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Optimizing ciprofloxacin removal through regulations of trophic modes and FNA levels in a moving bed biofilm reactor performing sidestream partial nitritation

Yifeng Xu [a,b], Xi Wang [b], Ying Gu [b], Chuanzhou Liang [a,b], Wenshan Guo [c], Huu Hao Ngo [c], Lai Peng [a,b,*]

[a] Hubei Key Laboratory of Mineral Resources Processing and Environment, Wuhan University of Technology, Luoshi Road 122, Wuhan 430070, China
[b] School of Resources and Environmental Engineering, Wuhan University of Technology, Luoshi Road 122, Wuhan 430070, China
[c] Centre for Technology in Water and Wastewater, School of Civil and Environmental Engineering, University of Technology Sydney, Sydney, New South Wales 2007, Australia

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ABSTRACT

The performance of partial nitritation (PN)-moving bed biofilm reactor (MBBR) in removal of antibiotics in the sidestream wastewater has not been investigated so far. In this work, the removal of ciprofloxacin was assessed under varying free nitrous acid (FNA) levels and different trophic modes. For the first time, a positive correlation was observed between ciprofloxacin removal and FNA levels, either in the autotrophic PN-MBBR or in the mixotrophic PN-MBBR, mainly ascribed to the FNA-stimulating effect on heterotrophic bacteria (HB)-induced biodegradation. The maximum ciprofloxacin removal efficiency (~98 %) and removal rate constant (0.021 L g^{-1} SS h^{-1}) were obtained in the mixotrophic PN-MBBR at an average FNA level of 0.056 mg-N L^{-1}, which were 5.8 and 51.2 times higher than the corresponding values in the autotrophic PN-MBBR at 0 mg FNA-N L^{-1}. Increasing FNA from 0.006 to 0.056 mg-N L^{-1} would inhibit ammonia oxidizing bacteria (AOB)-induced cometabolism and metabolism from 10.2 % and 6.9 % to 6.2 % and 6.4 %, respectively, while HB-induced cometabolism and metabolism increased from 31.2 % and 22.7 % to 41.9 % and 34.5 %, respectively. HB-induced cometabolism became the predominant biodegradation pathway (75.9 %-85.8 %) in the mixotrophic mode. Less antimicrobial biotransformation products without the piperazine or fluorine were newly identified to propose potential degradation pathways, corresponding to microbial-induced metabolic types and FNA levels. This work shed light on enhancing antibiotic removal via regulating both FNA accumulation and organic carbon addition in the PN-MBBR process treating sidestream wastewater.

Introduction

Higher consumption of antibiotics always results in large amounts of unchanged residues excreting into wastewater, posing potential hazards on aquatic ecosystems (Ukić et al., 2019). Although wastewater treatment plants (WWTPs) could remove organic carbon, nitrogen and phosphorus efficiently, lower removal efficiencies were observed for most antibiotics, rendering WWTPs effluent as the major sources for antibiotic residues and their transformation products into the receiving aquatic environment. As a widely used fluoroquinolone antibiotic, ciprofloxacin was detected at a broad range of 15 ngL^{-1} to 31 mg L^{-1} in various types of wastewaters (Tran et al., 2018; Larsson et al., 2007; Nguyen et al., 2017). Apart from the mainstream wastewater, sludge digestion liquor (i.e., sidestream wastewater) containing high levels of ammonium (500–2000 mg-N L^{-1}) might be another important source of antibiotics (Jelic et al., 2012; Xu et al., 2022). Therefore, it is of significance to understand the fate and transformation of antibiotics during sidestream wastewater treatment processes.

During the conventional biological treatment processes for mainstream wastewater, adsorption onto the sludge was the predominant removal pathway of ciprofloxacin (Zhang et al., 2018), leaving ciprofloxacin as the unchanged form with potential threats to the

* Corresponding author at: Hubei Key Laboratory of Mineral Resources Processing and Environment, Wuhan University of Technology, Luoshi Road 122, Wuhan 430070, China.
E-mail address: lai.peng@whut.edu.cn (L. Peng).

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environment. Given that ciprofloxacin is less likely to be biodegraded in the activated sludge processes (Dorival-García et al., 2013), it is important to improve the biodegradation abilities for complete removal of ciprofloxacin in the WWTPs. Antibiotics removal efficiencies could be significantly enhanced in conventional nitrification processes (Kassotaki et al., 2016; Guo et al., 2023; Xu et al., 2016), due to the contribution by ammonia oxidizing microorganisms (AOMs)-induced cometabolic biodegradation (Li et al., 2022). The non-specific ammonia monooxygenase (AMO) could catalyze the biodegradation of a broad spectrum of aliphatic and aromatic compounds (Yu et al., 2018), thus increasing biodegradation potentials of recalcitrant antibiotics. However, relatively lower abundance and slower growth rate of AOMs in the conventional activated sludge systems might hinder the improvement of cometabolism for antibiotic removal. Contrarily, moving bed biofilm reactor (MBBR) showed advantages with higher retaining abilities for slow-growing microorganisms and higher biomass concentrations, which could be adopted as good "carriers" to enrich AOMs (Wolff et al., 2021).

Partial nitritation/anammox (PN/A) process has been widely applied in full-scale installations for sidestream nitrogen removal in low chemical oxygen demand (COD)/N ratio wastewaters (Wang et al., 2022), saving at least 60 % aeration and 100 % carbon consumption compared to the traditional nitrification/denitrification process (Lackner et al., 2014; Choi et al., 2019). Contradictory biodegradation efficiencies were reported for different types of pharmaceuticals. For instance, 80 %−100 % removals were observed for sulphadiazine, atenolol, estrone, naproxen and ibuprofen (Xu et al., 2022; Alvarino et al., 2015; Li et al., 2020), while less than 10 % removals were achieved for carbamazepine and venlafaxine (Alvarino et al., 2015; Kassotaki et al., 2018). Ammonia oxidizing bacteria (AOB) were dominant species in the PN process as a result of stable suppression on nitrite oxidizing bacteria (NOB) via a variety of strategies such as short sludge retention time (Li et al., 2021), low dissolved oxygen (DO) (Laureni et al., 2019), high concentrations of free ammonia (FA) or free nitrous acid (FNA) (Wang et al., 2020). Enriched AOB communities in the PN processes might favor better biodegradation of antibiotics. AOB-induced metabolism were believed to be the major atenolol biodegradation mechanism in sidestream PN process (Xu et al., 2022), while AOB-induced cometabolism was considered as the predominant sulfadiazine biodegradation mechanism in mainstream PN/A process (Li et al., 2020). In addition, since heterotrophic bacteria (HB) proved their important roles in biodegradation of antibiotics in the conventional nitrifying activated sludge systems and PN activated sludge systems (Xu et al., 2022; Guo et al., 2023), different trophic modes (i.e., autotrophic, mixotrophic) might also impact antibiotic removal in the biofilm-based systems. Given the fact that total nitrogen could be removed at 90 % in the PN/A-MBBR process (Tao and Hamouda, 2019), the application of MBBR in sidestream PN processes might also exhibit superior performance in antibiotic biodegradation. However, such information is still limited so far. Although a recent study confirmed the regulation effects of FNA on atenolol biodegradation mechanisms (Xu et al., 2022), whether the similar FNA inhibition would occur for antibiotics in the PN-MBBR is not clear and the impact of exogenous organic carbon should also be investigated for better removal of antibiotics.

