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Anaerobically digested blackwater treatment by simultaneous denitrification and anammox processes: Feeding loading affects reactor performance and microbial community succession

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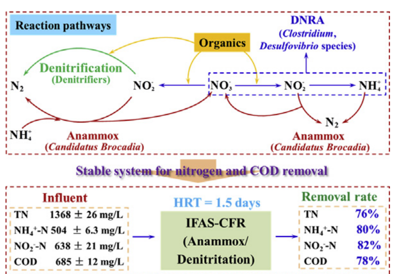
HIGHLIGHTS

- Denitrification-Anammox process showed a stable nitrogen and COD removal performance.
- Anammox and denitrification separately contributed to 44–48% and 52–56% of TN removal.
- Proteins were biodegraded preferentially to humic acid-like matters at high feeding loadings.
- Increasing feed load promoted the growth of anammox bacteria and denitrifiers in biofilm.
- Biofilm had higher nitrogen removal rate than suspended sludge, but opposite for COD removal.

GRAPHICAL ABSTRACT



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ABSTRACT

Source diverted blackwater collected from toilets can be anaerobically digested to recover energy. The anaerobically digested blackwater (ADB) contains high levels of ammonium and low carbon to nitrogen (C/N) ratio. In the present study, ADB was treated by a two-stage nitritation-denitrification/anammox process in an integrated fixed film activated sludge-continuous flow reactor (IFAS-CFR). NH_4^+-N, NO_2^--N, total nitrogen (TN), and chemical oxygen demand (COD) removal efficiencies were 80%, 82%, 76%, and 78%, respectively. Anaerobic ammonium oxidation (anammox) and denitrification contributed to 44–48%, and 52–56% of total nitrogen removal, respectively. Both of the protein- and humic acid-like matters were removed during the process. An increase in feed load promoted the sustained growth of anammox bacteria—*Candidatus Brocadia* in the biofilm, as well as an increase of denitrifiers (*Pseudomonas*, *Thermotonus*, *Phodanobacter*, *Caulobacter*) in both biofilm and suspended biomass, which

Abbreviations: ADB, anaerobically digested blackwater; AOB, ammonia oxidizing bacteria; COD, chemical oxygen demand; DNRA, dissimilation of nitrite or nitrate reduction to ammonium; DO, dissolved oxygen; EEA, excitation-emission area; EEM, excitation-emission matrix; Ex/Em, excitation/emission wavelengths; FRI, fluorescence regional integration; HRT, hydraulic retention time; IFAS-CFR, integrated fixed film activated sludge-continuous flow reactor; IFAS-SBR, integrated fixed-film activated sludge-sequencing batch reactor; ON, organic nitrogen; TN, total nitrogen; TSS, total suspended solids; UASB, up-flow anaerobic sludge blanket; VSS, volatile suspended solids.

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Denitrification
Anammox
Fluorescence regional integration (FRI)
High-throughput sequencing

remained higher in the suspended biomass than in biofilm. Overall, biofilm had higher nitrogen removal efficiency than suspended biomass, while suspended biomass had a higher COD removal efficiency than biofilm.

1. Introduction

Blackwater, which is comprised by toilet waste containing faeces, urine, toilet paper, and flush-water, accounts for 15–30% of domestic wastewater (Jefferson et al., 2000; Palmquist and Hanæus, 2005). Anaerobic digestion, which converts organic matter to methane, can be applied to recover energy from blackwater (Rajagopal et al., 2013). However, anaerobically digested blackwater (ADB) contains high amounts of ammonium (1400 mg NH_4^+-N/L), moderate amounts of chemical oxygen demand (COD, ~1400 mg COD/L), and a relatively low carbon to nitrogen (C/N) ratio, which can result in adverse effects on public health and the environment without appropriate treatment (Gao et al., 2019). Thus, nitrogen and organics must be removed from ADB before it is released to the environment.

Although nitrification and denitrification can be used for nitrogen and organics removal, these processes demand high oxygen levels and an external organic carbon source (Du et al., 2014, 2016; Cao et al., 2019). Anaerobic ammonium oxidation (anammox), oxidizes ammonium (Eq. (1)) without the need for added oxygen or carbon and has thus recently received much attention. Anammox processes can be combined with nitritation processes (Eq. (2), ammonia oxidizing bacteria (AOB) partial nitrification of NH_4^+ to NO_2^-) to reduce aeration and external carbon needs by 60% and 100%, respectively while producing 90% less sludge, as compared to the conventional nitrification/denitrification processes (Du et al., 2016). In the presence of organic carbon, denitritation (Eq. (3)) uses heterotrophic denitrifiers to reduce NO_2^- and organic substances. The NO- 3 produced by anammox can also be reduced to N_2 by denitrifiers (Du et al., 2016; Zhou et al., 2018; Meng et al., 2019). A process that combines nitritation, denitrification, and anammox has the potential to remove nitrogen and organic matter from ADB.

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \tag{1}$$

$$NH_4^+ + 1.38O_2 + 1.98HCO_3^- \rightarrow 0.018C_5H_7O_2N + 0.98NO_2^- + 1.04H_2O + 1.89H_2CO_3 \tag{2}$$

$$NO_2^- + 1.375\,H^+ + 0.375CH_3COO^- \rightarrow 0.5\,N_2 + 1.25H_2O + 0.75CO_2 \tag{3}$$

In order to meet discharge regulations of COD and nutrients, further treatment of ADB is often required. However only limited studies have been reported to date on the treatment of ADB. Vlaeminck et al. (2009) investigated the application of one-stage partial nitritation and anammox process in a rotating biological contactor for treating ADB and found that a stable nitrogen removal rate and efficiency of 700 mg/L/d and 76%, respectively, were reached over 5 months. By using an algal treatment system fed with ADB, de Wilt et al. (de Wilt et al., 2016) found that both PO_4–P and NH_4–N were nearly completely removed, and 60–100% of micropollutants were removed. The only other study on the biological nitrogen removal from ADB used a combination of a partial nitrification (SHARON) and anammox process (Van Dongen et al., 2001), and reported 80% N reduction. However, none of these studies discussed about the fate of COD in ADB. The presence of organic matter in ADB is inevitable and may cause inhibitory effects for anammox at high concentrations. Thus, it's important to achieve the simultaneous removal of ammonia and COD from ADB.

