fluctuation of R_S and R_P were suggestive of disordered anammox metabolic pathways. In addition, the SAA of sludge decreased to 398.35 mg N g^{-1} VSS d^{-1}, which was only 63% of the initial level (Fig. 3).

3.3. Effluent fluorescence component variation

EEM coupled with PARAFAC analysis has been widely applied for bioreactor effluent analysis due to its sensitivity in identifying fluorescence substance (Wen et al., 2003; Maqbool et al., 2017; Qian et al., 2019). Operation condition change or toxic substance stress commonly cause interference on microbial metabolism balance and result in soluble microbial products variation (Zhang et al., 2019). According to previous studies, the operation status of anammox bioreactor can be effectively reflected *via* tracking effluent fluorescence component variation (Lu et al., 2017; Li et al., 2020). Herein, the effluent EEM spectra were collected by scanning at excitation wavelengths of 200–450 nm and then PARAFAC analysis

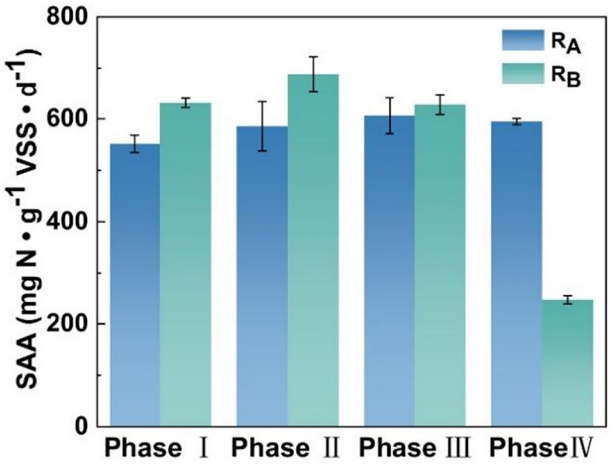

Fig. 3. Long-term effects of BPA on anammox granules: variation in specific anammox activity (SAA) during each phase.

was performed. There were two components centered at excitation/emission (Ex/Em) = 275/300 (Component 1), (250, 280)/400 (Component 2) nm in bioreactor effluent (Fig. 4a–d), which corresponds to protein-like and humic-like substances, respectively (Li et al., 2020).

The fluorescent scores of two components in reactor R_A were found to be slightly varied across the entire operation period (Fig. 4e). In comparison, the effluent fluorescent substances of R_B showed a similar variation during phases I–III (Fig. 4f). However, under 10 mg L^{-1} BPA stress, the relative content of protein-substance (component 1) in effluent increased, whose variation amplitude was much greater than that of humic-like substances (component 2). A similar result was observed when low temperature as a stimulator imposed on anammox bioreactor (Li et al., 2020). As a sensitive indictor, effluent protein-like fluorescence intensity is positively related to the anammox sludge activity. Herein, 10 mg L^{-1} BPA would adversely affect the sludge activity and interfere microbial metabolism, which resulted in effluent protein-like component decline.

3.4. Microbial community shift and functional genes response

Based on the results of α-diversity (Table 1), it was found that both the Chao1, ACE and Shannon indexes declined with the increased BPA dosage. The dominant phyla found in bioreactors were *Planctomycetes*, *Proteobacteria*, *Chloroflexi*, *Bacteroidetes*, and *Acidobacteria* (Fig. 5a). Among them, *Planctomycetes* decreased from 23% to 17% while *Chloroflexi* increased from 23% to 31% during the phase II-IV respectively, under BPA stress. *Chloroflexi* was widely reported as the coexistent bacteria in anammox system, which was able to survive by using lytic cell as substrate (Kindaichi et al., 2012; Li et al., 2020; Fu et al., 2021). High concentration of BPA would possible induce inhibition on autotrophic bacteria and lead to soluble organic matter increase (Fig. 4f), which might support the growth of *Chloroflexi*.