This work aimed to investigate the removal performance of ciprofloxacin in the MBBR system treating sidestream wastewater. The effects of FNA and trophic modes on ciprofloxacin removal were explored by assessing contributions from microbial-induced metabolic types including AOB-induced cometabolism, AOB-induced metabolism and HB-induced metabolism. Moreover, ciprofloxacin transformation products and pathways were identified. This study might provide guidance for the application of PN-MBBR in removing antibiotics and ammonium simultaneously during the sidestream treatment processes.

Results and discussion

Negligible removal of ciprofloxacin by sorption in the sidestream PN-MBBR

As demonstrated in Fig. S1, insignificant removal efficiencies (~9 %) were achieved in the sterilized control, regardless of the effect of the FNA levels (0–0.056 mg-N L^{-1}). By contrast, ciprofloxacin was removed up to 5 % after 120 min in the blank-carrier control (Fig. S1). Given negligible contribution from photodegradation, volatilization and hydrolysis (Xu et al., 2023), sorption onto the sterilized biomass (4.59 ± 0.13 g SS L^{-1}) in the biofilm might contribute 4 % of ciprofloxacin removal in the sidestream PN process. Interestingly, ciprofloxacin prone to be removed via sorption onto different biomass types (e.g., flocculent activated sludge, biofilm, granular sludge) in the conventional mainstream treatment process (Xu et al., 2023; Amorim et al., 2014; Jia et al., 2012). Relatively higher sorption efficiencies (~50 %) were reported for ciprofloxacin, accompanied with its lower biodegradation potential (9 %−42 %) in the nitrifying-MBBR system (Xu et al., 2023). The discrepancy in sorption abilities might be related with the abundance of NOB, which could secrete a large quantity of extracellular polymeric substances (EPS) in favor of adsorption (Shao et al., 2019). NOB were largely suppressed in the PN-MBBR, resulting in lower EPS contents compared with the conventional activated sludge or nitrifying biofilms. Similarly, in response to the switching in dominant conditions from nitrification to nitritation, there was a significant decrease in EPS production in an integrated fixed-film activated sludge reactor (Shao et al., 2019). Besides, the production of total EPS showed no significant difference ($p > 0.05$) between the beginning (total polysaccharides of 3.73 mg g^{-1} SS and total protein of 0.24 mg g^{-1} SS) and the end (total polysaccharides of 2.77–3.65 mg g^{-1} SS and total protein of 0.17–0.20 mg g^{-1} SS) of the tests (Fig. S2), which further suggested that sorption was not the primary pathway to remove ciprofloxacin.

Although sorption was the major removal route for hydrophobic contaminants, such as fluoroquinolones and tetracyclines in the conventional wastewater treatment processes (Kim et al., 2005; Cao et al., 2019), they could further be released from sludge under oxidation and heating conditions (Xu et al., 2020; Xu et al., 2013). Therefore, decreasing sorption potentials of ciprofloxacin from ~50 % in the nitrifying biofilms (Xu et al., 2023) to ~4 % in the PN biofilms might shed light on reducing the ecological risk of antibiotic residues in the sludge, which deserves further research.

FNA enhanced ciprofloxacin removal in the autotrophic sidestream PN-MBBR

As depicted in Fig. 1, average ciprofloxacin removal efficiencies in the autotrophic PN-MBBR were 17 %, 49 % and 56 % in the tests I-1, II-1 and III-1 at FNA concentrations of 0, 0.006 and 0.056 mg-N L^{-1}, respectively. Although the chemical reaction with nitrite at the utmost concentration (1331.36 mg-N L^{-1}) led to 2.3 % removal of ciprofloxacin (Fig. S3), nitrite accumulation could not reach such high levels for most batch tests. Considering the minimum concentrations of ciprofloxacin and nitrite for initiating chemical reactions, it was reckoned that nil contribution was from chemical reactions to ciprofloxacin removal. Then, an obvious positive correlation was observed between ciprofloxacin biodegradation and FNA levels in the autotrophic PN-MBBR, excluding the contributions from sorption (~9 %) and chemical reaction (~0 %).

This was contrary to our previous observation that FNA had an inhibiting effect on atenolol removal in the PN-activated sludge system, where atenolol biodegradation decreased from 67 % to 28 % with increasing FNA from 0.03 to 0.19 mg-N L^{-1} (Xu et al., 2022). The underlying microbial-induced metabolic types were further analyzed to reveal such FNA-regulating mechanisms on ciprofloxacin biodegradation. Due to the absence of external organic carbon source and limited

