

METAGENOMIC ANALYSIS OF BIOFLOC PRODUCED FROM FURFURAL RESIDUE WASTEWATER TREATMENT

Zhang Lei, Wang Feihong✉

Received on September 9, 2024
Presented by H. Najdenski, Member of BAS, on December 17, 2024

Abstract

Biofloc technology (BFT) is an environmentally-friendly and recyclable technology. This technology is widely used in the aquaculture industry and has vast prospects due to its ability to convert pollutants in aquaculture tail water into microbial protein feed. BFT was derived from the activated sludge method but in past studies it only had a role in aquaculture waste. In this study, we used metagenomic technology to study the microbial structure of bioflocs (BF) produced from furfural residue wastewater. The microbial structure is one of the most important parts of BF applications and must be distinguished from activated sludge. Metagenomic analysis is suitable to show us the essence of BF. *Bacillota* (27%), *Pseudomonadota* (18%), *Mucoromycota* (20%), and *Ascomycota* (15%) are the dominant microbes in BF, which are different from the microbiological compositions in CK group (*Pseudomonadota* accounts for 81% of total microorganisms). The heatmap shows a significant difference in BF and CK. The relative abundance of ARGs (Antibiotics resistance genes) in BF is also lower than CK, decreasing by 62.13%. This research shows that the BF produced by furfural residue wastewater contains the properties of BF from aquaculture. This result provides a theoretical basis for promoting BFT in organic wastewater from all fields in the future.

Key words: BFT, biofloc, metagenomic analysis, furfural residue wastewater

Introduction. Biofloc technology (BFT) is a strategic technology with environmentally friendly and agriculturally beneficial traits. It conforms to the

characteristics of the times and the requirements of development [1]. BFT has been applied in aquaculture systems for over seventy years. Essentially, BFT is a complex ecosystem that involves nutrition, animal healthcare, waste degradation, and water purification [2].

As a wastewater treatment, BFT is successful in removing ammonia and nitrate waste from shrimp farm wastewater and in maintaining nutrients and water quality at safe levels [3]. In addition to aquaculture wastewater treatment, we only found a case in treating the wastewater which contained high organic solids, nitrogen waste, and phosphorus in the food processing industry [4]. As a feedstuff, BF has been widely used in aquaculture, especially for shrimp and prawn [5, 6].

So, can we use BFT to transform other types of organic wastewater? There is no doubt about the question. But the first task is to distinguish BF and sludge. We also see the mixed usage of activated sludge in BFT. The most likely difference must be single-cell protein (SCP) and the productive strain. The common microorganisms used to produce SCP include mould, yeast, algae, and specific bacteria [7–9]. Furfural wastewater, a type of processing water, contains a lot of organic matter. In the straw biorefinery field, the treatment of processing water is a limiting technique. From the microbial structure of BF in other studies, most of them belong to SCP, while the sludge does not.

Metagenomic sequencing could theoretically obtain all microbial genome information within a sample, providing direct evidence to show the microbial composition. It is used to uncover microbial communities of activated sludge or biofloc [10, 11]. It is a suitable tool to distinguish between sludge and BF.

Materials and methods. Furfural residue (FR) was purchased from Bayan County Yuanda Furfural Co., Ltd. (Heilongjiang Province, China). Experimental wastewater production process: (1) FR was washed with water at a 1:10 ratio; (2) One hundred μm filter paper was used to achieve filtrate; (3) CaOH was added to adjust pH to 6–7; (4) Filter again to obtain experimental wastewater. The experimental wastewater simulated the high concentration organic wastewater produced in the process of detoxification of furfural residue. Biofloc (BF) sample: Add the marketable biofloc (M-BF) with a 0.1% dosage (0.1 g BF to 100 ml experimental wastewater in an Erlenmeyer flask). The Erlenmeyer flask was placed on a circumferential oscillator and run at 100 rpm for 3 h every day until the end of the experiment. The ambient temperature was 5–18 °C in March 2024. M-BF was purchased from Nissan Industries Co., Ltd. (Japan). The experiment lasted four weeks. BF was obtained by centrifugation at 6000 rpm for 10 min. CK sample: The same experimental procedure and environment were followed without adding M-BF.