In the present study, the second stage of a two-stage nitritation-anammox/denitritation process for ADB treatment was studied. An integrated fixed film activated sludge-continuous flow reactor (IFAS-CFR) was applied to carry out anammox/denitritation treatment. To evaluate how feed loading affected reactor performance and the microbial community, the long-term performance of the IFAS-CFR at different feed loadings was investigated. High-throughput sequencing of 16S rRNA gene amplicons was used to detect the succession of microbial communities in the IFAS-CFR; batch experiments were conducted to evaluate the removal efficiencies of nitrogen and COD by biofilm and suspended biomass during different phases of reactor operation.

2. Materials and methods

2.1. Experimental setup and operation

ADB used in this study was collected from a continuous operating up-flow anaerobic sludge blanket (UASB) reactors, then introduced into a laboratory-scale nitritation reactor, using integrated fixed-film activated sludge-sequencing batch reactor (IFAS-SBR) operation to oxidize NH_4^+-N to NO_2^--N. Detailed operation conditions of IFAS-SBR are provided in the supporting information. Water characteristics of ADB and IFAS-SBR effluent are provided in Table S1.

IFAS-SBR effluent was combined with ADB (with NO_2^--N [mainly from IFAS-SBR]: NH_4^+-N [mainly from ADB] mass ratio of 1.32) to feed an IFAS-CFR (Fig. 1). An air-tight seal of the feeding bottle was maintained by a rubber stopper containing one outlet from the liquid phase and one inlet to the headspace. The feeding bottle was sparged by pure N_2 gas for 30 min to removal the influent dissolved oxygen (DO). The IFAS-CFR, which is made from a plastic cylinder with a diameter of 0.12 m and a working volume of 1.0 L, was maintained at a hydraulic retention time (HRT) was maintained at 1.5 days by a constant influent rate of 27.8 mL/h. The surface of the IFAS-CFR was covered with silver foil to inhibit the growth of phototrophic microorganisms. The operation temperature was maintained at 35 °C with a silicone heat blanket with digital temperature control. The reactor was mixed using an agitator with a rotary speed of 30 rpm. The denitrification-anammox reactor was continuously operated for 96 days with stable performance.

2.2. Biomass inoculum and feed media

The IFAS-CFR was seeded with laboratory enriched anammox sludge. To investigate the effects of feed loading on denitrification-anammox performance, the IFAS-CFR was fed with a mixture of ADB and IFAS-SBR effluent supernatant at a ratio of 1:1.66 to achieve the NO_2^--N: NH_4^+-N mass ratio of 1.32:1. This was performed at various levels of dilution (with DI water) for step-wise reactor startup. Table 1 shows the parameters of feed media at different times.

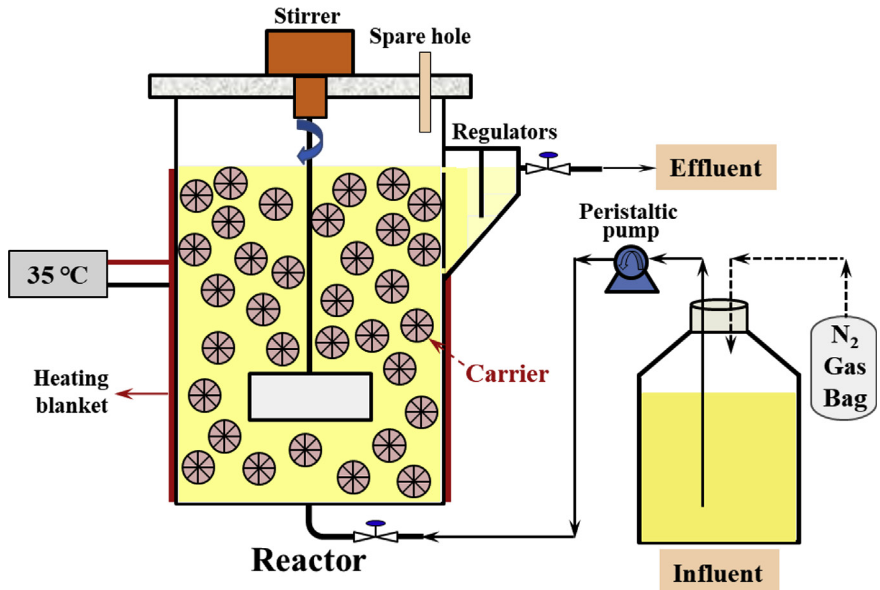

Fig. 1. Schematic diagram of denitrification-anammox treatment of anaerobic digested blackwater.

Table 1
Denitrification-anammox conditions over time [a].

Stage (day)	NH_4^+-N (mg/L)	NO_2^--N (mg/L)	NO_3^--N (mg/L)	TN (mg/L)	COD (mg/L)	TA	pH
1 (0–8)	52.3 ± 0.3	69.1 ± 0.8	1.02 ± 0.14	132.7 ± 14	113 ± 2.4	243.7 ± 12	7.2 ± 0.3
2 (9–33)	162 ± 2.8	214 ± 0.6	1.89 ± 0.28	418.3 ± 11	269 ± 28	804.5 ± 19	7.1 ± 0.2
3 (34–60)	254 ± 6.4	332 ± 12	3.72 ± 0.39	642.6 ± 15	348 ± 39	1183 ± 24	7.2 ± 0.2
4 (61–96)	504 ± 6.3	638 ± 21	5.67 ± 0.41	1368 ± 26	685 ± 12	2489 ± 23	7.2 ± 0.1

[a] TN, total nitrogen; COD, chemical oxygen demand; TA, total alkalinity.