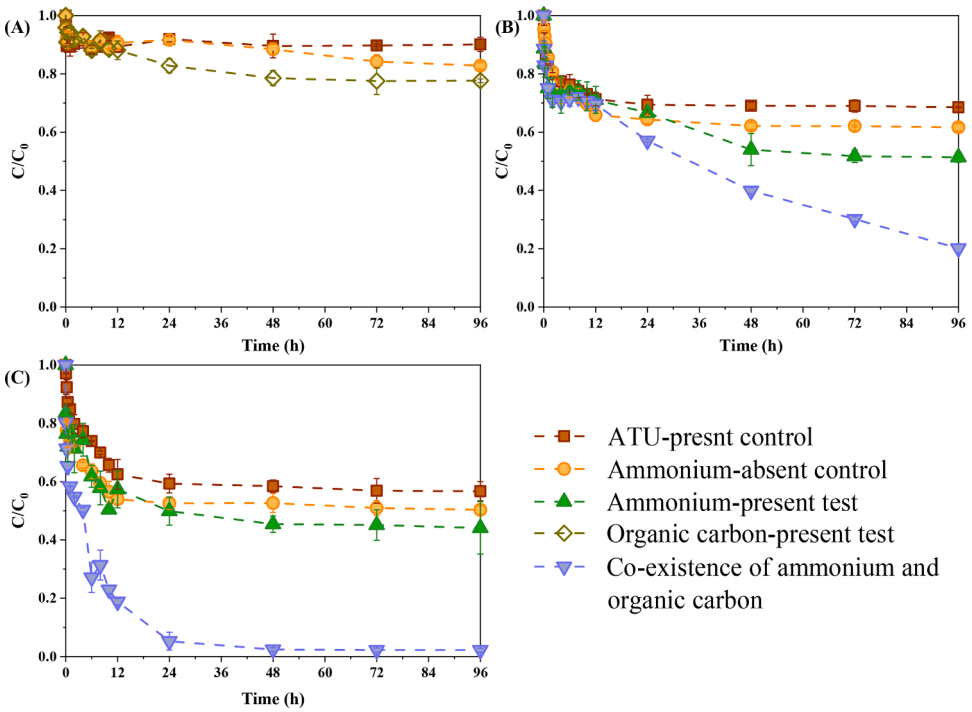

Fig. 1. Ciprofloxacin concentration profiles in controlled experiments under varying FNA levels: (A) 0 mg-N L^{-1}; (B) 0.006 mg-N L^{-1}; (C) 0.056 mg-N L^{-1}.

endogenous organic carbon availability during the autotrophic PN-MBBR (Xu et al., 2018), HB-induced cometabolic biodegradation was considered as negligible. As shown in Fig. 2A, the contributions from AOB-induced metabolism and HB-induced metabolism were 7.3 % and 1.1 %, respectively, in the test I-1 (FNA=0 mg-N L^{-1}). By contrast, the corresponding contributions were 6.9 %/22.7 % and 6.4 %/34.5 % in the test II-1 (FNA=0.006 mg-N L^{-1}) and test III-1 (FNA=0.056 mg-N L^{-1}), respectively. AOB-induced cometabolism was not present in the test without ammonium while its contribution to ciprofloxacin removal was 10.2 % and 6.2 % in the test II-1 and III-1, respectively.

The metabolic and cometabolic biodegradation by AOB were both inhibited along with the increasing FNA concentration from 0.006 to 0.056 mg-N L^{-1}, which was also consistent with the decreasing nitrite accumulation rates from 3.80 ± 0.06 mg-N g^{-1} SS h^{-1} to 0.31 ± 0.04 mg-N g^{-1} SS h^{-1} (Fig. S4) and the decreasing ammonia oxidation rates from 14.42 ± 2.11 mg-N g^{-1} SS h^{-1} to 4.01 ± 1.35 mg-N g^{-1} SS h^{-1} (Fig. S5). Such FNA inhibition on AOB-induced biodegradation is similar to our previous observation that AOB-induced metabolism dropped from 29 % to 16 % and AOB-induced cometabolism from 11 % to 8 % with FNA increasing from 0.03 to 0.19 mg-N L^{-1} during atenolol biodegradation in the PN-activated sludge system (Xu et al., 2022). FNA inhibition on AOB-induced metabolic types might result from decreasing biomass and AMO activities, lacking enough energy to maintain AOB growth and binding with the active sites of AMO (Duan et al., 2020; Musiani et al., 2021).

On the other hand, an increasing trend was observed for HB-induced metabolism from 1.1 % to 34.5 % when FNA varied from 0 to 0.056 mg-N L^{-1}. This might suggest the stimulating effect of FNA on growth and activity of heterotrophic microorganisms in the PN-MBBR, which eventually led to enhanced removal of ciprofloxacin under higher FNA concentrations. Apart from the autotrophic microorganisms (Text S1), heterotrophic bacteria including *Truepera* (23.4 %) and *Aequorivita* (11.3 %) were also abundant in the PN-MBBR (Fig. S6), which were different from corresponding portions in the nitrifying-MBBR (<0.1 % for *Truepera* and *Aequorivita*) and PN-activated sludge systems (3.2 % *Truepera* and 0 % *Aequorivita*) (Xu et al., 2022; Xu et al., 2023). Synergistic relationships between *Truepera* and *Nitrosomonas* were also confirmed during stable PN processes either under sidestream (Hu et al., 2023) or mainstream conditions (Yu et al., 2020). As an important thermophilic denitrifier, the higher abundance of *Truepera* in the PN-MBBR was ascribed to the medium temperature applied during reactor operation and batch test conduction (~33 °C) (Hu et al., 2023). The genus *Truepera*

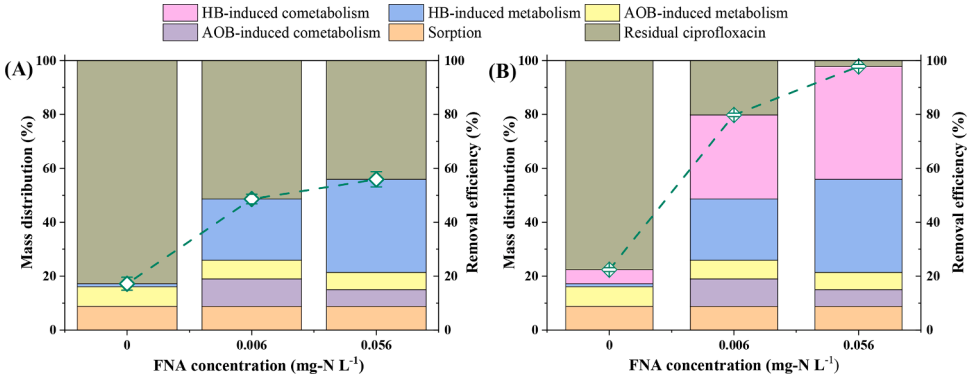

Fig. 2. Calculated mass distribution of ciprofloxacin under varying FNA levels in the (A) autotrophic PN-MBBR and (B) mixotrophic PN-MBBR.