Twenty mg samples were used for DNA extraction by a DNA extraction kit (Fastpure Microbiome DNA Isolation Kit, Vazyme, China) according to the manufacturer's protocol. The DNA concentration was measured using a NanoDrop

2000c spectrophotometer (Thermo Scientific, United States). The quality and quantity of the DNA extract were evaluated with gel electrophoresis and a Quantus fluorometer (Promega, Madison, WI, USA). The DNA sequencing libraries and subsequent Illumina HiSeq sequencing services were provided by Novogene (Beijing, China).

Results and discussion. Biodiversity analysis of CK and BF. In this study, we observed the dominance of the bacterial domain in the CK sample with a prevalence of 88%. We also observed the presence of other domains with an abundance of 0.008% and 1.7% for *archaea* and *viruses*, respectively. In CK, the first predominant phylum observed was *Pseudomonadota* with an abundance of 81%, and the second predominant phylum was *Gammaproteobacteria* (79%). Moreover, *Xanthomonadales* (38%), *Enterobacterales* (23%), *Pseudomonadales* (8%), *Burkholderiales* (1%), *Mucorales* (1%) can be seen in the forth loop. In Fig. 1A, we show a total of eight loops. From high to low, the dominant microbes were *Stenotrophomonas* (37%), *Stenotrophomonas maltophilia* (15%), *Pseudomonas* (8%), *Pantoea* (3%), *Cronobacter sakazakii* (2%), *Cronobacter dublinensis* (1%), *Burkholderiales* (1%), *Rhizopus arrhizus* (1%).

In contrast, BF has a high prevalence in the *Bacteria* and *Eukaryota* domain, with an abundance of 46% and 35% in Fig. 1B, respectively. Further segmentation shows that *Bacillota* (27%) and *Pseudomonadota* (18%) belong to *Bacteria*, *Mucoromycota* (20%) and *Ascomycota* (15%) belonged to *Eukaryota*. The species with an abundance of over 1% are listed as *Weissella paramesenteroides* (14%), *Mucor lusitanicus* (11%), *Clavispora lusitaniae* (11%), *Pediococcus* (8%), *Pantoea* (5%), *Pediococcus pentosaceus* (3%), and *Rhizopus arrhizus* (2%).

Xanthomonadales, *Pseudomonadales*, and *Burkholderiales*, which together make up 47%, are the common and dominant microbes in water treatment or natural water [12,13]. *Enterobacterales* are easily reproduced in untreated organic water, which is also related to straw [14]. *Bacillota* (27%) and *Pseudomonadota* in BF are similar to ATS-system (*Bacillota* 14–27%; *Pseudomonadota* 29–41%), which is used to treat wastewater from municipal wastewater, manure, and biogas plant effluent supernatant [15].

In the present study, we used cluster analysis and drew the heatmap in Fig. 2. It was observed that there was a two-level differentiation in CK and BF samples. The abundance of *Sphingobacteriia*, *Gammaproteobacteria*, and *Betaproteobacteria* in CK was significantly higher than in BF. These three types of bacteria are commonly found in wastewater treatment systems [16]. Correspondingly, the abundance proportion of *Caudoviricetes*, *Alphaproteobacteria*, *Ustilaginomycetes*, *Spirochaetia*, *Mucoromycetes*, *Saccharomycetes*, and *Bacilli* was higher in BF.

ARGs and biosynthesis of antibiotics. Figure 3 presents the relative abundance of ARGs (Antibiotics resistance genes). The average content of the ARGs in CK was 1611 ppm, which is 2.64 times higher than in the P group. The most abundant ARGs in the P group was the *vanT* gene in *vanG* clus-