Taxonomic details at the genus level show that the dominant anammox bacteria in two reactors were *Ca. Kuenenia* (Fig. 5b). Also, a high percentage of *Denitratisoma* was found and remained at a stable status during the entire operation period. The genera *Denitratisoma* was identified as capable of driving heterotrophic nitrogen removal (Ahmad et al., 2021). In this work, no external carbon source was supplied in two reactors, which indicates that available carbon content for heterotrophic nitrogen removal is limited. In addition, chemometrics calculation indicated that both the *Rs* and R_P matched well with the theoretical value of anammox reaction. Herein, the contribution of nitrogen removal through *Denitratisoma* might be limited, even its relative abundance was high. Comparing to a fluctuant abundance in control reactor (R_A), the relative abundance of *Ca. Kuenenia* remained unchanged in bioreactor (R_B) at the end of phases II and III. Whereas, it decreased from 18% to 16% when 10 mg L^{-1} BPA was dosed. Meanwhile, the relative abundance of *SBR1031* increased from 11% on day 48 to 15% on day 73, and finally stabilized at 20% at the end of phase IV. The genera *SBR1031* belongs to *Chloroflexi*, which was documented to have the ability of aromatic compound degradation (Colatriano et al., 2018) and possibly play an important role in eliminating extracellular peptides and cell materials (Zhao et al., 2018). Microbial redundancy analysis at phylum level showed that a clear separation along the RDA2 vector (45.3%), which was mainly due to the proliferation of *Sumerlaeota*, *Myxococcota*, *Acidobacteriota*, *Actinobacteriota*, and *DTB120* (Fig. 5c). At the genus level, BPA showed a negative influence on *Ca. Kuenenia* but a positive correlation with *SBR1031* (Fig. 5d). The above results implied that BPA was possible to induce anammox bacteria abundance decline while increase the heterotrophic bacteria richness. Heterotrophic microorganism was capable of removing death cell and other organics. Also, they might be beneficial to maintaining anaerobic microenvironment, which is favorable for anammox consortia stability. This was similar to the results of Su et al. (2022), who deduced that heterotrophic bacteria including *SBR1031* in sludge floc benefited for the stability of nitrogen removal system.

Three main genes including *hzs*A, *nir*S, and *hdh* play crucial roles in fulfilling anammox reaction. During the entire operation phases, the