was reported to be highly involved in biodegradation of polycyclic aromatic hydrocarbons and phenol (Luo et al., 2021; Lu et al., 2019) and dominated in removal of tetracycline and oxytetracycline (Wang et al., 2023), probably suggesting its important role in removal of ciprofloxacin in this PN-MBBR system. Beside the denitrification abilities, *Aequorivita* was also capable of degrading organic pollutants (Zheng et al., 2016). The sufficient availability of nitrite as electron acceptor and limited endogenous organic carbon from biomass decay or the only exogenous organic carbon (i.e., ciprofloxacin) as electron donor might facilitate the reduction of nitrite under lower DO concentrations (0.5–1.0 mg L^{-1}), supporting the growth and energy synthesis of heterotrophic denitrifiers such as *Truepera* and *Aequorivita*. The enhanced heterotrophic denitrifying activity under higher FNA concentrations of 0.056 mg-N L^{-1} could also be confirmed from the insignificant increase in nitrite concentrations after 48 h in Test III-1 while nitrate concentrations being close to 0 mg-N L^{-1} (Fig. S4C). By contrast, relatively weaker heterotrophic denitrifying activity was associated with the lower FNA concentration (0.006 mg-N L^{-1}). Therefore, the contributions from metabolic biodegradation by HB (mainly *Truepera*) were much higher in the presence of nitrite (FNA of 0.006 or 0.056 mg-N L^{-1}) than that in the absence of nitrite (FNA of 0 mg-N L^{-1}) (Fig. 2A). Furthermore, slight enhancement of HB-induced metabolic biodegradation from 22.7 % to 34.5 % might be due to the growth-stimulating effect by the acidic pH conditions on the involved heterotrophs (i.e., *Truepera*) (Mockaitis et al., 2020), which in turn improved its degrading potentials of ciprofloxacin. It was inferred that HB-induced biodegradation, instead of AOB-induced biodegradation, would be the predominant biodegradation mechanisms of ciprofloxacin during the sidestream PN-MBBR process.

Given relatively lower DO concentrations (0.5–1.0 mg L^{-1}) during reactor operation and batch test conduction facilitating the PN process by suppressing NOB, heterotrophic denitrifier *Truepera* still occupied a considerable fraction in the microbial communities of the biofilm. This is consistent with the previous literature that the coexistence of *Nitrosomonas* and obligatory aerobic or facultative anaerobic heterotrophs including *Armatimonadetes_gp5, Gemmo-bacter, Pseudomonas, Truepera, Luteimona* and *Shinella* was identified in the mainstream PN process at DO levels of 0.2 mg L^{-1} (Yu et al., 2020). Generally, AOB and HB had different affinities for oxygen with respective half saturation constant of 1.1 and 0.2 mg L^{-1} (Xu et al., 2018). Therefore, HB-induced biodegradation would be predominant over AOB-induced biodegradation as a result of better oxygen uptake capabilities. Compared with our previous work in the PN-activated sludge at DO levels of 2.5–3.0 mg L^{-1} (Xu et al., 2022), limited DO supply in the PN-MBBR could affect the activity of AOB because of the competition for oxygen between AOB and HB, thus reducing AOB-induced biodegradation of ciprofloxacin. Another possible explanation for FNA-inhibiting effect on AOB instead of HB might be the stratified structures of biofilm, exposing AOB in the surface layer to the FNA stress and protecting denitrifying HB in the inner layer from the FNA stress (Xu et al., 2023).

COD further enhanced ciprofloxacin removal in the mixotrophic sidestream PN-MBBR

To further investigate the role of HB in removing ciprofloxacin in the PN-MBBR, the follow-up experiments in the presence of exogenous organic carbon were conducted to unravel the contribution of HB-induced biodegradation under varying FNA accumulation levels as shown in Fig. 1. Nearly constant COD concentrations were controlled by providing exogenous glucose for Tests I-3, II-4 and III-4 as shown in Fig. S7. At the initial concentration of 1.0 mg L^{-1}, ~22 % ciprofloxacin was removed at a degradation rate of 0.0009 mg g^{-1} SS h^{-1} in Test I-3 under average FNA accumulation level of 0 mg-N L^{-1}, based on non-linear regression analysis (Fig. S8A). In contrast, ciprofloxacin removal efficiency was achieved at ~80 % in Test II-4, at an average degradation rate of 0.0018 mg g^{-1} SS h^{-1} (Fig. S8B). Similarly, ciprofloxacin experienced a rapid decrease at approximately 0.014 mg g^{-1} SS h^{-1} within 12 h in Test III-4 (average FNA level of 0.056 mg-N L^{-1}, Fig. S8C), obtaining an average removal efficiency of ~98 %.

Similar to the autotrophic conditions, ciprofloxacin removal also showed a positive relationship with the FNA levels in the mixotrophic PN-MBBR treating sidestream wastewater, owing to HB-induced biodegradation. Distinct with FNA inhibition on AOB-induced biodegradation, the proportions of HB-induced cometabolism in ciprofloxacin removal gradually increased from 5.2 %, 31.2 % to 41.9 % accompanied with the increasing FNA concentrations from 0, 0.006 to 0.056 mg-N L^{-1} (Fig. 2B). The predominant biodegradation mechanism changed with the varying FNA levels, with HB-induced cometabolism prevailing under higher FNA concentrations of 0.056 mg-N L^{-1}. This supported the coexistence of FNA and COD in improving the HB-induced cometabolism. Given the same COD concentration in all tests, it was speculated that FNA might be the determining factor for HB-induced cometabolism in the sidestream PN-MBBR. However, knowledge on the physiological properties of the involved HB genera (especially *Truepera*) was scarce so far, as well as their metabolism and cometabolism in pharmaceutical biodegradation (Albuquerque et al., 2005). One possible explanation for improving HB-induced cometabolism under higher FNA concentrations was that FNA could stimulate the activities of the enzymes within the heterotrophs, such as monooxygenases, dioxygenases, hydrolases and transferases (Kennes-Veiga et al., 2021). Nonetheless, this was contradictory to the conventional notion that FNA, as a biocidal agent, could inhibit the growth of many types of microorganisms (Duan et al., 2020). The underlying stimulating effect of FNA needs to be investigated in the future work.