2.3. Assay of nitrogen and COD removal in biofilm and suspension

Batch experiments were performed to investigate nitrogen and COD removal from biofilm and suspended sludge in different phases. The media was prepared by combining effluent supernatant from the IFAS-SBR with ADB (with the volume mixing ratio of 1.66:1) and diluting the mixture with DI water until the concentrations of NH_4^+-N, NO_2^--N, TN, and COD reached 50 mg N/L, 66 mg N/L, 138 mg N/L, and 72 mg COD/L, respectively. Sludge suspension (100 mL) and 20 carriers were taken out of the IFAS-CFR on days 1, 45, and 90. Carriers were washed three times using phosphate buffered saline (PBS) consisting of 2 mM Na_3PO_4, 4 mM Na_2HPO_4, 9 mM NaCl, and 1 mM KCl (Yu et al., 2008), and the suspension was centrifuged at 4500 rpm for 20 min at room temperature (21.8 °C). The washed carriers and collected sludge were added separately to flasks with a media volume of 100 mL; all reactions were performed in a shaking incubator (SK-71, JEIO TECH, Korea) at 180 rpm and 35 °C for 10 h. The removal (*Rl*) of inorganic nitrogen (InoN, where InoN = NH_4^+-N + NO_2^--N), organic nitrogen (ON, where ON = TN − [NH_4^+-N + NO_2^--N + NO_3^--N]), and COD were calculated using the following equation:

$$Rl = \frac{substrate\ consumption\ rate}{VSS} \tag{4}$$

where substrate consumption includes the removal of InoN, ON, and COD, and VSS represents the volatile suspended solids in the biomass.

2.4. Chemical analysis

COD concentrations were measured according to *Standard Methods* (APHA, 2005). The concentrations of NH_4^+-N, NO_3^--N, NO_2^--N, TN, and total alkalinity (TA) were measured using a benchtop spectrophotometer (DR 3900, HACH, USA) with the following HACH kits and reagents: NH_4^+-N kits, TNT (2–47 mg/L NH_3–N, HACH®, USA); NO_3^--N kits, TNT 835 (0.2–13.5 mg/L NO_3–N,HACH®, USA); NitriVer®2 Nitrite Reagent Powder Pillows (2–250 mg/L NO-2, HACH®, USA); Nitrogen (Total) kits, TNT 827 (5–40 mg/L N, HACH®, USA); and TA kits, TNT870 (25–400 mg/L $CaCO_3$, HACH®, USA). The effects of anammox and denitrification on the removal of TN without considering biomass synthesis were calculated as follows:

$$Anammox\ percentage\ (\%) = \frac{\left(NH_4^+ - N_{Inf}\ - NH_4^+ - N_{Eff}\right) \times (1 + 1.32\ - 0.26) \times 100\%}{TN_{Inf} - TN_{Eff}} \tag{5}$$

$$Denitrification\ percentage(\%) = 100\% - Anammox\ percentage\ (\%), \quad (6)$$

where NH_4^+-N_{Inf} and NH_4^+-N_{Eff} are the influent and effluent ammonium concentrations, respectively; and TN_{Inf} and TN_{Eff} are the influent and effluent TN concentrations, respectively.

Total suspended solids (TSS) and volatile suspended solids (VSS) in the suspension were analyzed in accordance with *Standard Methods* (APHA, 2005). The biomass concentration on the surface of carriers was measured according to our previous studies (Shao et al., 2017, 2018). The pH of the samples was measured with a pH meter (B40PCID, VWR, SympHony). The fluorescence spectrum of the samples was characterized using a excitation-emission matrix (EEM) fluorescence spectrophotometer (Varian Cary Eclipse, Agilent, Australia). The fluorescence spectra were collected every 10 nm over an emission range of 220–450 nm, and 10 nm over an emission range of 250–550 nm, with a 5 nm width of the excitation/emission slit and 1200 nm/min scanning speed. The DI water was used as the control. A fluorescence regional integration (FRI) technique was used to quantify EEM fluorescence (Chen et al., 2003; Zhou et al., 2017a). There are five regions in the EEM fluorescence spectrum: Regions I and II, with excitation/emission wavelengths (Ex/Em) of 200–250/280–330 nm and 200–250/330-380 nm, respectively, are related to aromatic proteins. Region III (Ex/Em 200–250/380-550 nm) is related to the fulvic acid-like matter. Region IV (Ex/Em > 250/280–380 nm) is related to protein-like matter. Region V (Ex/Em > 250/380–550 nm) is related to humic acid-like matter (Chen et al., 2003; Zhou et al., 2017b). OriginLab 8.1.5 software (OriginLab Corp, Northampton, USA) was used to calculate the cumulative volume beneath each excitation-emission area (EEA). Normalized excitation-emission area volumes ($\Phi_{i,n}$ and $\Phi_{T,n}$) and percent fluorescence response ($P_{i,n} = \Phi_{i,n}/\Phi_{T,n}$) were calculated by normalizing the cumulative EEA volumes to relative regional areas (nm^2) (Chen et al., 2003).

2.5. Microbial community analysis

Two carriers with biofilm attached in the IFAS-CFR were collected on days 1, 45, and 90 and cut into small fractionlets before DNA extraction. Because no suspended biomass was inoculated to the bioreactor, suspended sludge (2 mL) was collected on days 45 and 90 (and not on day 1). Suspensions were centrifuged at 6000 rpm for 10 min at 4 °C. The pellets were collected after centrifugation and the carrier fractionlets were used for genomic DNA extraction using a DNeasy PowerSoil Kit (Qiagen Inc., Toronto, Canada) according to the manufacture's protocol.