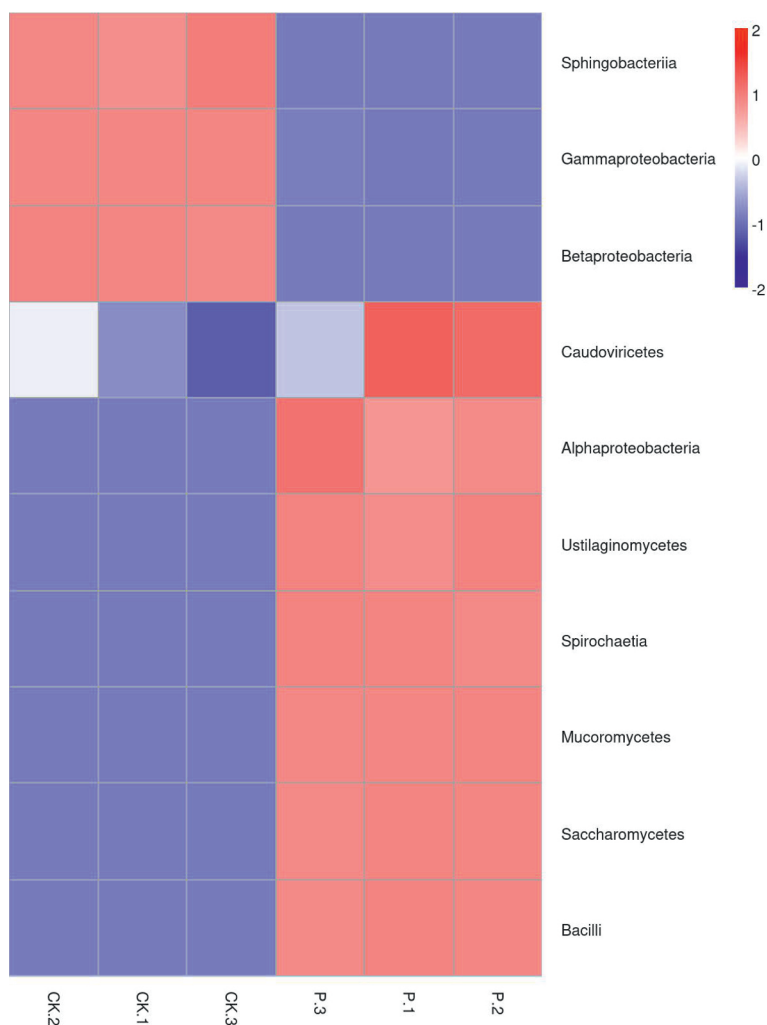


Fig. 2. Heatmap for the microbial community in phylum level

ter (143.41 ppm), 17.3 times higher than CK. Ranging from the top 2 to 10 in the P group were *Escherichia coli* EF-Tu mutants conferring resistance to Pulvomycin (65.90 ppm), *adeF* (33.73 ppm), *emrR* (32.21 ppm), *Klebsiella pneumoniae* KpnF (31.37 ppm), *Klebsiella pneumoniae* KpnH (28.17 ppm), *Klebsiella pneumoniae* KpnE (27.84 ppm), *msbA* (26.84 ppm), *Haemophilus influenzae* PBP3 conferring resistance to beta-lactam antibiotics (22.76 ppm), *Escherichia coli* AcrAB-TolC with *MarR* mutations conferring resistance to ciprofloxacin and tetracycline (14.24 ppm). In CK, the top 10 was *adeF* (123.85 ppm), *qacJ* (123.28 ppm), *smeR* (107.22 ppm), *vanY* gene in *vanM* cluster (101.85 ppm), *Escherichia coli* EF-Tu mutants conferring resistance to pulvomycin (97.87 ppm), *smeE* (98.98 ppm), *smeF* (80.66 ppm), AAC(6)-Iak (77.82 ppm), *Klebsiella pneumoniae* KpnF (47.92 ppm), *Klebsiella pneumoniae* KpnE (42.05 ppm).