Obviously, higher ciprofloxacin removal efficiencies and rates were achieved in the presence of COD than those in the absence of COD for each FNA accumulation level (Figs. 1 and 2). The increasing trend along with FNA levels was intensified under exogenous organic carbon availability (Fig. 2), due to FNA-stimulating effects on HB-induced biodegradation. Compared with the autotrophic MBBR, the mixotrophic MBBR would perform better in the removal of antibiotics during sidestream PN processes, owing to the significant contribution from HB-induced cometabolism. Similarly, a significant increase in contribution of HB was observed during cefalexin biodegradation by a nitrifying sludge system, while AMO subunit A (*amoA*)-AOB expression exhibited distinct attenuation (Guo et al., 2023). Acetate addition also led to significant enhancements in the biotransformation rates of acetaminophen and anhydro-erythromycin from 852 to 1950 μg g^{-1} VSS d^{-1} and from 195 to 280 μg g^{-1} VSS d^{-1}, respectively, suggesting the important role of HB-induced cometabolism in enhancing antibiotic removal (Lorena et al., 2021). In this study, HB became the major player (75.9–85.8 %) among other microorganisms in biodegradation of ciprofloxacin in the sidestream mixotrophic PN-MBBR with COD/N ratios of 0.34 (Fig. 2B). However, in a mainstream continuous MBBR, increasing COD/N ratios (5–100) would result in the prevalence of heterotrophic conditions associated with higher biofilm concentrations, while much higher biodegradation of diclofenac, ibuprofen and carbamazepine was achieved under low COD/N ratios (0.125–0.5) due to the abundance of *amo*A gene and contribution of AOB-induced cometabolism (Ahmadi et al., 2023). The discrepancy might be due to large proportions of HB genera (>43.5 %) in the microbial communities of the biofilm system treating sidestream wastewaters. Despite the improvement on antibiotic degradation by the diverse microbial communities, how to control or exploit potentials of the domesticated microorganisms should also be further elucidated.

Ciprofloxacin transformation pathways in sidestream PN-MBBR

Generally, no ciprofloxacin transformation products (TPs) were detected at the beginning of the experiments. The metabolites denominated with their molecular mass, including TP136, TP160, TP162, TP198, TP208, TP214, TP226 and TP310, were first detected in this study (Fig. S9). For instance, TP160 and TP162 were detected in the

batch tests with allylthiourea (ATU), probably formed based on the heterotrophic activity. TP310 and TP214 might originate from HB-induced cometabolic biodegradation, despite producing at 0.056 mg-N L^{-1} and 0.006 mg-N L^{-1} of FNA levels, respectively. Owing to the presence of ammonium, TP198, TP208 and TP226 were the exclusive products of AOB-induced cometabolic biodegradation at FNA concentration of 0.006 mg-N L^{-1}, and TP136 formed at 0.056 mg-N L^{-1}.

The tentative structures of TPs were identified according to the fragmentation patterns in Fig. S10. Thereafter, possible biotransformation pathways of ciprofloxacin in the sidestream PN-MBBR process were proposed in Fig. 3. Cleavage of the piperazine ring or the fluorine substituent was involved in the formation of less toxic TPs, as a result of losing the antibacterial groups (Amorim et al., 2014). The major ciprofloxacin transformation pathways were: *a)* N-dealkylation was the most common reaction catalyzed by AOB and HB, yielding TP160, TP214, TP208 and TP226. As a frequently reported reaction in mammalian metabolism systems, N-dealkylation also occurred in the biotransformation of amine-containing micropollutants in activated sludge (Gulde et al., 2016), which could be catalyzed by monooxygenase, cytochrome P450 or monoamine oxidase-w-Transaminase cascade (Xu et al., 2017). *b)* Deamination was involved in the formation of TP136 and TP310, catalyzed by AOB and HB, respectively. This reaction was also common for amine-containing compounds including sulfonamides and atenolol (Xu et al., 2022; Zhou et al., 2019). As the potential enzyme for deamination reaction, deaminases were mostly encoded in the genomes of *Nitrosomonas* or *Truepera* (Zhou et al., 2019). *c)* Defluorination would lead to the formation of TP136, TP160 and TP162 in the PN-MBBR. AMO could catalyze the reductive dehalogenation on nitrapyrin at low DO concentrations and in the presence of hydrazine (Vannelli and Hooper, 1993). The intracellular cytochrome P450 enzymes were also responsible to catalyze defluorination during ciprofloxacin biodegradation (Fang et al., 2021). *d)* Decarboxylation uniquely belonged to HB-induced metabolic biodegradation, producing TP160 and TP162. Similar reaction was also reported in biotransformation of ciprofloxacin by the sulfate-reducing bacteria-enriched sludge (Fang et al., 2021). *e)* Hydroxylation was an important reaction catalyzed by monooxygenase (Amorim et al., 2014), probably resulting in TP310 by heterotrophs. *f)* Addition and reduction reactions were also observed to occur on ciprofloxacin, which were also documented in aerobic experiments by heterotrophic enzymes (Kennes-Veiga et al., 2022) and the reduction of benzaldehyde to benzyl alcohol by *Nitrosomonas europaea* (Keener and Arp, 1994).

Overall, the formation of TPs was related to microbial-induced metabolic types and FNA levels. The possible reason might lie in the different enzyme systems of HB and AOB. For instance, AMO could catalyze the biodegradation of various organic substrates in the presence of ammonium (Yu et al., 2018). In this study, TPs observed only in the presence of ammonium might indicate the importance of AOB-induced cometabolism over AOB-induced metabolism in terms of transforming ciprofloxacin, which is consistent with our previous observation in the nitrifying MBBR (Xu et al., 2023). Besides, both TP162 and TP310 might be intermediates rather than ultimate products with an increase-decrease time-dependent tendency, probably suggesting the mineralization abilities of HB (Alvarino et al., 2016). In addition, the loss of the piperazine ring or the fluorine substituent in ciprofloxacin also reflected the potential of MBBR in reducing antimicrobial activities of antibiotics in the sidestream PN process.