Forward primers (515) (5′- ACA CTG ACG ACA TGG TTC TAC AGT GYC AGC MGC CGC GGT AA -3′) and reverse primers (806) (5′- TAC GGT AGC AGA GAC TTG GTC TGG ACT ACN VGG GTW TCT AAT -3′) were used to amplify 16S rRNA genes as previously described (Apprill et al., 2015; Parada et al., 2016). DNA amplicon samples were sent to McGill University and the Génome Québec Innovation Centre (Montréal, Québec, Canada) for barcoding and sequencing on an Illumina Miseq PE250 platform. The demultiplexed raw sequences containing forward and reverse sequences were paired and quality-filtered using the "DADA2" algorithm (Callahan et al., 2016) in Qiime 2 pipelines, version 2018.8 (Caporaso et al., 2010). The GreenGenes reference database, version 13_5, was used to assign taxonomy with 99% similarity (McDonald et al., 2012; Werner et al., 2012).

2.6. Statistical analysis

Each sample from the IFAS-CFR was assayed three times at the times noted to measure COD, TSS, VSS, TN, NH_4^+-N, NO_3^--N, NO_2^--N, TA, and pH. The results are expressed as the mean and standard deviation (mean ± SD) of the three time determinations. Influent and effluent from the IFAS-CFR were assayed at the times noted one time for 3D-EEM. OriginLab 8.1.5 software (OriginLab Corp, Northampton, USA) and Pearson's correlation coefficient (R^2) were used to identify and estimate the correlation between two parameters. Correlations were considered statistically significant when the confidence interval was higher than 95% ($P < 0.05$).

3. Results and discussion

3.1. Nitrogen removal characteristics

Fig. 2 (a) and (b) show the quantities of NH_4^+-N and NO_2^--N, respectively, removed from anaerobic digested blackwater by denitrification-anammox. In stage I, the effluent concentrations of NH_4^+-N and NO_2^--N gradually decreased and then remained stable. In stage II, the effluent concentrations of NH_4^+-N and NO_2^--N dramatically increased initially, then gradually decreased, then remained fairly stable. In stage III, the effluent concentrations of NH_4^+-N and NO_2^--N increased slightly over a short-time, then decreased and remained stable with a removal rate of 84% and 87%, respectively. In stage IV, effluent concentrations for NH_4^+-N and NO_2^--N dramatically increased in the initial 3 days followed by a gradual decrease. The removal rates of NH_4^+-N and NO_2^--N were 80% and 82%, respectively. Increasing the feed loadings of nitrogen and COD reduced the removal rate of NH_4^+-N and NO_2^--N initially, which is consistent with previous studies (Du et al., 2016; Meng et al., 2019). However, the nitrogen removal rate recovered quickly within only 3–5 days. The NO_3^--N output gradually increased and remained stable at about 8 mg/L in stage I. A further increase in the NH_4^+-N feed in stages II and III caused effluent NO_3^--N to gradually increase before stabilizing at 74 mg/L in stage IV.

The variations in TN, NH_4^+-N, and NO_2^--N removal were similar throughout the IFAS-CFR process (Fig. 1 [e]). When the TN concentration was increased from stage I to stage III, the effluent TN gradually increased before stabilizing. The TN removal rate initially decreased from 79% to 65% and then gradually increased and remained stable at 78%. In stage IV, the removal rate of TN dramatically decreased initially, then gradually increased and remained stable at about 76%. The contributions of denitrification and anammox responsible for TN removal were used to evaluate the stability of nitrogen removal (Du et al., 2016; Meng et al., 2019). TN removal was mainly due to 52%–58% denitrification and 42%–48% anammox. When the TN concentration was increased from 630 to 1350 mg/L, the average TN removal by anammox decreased from 47% to 42%, and the average TN removal by denitrification increased from 53% to 58%, indicating that denitrifiers were dominant in the removal of TN. The inhibition of anammox in the presence of organic substances has been observed in previous studies (Kumar and Lin, 2010; Tang et al., 2010; Du et al., 2016), as denitrifiers have a higher growth rate than anammox bacteria. Our results showed that although an increase in feed loading reduced anammox activity and improved the denitrification efficiency, denitrifiers and anammox bacteria could coexist harmoniously, allowing the reduced nitrogen removal efficiency to recover quickly and then stabilize. Thus denitrification-anammox is a stable system for the anaerobic digestion of blackwater.

3.2. Organic compound removal characteristics

Fig. 3(a) shows COD removal by denitrification-anammox during the anaerobic digestion of blackwater. In stage I, the COD output gradually decreased, then remained stable with a removal rate of