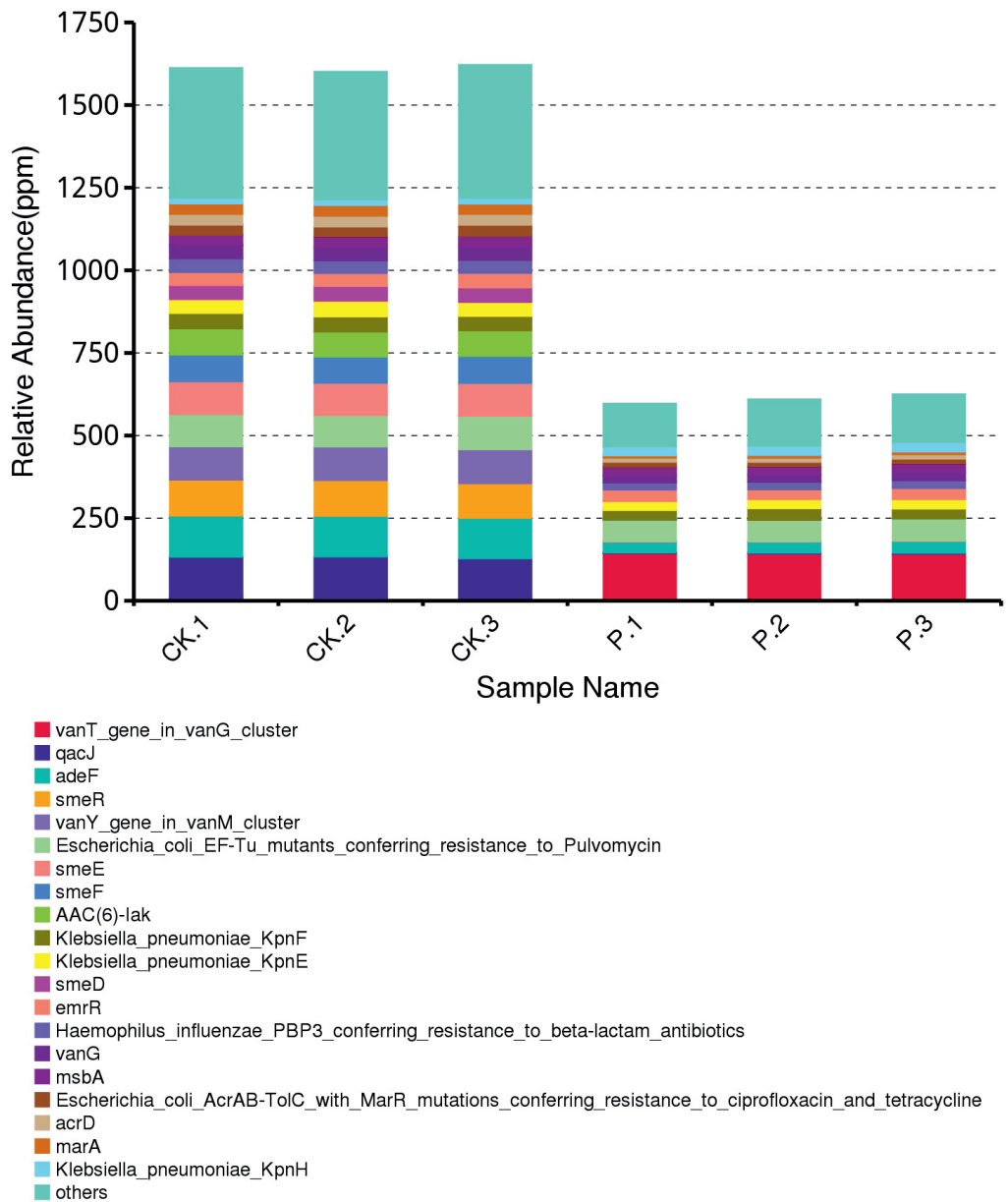


Fig. 3. Relative abundance of ARGs in CK and BF

It can be seen from Fig. 4 that activated sludge contains a large number of antibiotic synthesis pathways, which are highly correlated with its abundant ARGs. BF had fewer antibiotic syntheses and less ARGs content [18]. The inhibition of activated sludge rich in antibiotics to some functional bacteria is significant. It is not clear whether the functional BF bacteria can tolerate the harmful substances