Environmental relevance and implications

Distinct with the suspended-growth system (e.g., activated sludge) suffering from FNA inhibition (Laloo et al., 2018), the performance of attached-growth system (e.g., biofilm) was improved under FNA stress, rendering the MBBR as an alternative performing the PN process to

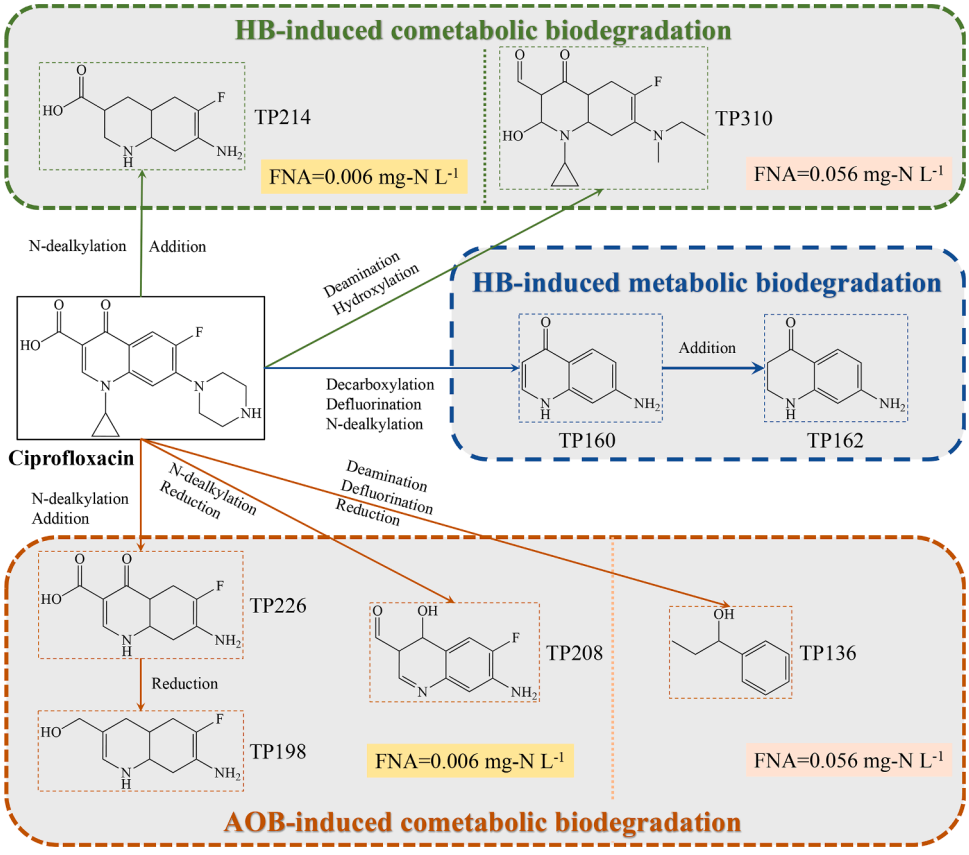

Fig. 3. Proposed biotransformation pathways of ciprofloxacin in the sidestream PN-MBBR process.

enhancing simultaneous removal of nitrogen and antibiotics when treating sidestream wastewater. Compared with suspended sludge, retention of biomass could be easily achieved by attaching to carriers in the MBBR, resulting in diverse functional microbial communities, which in turn provided higher biotransformation potentials for recalcitrant compounds (Bramha et al., 2022). Complex and layered biofilm systems were more viable to increase biodiversity for nitritation/denitritation or PN/A processes (Zou et al., 2020; Regmi et al., 2016). Removal of antibiotics and nutrients was also influenced significantly by carrier types and carrier surface properties, e.g., surface coatings (Morgan-Sagastume, 2018), redox mediators (Zhang et al., 2022), which could accelerate biological process effectively.

In addition, ciprofloxacin removal was significantly improved in the mixotrophic PN-MBBR at FNA concentration of 0.056 mg-N L^{-1}, being removal efficiency (~98 %) and removal rate constant (0.021 L g^{-1} SS h^{-1}) 5.8 and 51.2 times higher than corresponding values (~17 % and 0.00041 L g^{-1} SS h^{-1}) in the autotrophic PN-MBBR at 0 mg FNA-N L^{-1}. The practical application challenges are raised in terms of increasing FNA levels and dosing organic carbon sources. First, two-stage PN/A or nitritation/denitritation biofilm-based processes would perform better in removal of antibiotics than the corresponding one-stage processes. Relatively higher concentrations of nitrite were present in the PN period of two-stage processes, while much lower nitrite was achieved due to the nitrite scavenging effect of anammox and denitrifying activity of HB in one-stage processes (Wang et al., 2022). Thus, enhancing antibiotic removal could be achieved in the two-stage biofilm-based processes, benefiting from higher FNA levels to stimulate HB-induced biodegradation. Second, dosing commercial organic carbon sources is costly and unsuitable for optimizing antibiotic removal in the full-scale sidestream PN-MBBR process. By contrary, short-chain volatile fatty acids (VFAs) produced during anaerobic fermentation of various sources (e.g., sewage sludge, food waste, industrial sludge) could be an alternating internal carbon source for growth of HB (Kang et al., 2023), thereby creating mixotrophic conditions for removing antibiotics. Acetate was the most efficient VFA for increasing HB activity and for maximizing nitrite accumulation (Le et al., 2019; Adav et al., 2010). Taken together, FNA accumulation and VFA production can be achieved via regulating PN-related and sludge fermentation-related operating parameters, finally leading to a sustainable and efficient biofilm-based technology in simultaneous removal of nitrogen and antibiotics in sidestream wastewater (Fig. 4).

Conclusions

This study provides guidance in optimizing ciprofloxacin removal through combing regulations of FNA and trophic modes in the PN-MBBR treating sidestream wastewaters. As a result of FNA-stimulating effect on HB-induced biodegradation, ciprofloxacin removal was enhanced with increasing FNA levels in either autotrophic or mixotrophic PN-MBBR. In the mixotrophic mode, exogenous COD supply could further improve ciprofloxacin removal. As FNA increased from 0.006 to 0.056 mg-N L^{-1}, AOB-induced biodegradation was inhibited, while HB-induced biodegradation significantly increased, rendering HB-induced cometabolism as the predominant mechanism under higher FNA stress. Furthermore, various transformation products without piperazine ring or fluorine substituents suggested the feasibility of PN-MBBR in eliminating antimicrobial activities of antibiotics during the sidestream treatment.