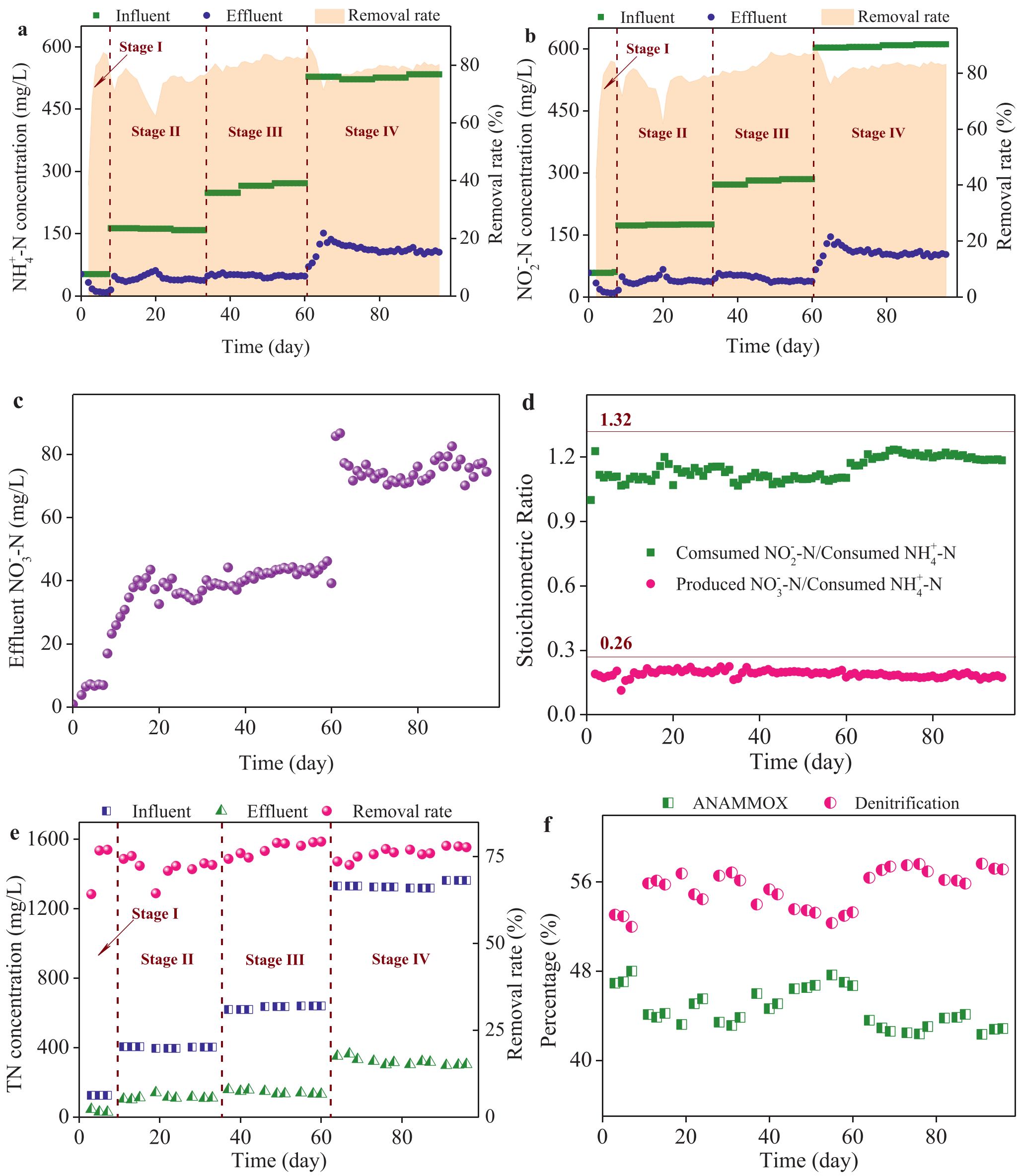

Fig. 2. Denitrification-anammox performance in the treatment of anaerobic digested blackwater; (a) NH_4^+-N, (b) NO_2^--N, the concentration of (c) NO_3^--N, (d) the ratio of NO_2^--N consumption to NH_4^+-N removal and the ratio of NO_3^--N production to NH_4^+-N consumption, (e) the removal of TN and (e) percentage of Anammox and denitrification contribution on TN removal in the denitrification- Anammox process during the long-term operation.

66%. When the input COD was increased from 250 to 405 mg/L (Stage II and III), the effluent COD initially increased, then gradually decreased, then remained stable with an average removal of 75%. In stage IV, the COD output dramatically increased initially, and then gradually decreased before stabilizing at a removal rate of approximately 75%.

Fig. 3(b—d) depict EEM fluorescence spectra of reactor influent, the normalized excitation-emission area volumes ($\Phi_{i,\ n}$) of the five regions, and the distribution of FRI, respectively. Humic acid-like substances (40%), protein-like substances (26.5%), and fulvic acid-like substances (24.5%) depicted in regions V, IV, and III, respectively, were the predominant organic substances in the EEM spectra of the anaerobically digested blackwater. Throughout the digestion, when the input $\Phi_{i,\ n}$ of regions III, IV and V were dramatically increased on days 18 and 60, the effluent $\Phi_{i,\ n}$ for humic acid-like and fulvic acid-like matter initially increased dramatically at both feeding times and then gradually decreased until it remained stable; however, the effluent $\Phi_{i,\ n}$ for protein-like substances increased only in the feeding time of day 18, then gradually decreased before stabilizing. Most of the aromatic proteins (regions I and II) were removed completely, although the influent concentrations in both of the regions gradually increased. The influent $P_{i,n}$ proportions for all five regions remained relatively stable at feeding times. With low COD loading (Stage I), the effluent proportions ($P_{i,n}$) for all five regions were approximately the same as the influent proportions ($P_{i,n}$), indicating that all the organic matter was being simultaneously biodegraded. However, when the COD loadings were gradually increased, the effluent proportion ($P_{i,n}$) for protein-like substances (region IV) continuously decreased, and aromatic proteins in regions I and II were almost non-detectable in the later stage. Results showed that humic acid-like, fulvic acid-like, and protein-like substances could be simultaneously removed under low COD loadings, however, protein-like substances were biodegraded preferentially at high COD loading conditions.