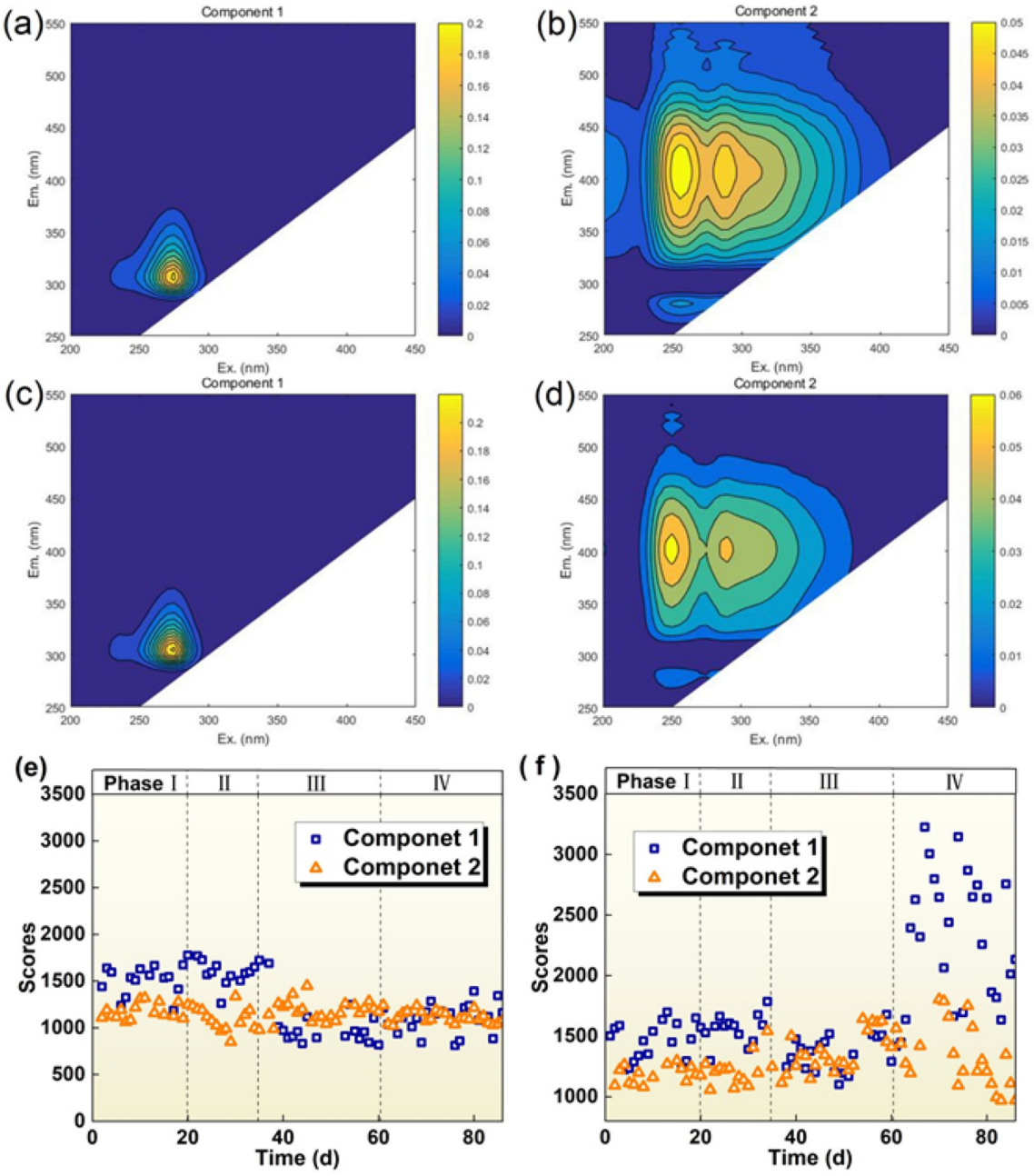

Fig. 4. The effluent fluorescence substances of R_A (a, b) and R_B (c, d) identified by PARAFAC approach, and fluorescent scores of R_A (e) and R_B (f).

abundance of the above genes in control reactor maintained at a stable level (Fig. 6a). In comparison, the abundance of *hdh* decreased from 3.5×10^6 to 2.7×10^6 copies g^{-1} vss when BPA increased from 1 to 3 mg L^{-1} (Fig. 6a). When the concentration of BPA was 10 mg L^{-1}, *hdh* decreased to 1.7×10^6 copies g^{-1} vss. In the meantime, the abundance of *hzs*A significantly reduced from 3.5×10^6 to 2.2×10^6 copies g^{-1} vss. A minor gene abundance variation during phase II and III was in accordance with the reactor performance, which implied a negligible influence of 1 and 3 mg L^{-1} BPA on anammox bioreactor. Under 10 mg L^{-1}, both the abundances of *hzs*A and *hdh* decreased, indicating high concentration BPA would induce damage to the functional genes and result in a deteriorated operation performance. Also, this work found that the *hdh* was preferentially decreased than that of *hzs*A. However, the behind mechanism was unclear, which still required further investigation.

Table 1
Microbial community richness and diversity in R_A (control) and R_B (BPA dosed) at phases II, III, and IV.

Sample	Chao1	ACE	Shannon	Simpson	Coverage
R_A-II	361	367	3.32	0.10	0.9989
R_A-III	360	366	3.75	0.05	0.9991
R_A-IV	407	407	3.94	0.04	0.9992
R_B-II	391	379	3.73	0.06	0.9991
R_B-III	387	377	3.73	0.06	0.9988
R_B-IV	358	364	3.60	0.06	0.9992

4. Conclusions

In this work, the response of anammox sludge to short- and long-term BPA stress was evaluated. It was found that 1 and 3 mg L^{-1} BPA exhibited a low acute toxicity while 10 mg L^{-1} BPA would significantly inhibit sludge activity. Continuous flow experiment revealed that 10 mg L^{-1} BPA would