Materials and methods

Chemicals

Ciprofloxacin (≥98 %) and ATU (≥98 %,) were purchased from Sigma-Aldrich, China. Acetonitrile (HPLC grade) was offered by Thermo Fisher, USA. Other chemicals were provided by Sinopharm, China. Ciprofloxacin stock solution at 100 mg L^{-1} was prepared in 0.2 M HCl and stored at −20 °C until use.

Setup and operation of PN-MBBR

The laboratory-scale MBBR was set up in a 2-L cylindrical reactor, containing 265 carriers at 40 % filling ratio for biofilm incubation. K5 was selected as the carrier due to its relatively larger specific surface area (800 m^2/m^3) (Morgan-Sagastume, 2018), easily shedding of aged biofilm (Forrest et al., 2016) and better pollutant removal capacity (Arabgol et al., 2020). The MBBR was operated with programmed

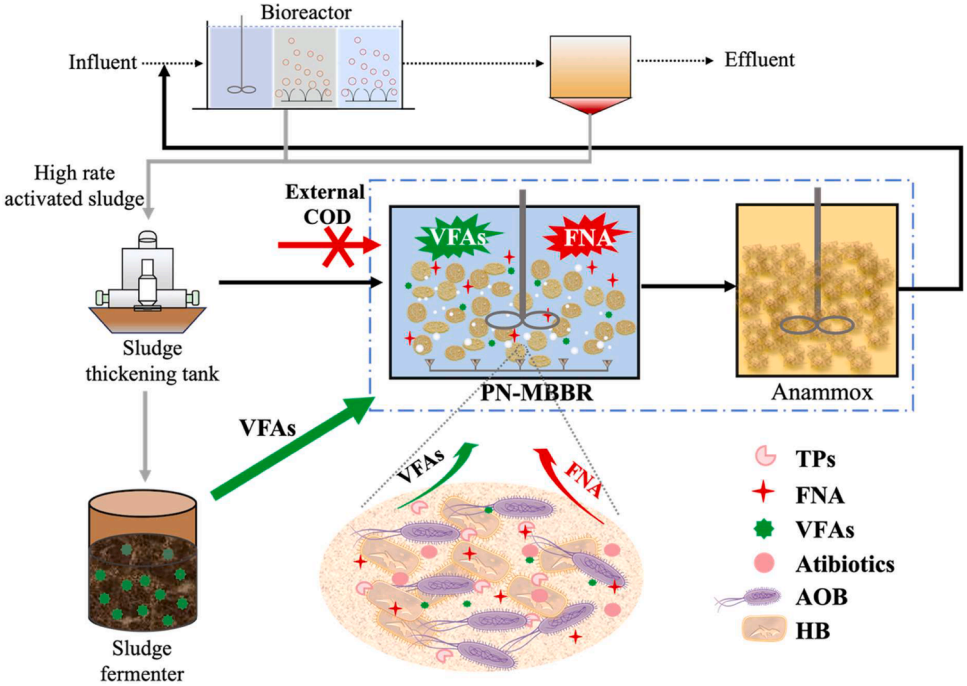

Fig. 4. The conceptual VFAs-FNA-based regulation strategies with MBBR-supported PN process for improving antibiotic removal in the sidestream wastewater.

aeration controllers to maintain the DO concentrations at 0.5–1.0 mg L^{-1} and to ensure efficient homogenization. A thermostatic water bath was coupled with the MBBR to control the nearly constant temperature at 33 ± 2 °C via the water jacket. During the enrichment stage (0–32 d), ammonium concentration in the influent was gradually increased from 300 mg-N L^{-1} to 1000 mg-N L^{-1}. During the stable stage (33–150 d), hydraulic retention time was designed at 12 h, comprising two operational cycles to achieve the PN process. Each cycle consisted of 40-min feeding, 305-min aerobic reaction and 15-min decanting. The synthetic wastewater constituents could be referred to the previous work (Xu et al., 2023). The detailed performance of the PN-MBBR is described in Fig. S11 in the Supporting Information. Before the batch tests, MBBR was run stably for >150 days with the average suspended solids (SS) concentrations of 5.68 ± 0.22 g L^{-1} and the average FNA level of 0.006 mg-N L^{-1}, achieving the effluent nitrite to ammonium ratio at 1.2 ± 0.4 and nitrite accumulation ratio over 80 %. *Nitrosomonas* was the sole genus of AOB (17.6 %) in the PN-MBBR according to microbial community analysis (Fig. S6).

Ciprofloxacin removal tests

The cultivated biofilm carriers were withdrawn randomly from the PN-MBBR into the 500 mL beakers with a filling ratio of 40 % and then washed with 0.1 M phosphate buffer (pH 7.5) to remove the residual substrates prior to the batch tests. To enhance the detection signals of the transformation products, the initial concentration of ciprofloxacin was set as 1.0 mg L^{-1}, which would not affect nitrification as well (Yi et al., 2017). Two series of experiments were designed to study the effect of FNA and organic carbon on removal of ciprofloxacin. For first series (i.e., autotrophic PN-MBBR), removal of ciprofloxacin was assessed at FNA concentrations of 0, 0.006 and 0.056 mg-N L^{-1} according to FNA accumulation levels in Test I-1, II-1 and III-1 as shown in Table 1. During the tests, ammonium bicarbonate was added to control constant ammonium concentrations (500 mg-N L^{-1}) according to the consumption rate. For each FNA accumulation level, additional control experiments were conducted to assess the contribution of AOB-induced metabolism and HB-induced metabolism by omitting ammonium during the entire period and by adding ATU at the beginning, respectively. ATU did not affect enzymes that were essential in energy conservation and central metabolism of heterotrophic bacteria (Men et al., 2017), therefore they could still remain active in the biofilm. For second series (i.e., mixotrophic PN-MBBR), according to sidestream characteristics (biodegradable COD of 136–221 mg L^{-1}) (Devos et al., 2023), organic carbon was provided at 170 mg L^{-1} at the beginning of tests to evaluate the effect of COD on the removal of ciprofloxacin in the PN-MBBR (Table 1). Glucose was chosen as the external organic carbon source considering the commercial availability and cost-effectiveness (Liu et al., 2022). Throughout the experiments, the batch beakers were all wrapped in aluminum foil to avoid photodegradation. DO and temperature were controlled same as the PN-MBBR, i.e., 0.5–1.0 mg L^{-1} and ~33 °C. Based on weight difference, deionized water was added before sampling to replenish the water loss due to evaporation. All batch tests were carried out in duplicate.