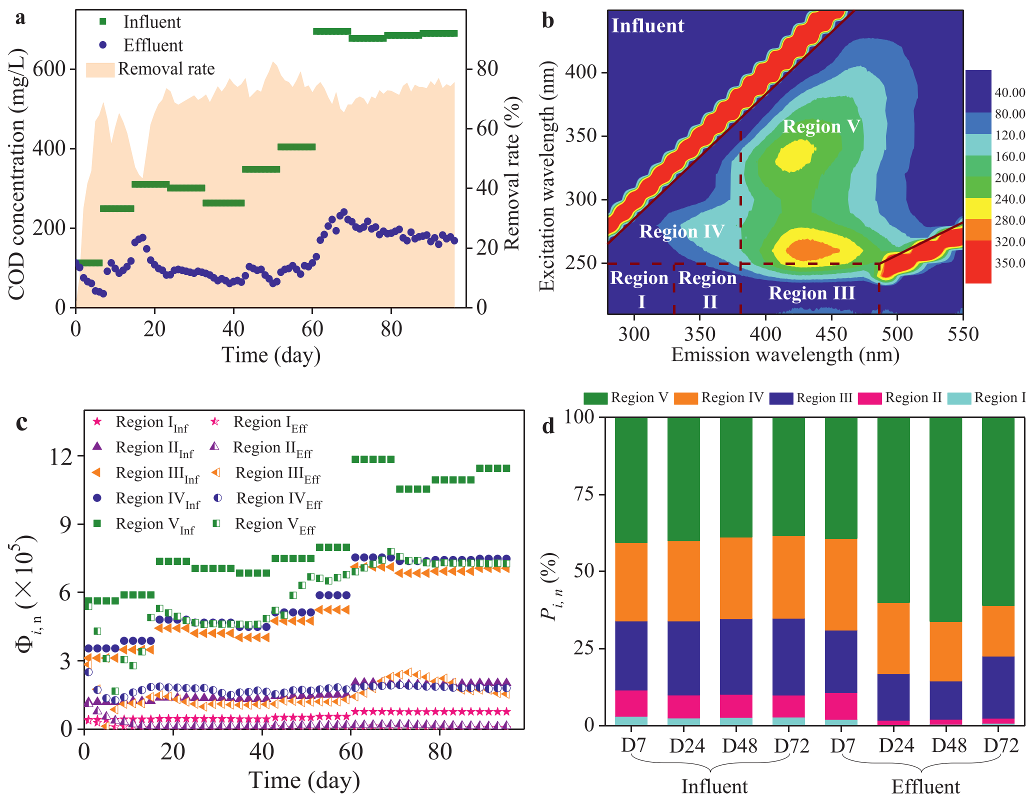

Fig. 3. (a) COD removal, (b) EEM fluorescence spectra of reactor influent at day 1, (c) variation of the normalized excitation-emission area volumes ($\Phi_{i,\,n}$) of the five regions, and (d) distribution of FRI in the anaerobic digested blackwater treated by denitrification-anammox process at the noted times. D7, D24, D48 and D72 ≡ days 7, 24, 48 and 72, respectively.

3.3. Removal of nitrogen and COD by biofilm and suspended sludge

Fig. 4(a) shows the IFAS-CFR removal of organic and inorganic nitrogen by biofilm and suspended sludge. The removal of TN was much higher by biofilm than by suspended sludge, indicating that biofilm played a prominent role in the removal of nitrogen. For biofilm, the removal of InoN was much higher than the removal of ON; the removal of ON gradually increased, however, the removal of InoN slightly decreased. For suspended biomass, the removal of InoN and ON was similar at all the sample times, and both removals gradually increased throughout the digestion. Results in this study found that biofilm plays a prominent role in the removal of nitrogen. This is consistent with Cao et al. (2019), who found that the enrichment of functional microbes leads to an increase in the nitrogen reduction rate. When increasing the feed loadings, the removal of ON by both biofilm and suspended sludge gradually increased; InoN removal by suspended biomass also increased, but at a slightly reduced rate compared to InoN removal by biofilm.

Fig. 4(b) represents the removal of COD by biofilm and suspended sludge. The removal of COD by biofilm was lower than the removal of COD by suspended sludge. This difference in COD removal increased over the term of operation, indicating that suspended sludge played a more prominent role than biofilm in the removal of COD. In biofilm, COD removal capacity gradually increased; however, it dramatically increased in suspended biomass. Results indicated that increasing the influent COD loading could dramatically improve the COD removal in suspended sludge. As denitrifiers contribute to the removal of organic matter (Du

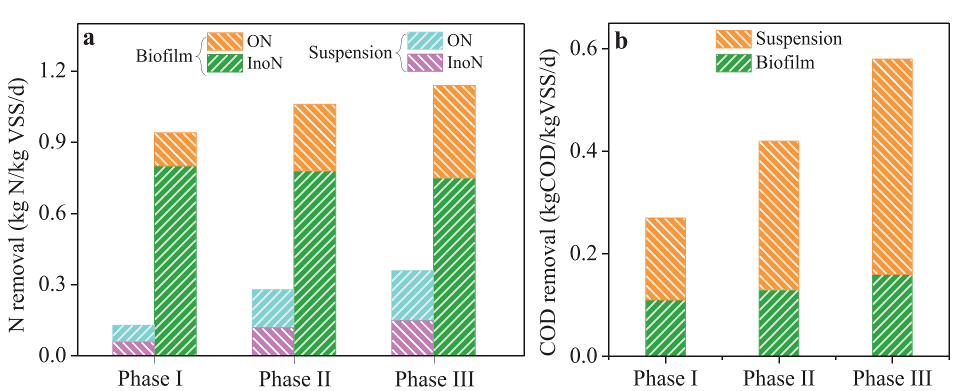

Fig. 4. Consumption rate of (a) organic nitrogen (ON) and inorganic nitrogen (InoN), (b) COD in both biofilm and suspension in phases I – III. Phases I, II, and III represent the times on days 1, 45 and 90, respectively.

et al., 2014; Meng et al., 2019), the removal of organics should be increased as functional bacteria accumulate in both biofilm and suspended sludge.