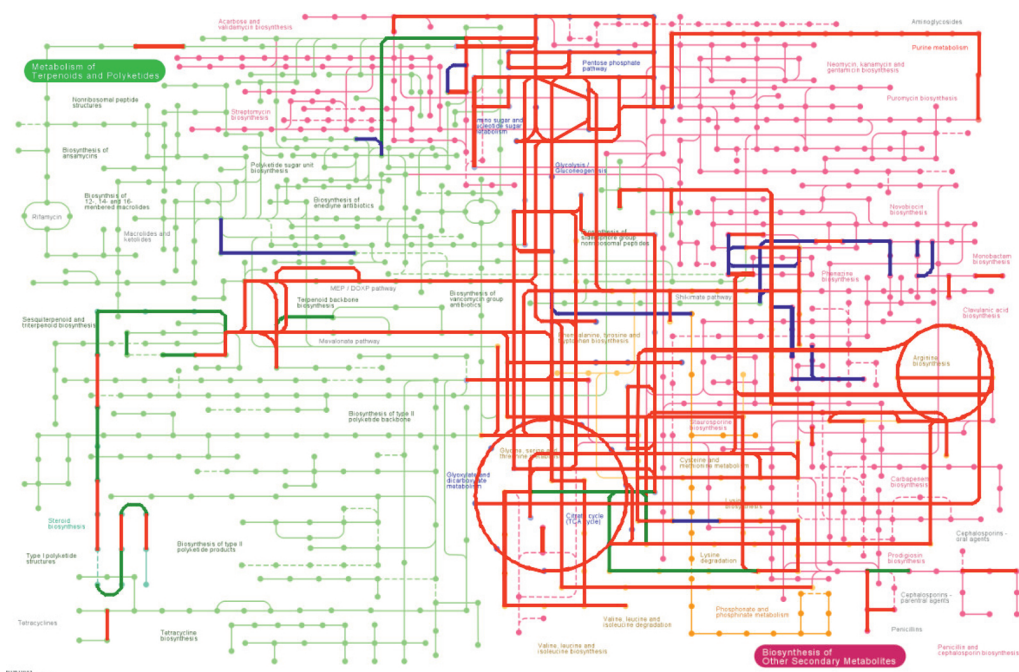


Fig. 4. Comparison of CK and BF in Global pathway map of biosynthesis of antibiotics

in the FR wastewater, and thus occupy the population advantage in the early stage, or BF functional bacteria natural inhibition of harmful bacteria.

We can see the biosynthesis of various antibiotics by KEGG in Fig. 4. The crude blue line represents the same biosynthesis, the crude red line belongs to the CK and the green line belongs to P group. As we can see, the crude red line occupies about 50% of the map. The points belonging to the BF are exclusive and correspond to the biosynthesis of type I polyketide structures, steroids, sesquiterpenoid, triterpenoids, and terpenoid backbones.

Whether sludge or biofloc, they are complex microbial systems. In particular, antibiotic-producing bacteria and antibiotic-resistant bacteria compete with each other for survival. The misuse of human antibiotics directly leads to an increase in microbial resistance. ARGs are ubiquitous in a wide variety of organisms [17]. However, the relative abundance in BF was lower than in CK which could be an advantage of BF. If we use the active microorganisms in BF, we should be cautious of the ARGs. In the previous study of biofloc, we found that there were very few studies on ARG. SU et al. [18] studied four resistance genes, *tetX*, *floR*, *sul1* and *sul2*, in water, BF and shrimp gut. The results showed that the flocs had more types of ARGs, but their content was low. Additionally, through digestion, some of the resistance genes are broken down. The biological flocs in this study were cultured in furfural residue washing solution, and their living environment is vastly different from aquaculture wastewater and even natural water.

Conclusions. Metagenomic analysis showed that the dominant microbes are *Bacillota* (27%), *Pseudomonadota* (18%), *Mucoromycota* (20%) and *Ascomycota* (15%) in the phylum in BF. At the species level, they were *Weissella paramesenteroides* (14%), *Mucor lusitanicus* (11%), *Clavispora lusitaniae* (11%), *Pediococcus* (8%), *Pantoea* (5%), *Pediococcus pentosaceus* (3%), *Rhizopus arrhizus* (2%). From the PCoA and heatmap, we find that the BF is different from CK (sludge) in microbial communities. Compared to CK, BF is more similar to existing reports about the metagenome of BF for fodder. In addition, the dominant microbes may have a good effect on soil due to biomass degradation.

ARGs and biosynthesis pathways of antibiotics proved that the BF was better than the sludge (the relative abundance decreased by 62.13%). The *VanT* gene in *vanG* cluster (143.41 ppm) was the most ARGs in BF. The last 9 ARGs were between 70–20 ppm but the top 10 ARGs in CK were between 130–40 ppm. The BFT was suitable to replace the activated sludge method in some fields, but the challenge of ARGs was still present.