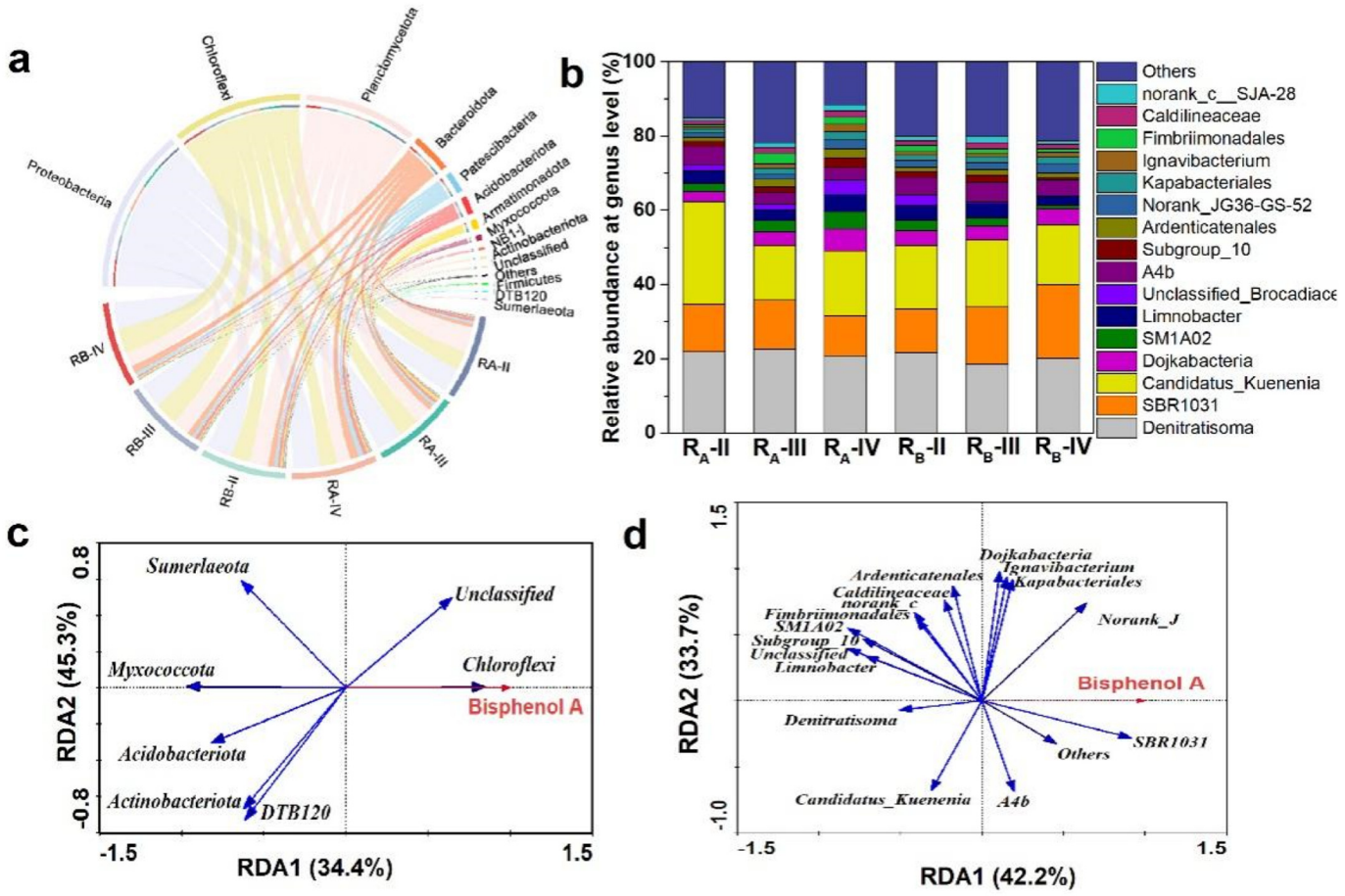

Fig. 5. Microbial community shift under the BPA stress. Microbial community of anammox granule at phylum (a) and genus level (b); redundancy analysis (RDA) of the community evolution at phylum (c) and genus level (d).

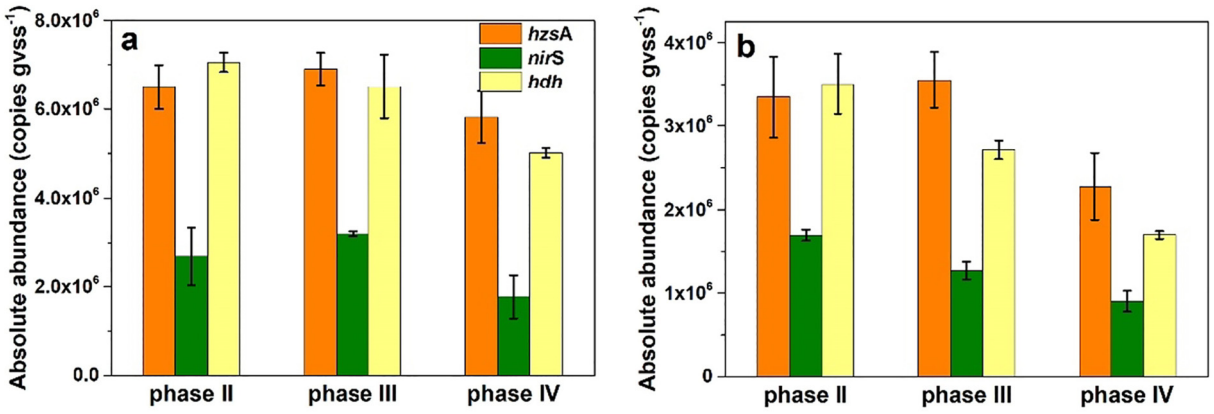

Fig. 6. Quantification results of anammox related functional genes at different operation phases in control (a) and BPA stressed (b) reactors.

deteriorate bioreactor performance and both the NRE, NRR and SAA showed a clear decline. High concentration BPA would interfere microbial metabolism and result in effluent protein-like substance increase. Anammox bacteria abundance declined while heterotrophic bacteria richness increased after exposing sludge at BPA. At the genus level, BPA showed a negative influence on *Ca. Kuenenia* but a positive correlation with *SBR1031*. Also, high content BPA was found to reduce the abundances of *hdh* and *hzs*A.

CRediT authorship contribution statement

Jing-Peng Li: Methodology, Investigation, Writing - original draft.
Qi Liu: Methodology, Investigation, Writing - original draft.
Ye-Nan Gu: Methodology, Investigation, Validation, Visualization.
Shi-Xu Wang: Writing - review & editing.
Gui-Feng Li: Methodology.
Nian-Si Fan: Writing - review & editing.

Bao-Cheng Huang: Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing.

Ren-Cun Jin: Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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