3.4. Biomass characteristics in the reactor

3.4.1. Biomass concentrations in biofilm and suspensions

Table 2 shows the concentrations of TSS and VSS and the ratio of VSS/TSS in biofilm and suspension on days 1, 45, and 90. In biofilm, both TSS and VSS concentrations gradually increased, and the ratio of VSS/TSS remained stable at 0.72 throughout the reactor operation. At the beginning of reactor operation, TSS and VSS concentrations in suspensions were low, however, TSS and VSS concentrations increased to 1.13 ± 0.09 g/L and 0.81 ± 0.06 g/L, respectively, after 90 days; the ratio of VSS/TSS also increased from 0.65 (day 1) to 0.71 (day 45) and to 0.72 (day 90). Results indicate that increasing the feed loading could improve the growth of biomass in both biofilm and suspended sludge, which could be attributed to the growth of both denitrifiers and anammox bacteria, as both organic and inorganic carbon can be electron donors (Kuenen, 2008; Cao et al., 2019). Moreover, the increased biomass in both biofilm and suspended could also improve the removal of nitrogen and organic matter (Du et al., 2014; Cao et al., 2019; Meng et al., 2019), leading to a stable performance of the IFAS-CFR for the treatment of ADB.

3.4.2. Microbial community characteristics

High-throughput sequencing of 16S rRNA gene amplicons was used to identify the microbial community in the IFAS-CFR. Fig. 5 shows the microbial community distribution at family level and genus level. Fig. S2 represents the taxonomic classification at the phyla level. Proteobacteria, which has been shown to contain most of the denitrifiers (Palmer and Horn, 2012), is the dominate phyla with a relative abundance of 47.9–52.4%, followed by Actinobacteria (10.0–19.6%), Bacteroidetes (9.9–16.8%) and Gemmatimonadetes (3.0–11.0%). Planctomycetes, the phylum of anammox bacteria (Cao et al., 2016, 2019), was also detected in both biofilm and suspended sludge throughout the denitrification-anammox process. Chloroflexi, with a relative abundance of 0.5–2.5% in this study, has commonly been found to be responsible for the biodegradation of organic matter in anammox processes (Miura et al., 2007). In the IFAS-CFR, the predominant families included *Hyphomicrobiaceae*, *Comamonadaceae*, *Xanthobacteraceae*, *Gordoniaceae*, *Gemmatimonadetes*, *Chitinophagaceae*, *Nocardiaceae*, *Trueperaceae*, and *Cryomorphaceae* (Fig. 5(a)). Brocadiaceae, the family of *Candidatus Brocadia* representing Anammox bacteria (Oshiki et al., 2011), was detected with a relative abundance lower than 1.0%.

Denitrifiers and anammox bacteria are the functional microorganisms in the IFAS-CFR responsible for removing nitrogen and organic matter. In this study, only one member of the anammox bacteria genera, *Candidatus Brocadia*, was detected in both biofilm and suspended sludge in the IFAS-CFR. The initial proportion of *Candidatus Brocadia* in biofilm was 0.35%, but gradually increased to about 0.92% after 90 days. The relative percentage of *Candidatus Brocadia* in the suspended sludge increased to 0.31% after 45 days, but nearly disappeared after 90 days. Increases in nitrogen and COD loadings promoted the sustained growth of anammox bacteria in the biofilm, leading to increased nitrogen removal (Fig. 4(a)). *Candidatus Brocadia* within suspensions increased at low feed loadings, but dramatically decreased and had almost disappeared at a COD concentration of 680 mg/L, which is consistent with previous studies showing that COD concentrations higher than 500 mg/L have been shown to completely inhibit anammox metabolism (Leal et al., 2016; Gu et al., 2018). While treating wastewater rich in NH+ 4-N and lacking in COD, Meng et al. (2019) found that *Candidatus Brocadia* was the dominant anammox species in the UASB, with the proportions of 0.16–1.58% at different temperatures. Cao et al. (2019) found that *Candidatus Brocadia* was the predominant anammox bacteria, decreasing from 0.66% to 0.35% as the influent nitrogen loading gradually increased in a partial denitrification-anammox system. Anammox bacteria and denitrifiers can coexist over long term operation in the presence of organic matter, but denitrifiers compete with anammox bacteria for electron acceptors, and the relatively low growth rate of anammox bacteria makes them less competitive than denitrifiers (Kumar and Lin, 2010; Du et al., 2014). Moreover, The typical detected microorganisms functional for dissimilatory nitrate reduction to ammonium were *Clostridium* and *Desulfovibrio* species in this study, both of which have been reported to achieve the dissimilation of nitrite or nitrate reduction to ammonium (DNRA) (Hasan and Hall, 1975; Mitchell et al., 1986). In biofilm and suspended sludge, both of the functional microorganisms gradually decreased.

Most of the microorganisms in the phylum Proteobacteria are denitrifiers, such as the genera *Pseudomonas*, *Thermotonus*, *Phodanobacter*, and *Caulobacter* (Mergaert et al., 2003; Prakash et al., 2012; Hester et al., 2018). In this study, the above genera gradually increased in both biofilm and suspensions throughout the digestion, leading to the increased removal of TN and COD (Fig. 4(a) and [b]). As the relative percentage of the above genera was higher in suspensions than in biofilm, COD removal was higher in suspensions than in biofilm (Fig. 4(b)).