To determine whether accumulated nitrite would affect ciprofloxacin removal via chemical reactions, 1.0 mg L^{-1} ciprofloxacin and different levels of nitrite were added into aqueous solution without biomass (Table S1). Besides, the sterilized-control tests were carried out to assess the contribution of adsorption to ciprofloxacin by inactivating biofilms at 121 °C and 101 kPa for 30 min. Blank carrier-control tests were conducted to assess the adsorption of ciprofloxacin onto the carrier without biofilm. Hydrolysis-control tests were conducted to assess ciprofloxacin removal in Milli-Q water without any biofilms or carries.

Chemical analysis

All the samples were centrifuged at 12,000 rpm for 30 min to acquire supernatant for analysis of nitrogen species, COD and ciprofloxacin. Nessler's reagent colorimetric method, N-(1-naphthyl)-ethylenediamine dihydrochloride colorimetric method and ultraviolet spectrophotometry were used to analyze the concentrations of ammonium, nitrite, and nitrate, respectively, by UV–Visible spectroscopy (Metash UV5500PC). COD test kit (LH-YDE-100, China) was used to measure COD concentrations. The Lowry method and the anthrone sulfuric acid method were adopted to determine the concentrations of EPS contents (i.e., protein and polysaccharide) (Felz et al., 2019). The concentrations of FA and FNA were calculated using the Eqs. (1), (2), in which $C_{NH_4^+-N}$ is the concentration of ammonium (mg-N L^{-1}), $C_{NO_2^--N}$ is the concentration of nitrite (mg-N L^{-1}) and T is the temperature (°C). Statistical significance was confirmed by analysis of variance (ANOVA) in SPSS (IBM SPSS Statistics 25). Statistically significant differences were considered when $p < 0.05$.

$$FA = \frac{C_{NH_4^+-N} \times 10^{pH}}{e^{6344/(273+T)} + 10^{pH}} \quad (1)$$

$$FNA = \frac{C_{NO_2^--N}}{e^{-2300/(273+T)} \times 10^{pH}} \quad (2)$$

Concentrations of ciprofloxacin were determined using a high-performance liquid chromatography (HPLC) (Agilent 1260, USA) equipped with a diode array detector. The Agilent ZORBAX SB-C18 column (4.6 mm × 150 mm, 5 μm) was kept in a column oven at 30 °C. The mobile phase consisted of 82 % Milli-Q water (with 3 % formic acid) and 18 % acetonitrile. The flow rate was set as 1 mL min^{-1} at the injection volume of 10 μL. High performance liquid chromatography tandem quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF-MS/MS, 1290II/6545B, Agilent) was used to analyze ciprofloxacin transformation products. ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size) was installed for sample separation. The sample (2 μL) was injected at a flow rate of 0.2 mL min^{-1}. The gradient elution program was initiated with Milli-Q water (A) and methanol (B) as mobile phases. The specific program was: 10 % B (0–2 min); 10 % B-60 % B (2–5 min); 60 % B (5–8 min); 60 % B-90 % B (8–12 min); 90 % B

Table 1
Operational conditions for ciprofloxacin biotransformation tests under varying FNA accumulation levels and different trophic modes.

Series	Tests	FNA (mg-N L^{-1})	COD (mg L^{-1})	pH	Ammonium (mg-N L^{-1})	Nitrite (mg-N L^{-1})	ATU (mg L^{-1})
Autotrophic PN-MBBR	I-1	0	0	8.0–8.5	0	0	0
	I-2	0	0	8.0–8.5	0	0	100
	II-1	0.006	0	8.0–8.5	500	0	0
	II-2	0.006	0	8.0–8.5	0	306.90	100
	II-3	0.006	0	8.0–8.5	0	306.90	0
	III-1	0.056	0	6.0–6.5	500	0	0
	III-2	0.056	0	6.0–6.5	0	80.26	100
	III-3	0.056	0	6.0–6.5	0	80.26	0
Mixotrophic PN-MBBR	I-3	0	170	8.0–8.5	0	0	0
	II-4	0.006	170	8.0–8.5	500	0	0
	III-4	0.056	170	6.0–6.5	500	0	0

(12–15 min); 90 % B-10 % B (15–16 min) and 10 % B (16–19 min). Positive electrospray ionization (ESI+) mode was applied in the mass spectrometer. The potential transformation products were screened based on the full scan mode, followed by the product ion mode for structure identification. The detailed mass spectrometry settings included drying gas (temperature of 320 °C, flowrate of 8 L min^{-1}), nebulizer (35 psi) and nozzle voltage (1000 V). The scanning mass range was set from *m/z* 40 to *m/z* 700. Based on the peak shape of the chromatogram, the transformation products were recognized as appearing in the biotransformation tests but not in the control groups, and showing a time-dependent increasing or increasing-decreasing trend. The product ion scan mode (MS^2) was conducted by applying collision energies at three levels of 10, 20 and 40 eV. The specific structure of each transformation product was further identified by deciphering the characterized molecular ion peaks, analyzing the belongings to each mass loss among molecular ions and fragmentation ions, and comparing with the reported degradation products in the literature.

Weight difference was measured to confirm the biofilm biomass concentration (Liang et al., 2019). In brief, three carries were taken out randomly and baked at 105 °C for over 24 h in order to dry completely prior to weighing. The carriers were then immersed in 2 M NaOH solution for at least 1 h under ultrasonic conditions. Subsequently, 5 % hydrochloric acid solution and deionized water were used to wash the dried carriers until the biofilm was completely removed, followed by baking at 105 °C for 24 h. The carriers were weighed again to calculate the weight difference as the biofilm biomass concentration.

CRediT authorship contribution statement

Yifeng Xu: Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Xi Wang:** Writing – original draft, Visualization, Investigation, Data curation. **Ying Gu:** Methodology, Investigation. **Chuanzhou Liang:** Supervision, Methodology. **Wenshan Guo:** Writing – review & editing, Supervision. **Huu Hao Ngo:** Writing – review & editing, Supervision. **Lai Peng:** Writing – review & editing, Supervision, Project administration, Methodology, 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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2024.100216.

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