REFERENCES

- [1] NISAR U., D. PENG, Y. MU, Y. SUN (2022) A solution for sustainable utilization of aquaculture waste: a comprehensive review of biofloc technology and aquamimicry, *Front. Nutr.*, **8**, <https://doi.org/10.3389/fnut.2021.791738>.
- [2] AHMAD I., A. M. BABITHA RANI, A. K. VERMA, M. MAQSOOD (2017) Biofloc technology: an emerging avenue in aquatic animal healthcare and nutrition, *Aquac. Int.*, **25**, 1215–1226.
- [3] SUWARTHA N., D. R. PUJIASTUTI (2017) Enhancing removal efficiency of ammonia and nitrate in shrimp farm wastewater using biofloc technology and effective microorganism S4 (EM4), *Int. J. Technol.*, **6**, 1021–1030.
- [4] KUHN D. D., A. L. LAWRENCE, J. CROCKETT, D. TAYLOR (2016) Evaluation of bioflocs derived from confectionary food effluent water as a replacement feed ingredient for fishmeal or soy meal for shrimp, *Aquaculture*, **454**, 66–71.
- [5] NETO H. S., S. T. SANTAELLA, A. J. P. NUNES (2015) Bioavailability of crude protein and lipid from biofloc meals produced in an activated sludge system for white shrimp, *Litopenaeus vannamei*, *Rev. Bras. Zootec.*, **44**(8), 236–275.
- [6] PÉREZ-ROSTRO C. I., J. A. PÉREZ-FUENTES, M. P. HERNÁNDEZ-VERGARA (2014) Biofloc, a technical alternative for culturing Malaysian prawn *Macrobrachium rosenbergii*, In: *Sustainable Aquaculture Techniques*, Rijeka, IntechOpen, 87–104, <https://doi.org/10.5772/57501>.
- [7] BAJIĆ B., D. VUČUROVIĆ, P. VASIĆ et al. (2023) Biotechnological production of sustainable microbial proteins from agro-industrial residues and by-products, *Foods*, **12**(1), 107.
- [8] SÁNCHEZ-MUROS M. J., P. RENTERIA, A. VIZCAINO, F. G. BARROSO (2020) Innovative protein sources in shrimp (*Litopenaeus vannamei*) feeding, *Rev. Aquac.*, **12**(1), 186–203.

- [9] MATASSA S., N. BOON, I. PIKAAR, W. VERSTRAETE (2016) Microbial protein: future sustainable food supply route with low environmental footprint, *Microbial Biotech.*, **9**(5), 568–575.
- [10] IBARBALZ F. M., E. ORELLANA, E. L. M. FIGUEROLA, L. ERIJMAN (2016) Shotgun metagenomic profiles have a high capacity to discriminate samples of activated sludge according to wastewater type, *Appl. Environ. Microbiol.*, **82**(17), 5186–5196.
- [11] CHEN X., Z. HE, J. ZHAO et al. (2022) Metagenomic analysis of bacterial communities and antibiotic resistance genes in *Penaeus monodon* biofloc-based aquaculture environments, *Front. Mar. Sci.*, **8**, 762345.
- [12] ATASHGAHI S., R. AYDIN, M. R. DIMITROV et al. (2015) Impact of a wastewater treatment plant on microbial community composition and function in a hyporheic zone of a eutrophic river, *Sci. Rep.*, **5**, 17284.
- [13] SETH N., S. VATS, S. LAKHANPAUL et al. (2017) Microbial community diversity of an integrated constructed wetland used for treatment of sewage, *Front. Microbiol.*, **15**, 1355718.
- [14] ELLIOTT J. A. K., C. KROHN, A. S. BALL (2024) Diversity of microbial communities in trade wastes – implications for treatments and operations, *Appl. Microbiol.*, **4**, 682–703.
- [15] BLIFERNEZ-KLASSEN O., J. HASSA, D. L. REINECKE et al. (2023) Microbial diversity and community structure of wastewater-driven microalgal biofilms, *Microorganisms*, **11**, 2994.
- [16] SHI L., N. LIU, G. LIU, J. FANG (2021) Bacterial community structure and dynamic changes in different functional areas of a piggery wastewater treatment system, *Microorganisms*, **9**, 2134.
- [17] ZHAO Y., Q. E. YANG, X. ZHOU et al. (2021) Antibiotic resistome in the livestock and aquaculture industries: Status and solutions, *Crit. Rev. Environ. Sci. Technol.*, **51**(19), 2159–2196.
- [18] SU H., T. XIA, W. XU et al. (2023) Temporal variations, distribution, and dissemination of antibiotic resistance genes and changes of bacterial communities in a biofloc-based zero-water-exchange mariculture system, *Ecotoxicol. Environ. Saf.*, **256**, 114904.
- [19] WANG P., X. LI, S. CHU et al. (2022) Metatranscriptomic insight into the effect of antibiotic exposure on performance during anaerobic co-digestion of food waste and sludge, *J. Hazard. Mater.*, **423**, Part B, 127163.

Yancheng Institute of Technology, Yancheng, 224051, China
e-mail: BE-science@outlook.com, wfh82090446@sohu.com