3.5. Mechanisms for simultaneous removal of nitrogen and organics in the IFAS-CFR

Fig. 6 Proposed mechanisms for simultaneous removal of nitrogen and organics, as well as the synthesis of their removal efficiency in IFAS-CFR. Through the four stages of IFAS-CFR operation, denitrification-anammox was used to treat ADB with a low C/N ratio. Anammox bacteria utilized NH_4^+-N/NO_2^--N with molar ratios of 1:1.32, producing 0.26 mol of NO_3^--N (Van de Graaf et al., 1995). Heterotrophic denitrifiers achieved the simultaneous removal of NO_2^- and organic matter, and the NO_3^- produced during anammox was reduced to N_2 (Du et al., 2016; Meng et al., 2019). The following removal rates were achieved in the IFAS-CFR: NH_4^+-N (80%), NO- 2-N (82%), TN (76%), and COD (78%). However, the average ratio of NO_2^--N consumption to NH_4^+-N removal (R_c) was 1.15 throughout

Table 2
TSS, VSS, and VSS/TSS in biofilm and suspension on days 1, 45, and 90.[a].

	Unit	Day 1		Day 45		Day 90	
		Biofilm	Suspension	Biofilm	Suspension	Biofilm	Suspension
TSS	g/L	1.86 ± 0.12	0.043 ± 0.006	2.04 ± 0.14	0.38 ± 0.02	2.21 ± 0.08	1.13 ± 0.09
VSS	g/L	1.34 ± 0.07	0.028 ± 0.004	1.45 ± 0.11	0.27 ± 0.04	1.59 ± 0.13	0.81 ± 0.06
VSS/TSS	–	0.72	0.65	0.71	0.71	0.72	0.72

[a] TSS, total suspended solids; VSS, volatile suspended solids.

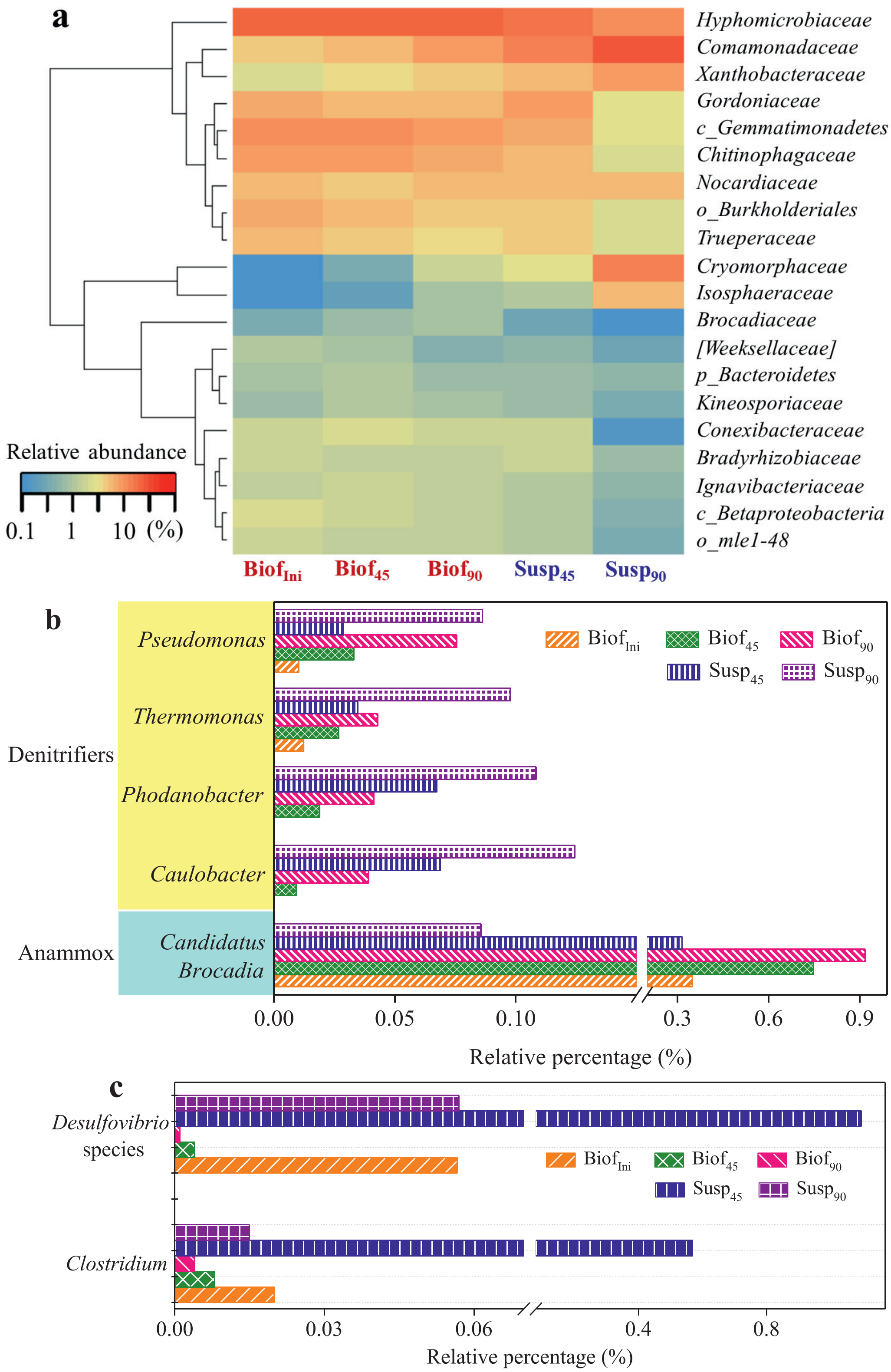

Fig. 5. Distribution of microbial community at (a) family level, (b) genus lever and (c) bacteria functional for dissimilatory nitrate reduction to ammonium (DNRA) with the samples taken from denitrification-Anammox system revealed by high-throughput sequencing of 16S rRNA gene amplicons. The genera with the relative abundance of over 0.5% in all samples are shown. $Biof_{Ini}$, $Biof_{45}$ and $Biof_{90}$ represents the biofilm samples in carrier taken on day 1, 45 and 90, respectively; $Susp_{45}$ and $Susp_{90}$ represents the suspension taken from reactor at day 45 and 90, respectively.

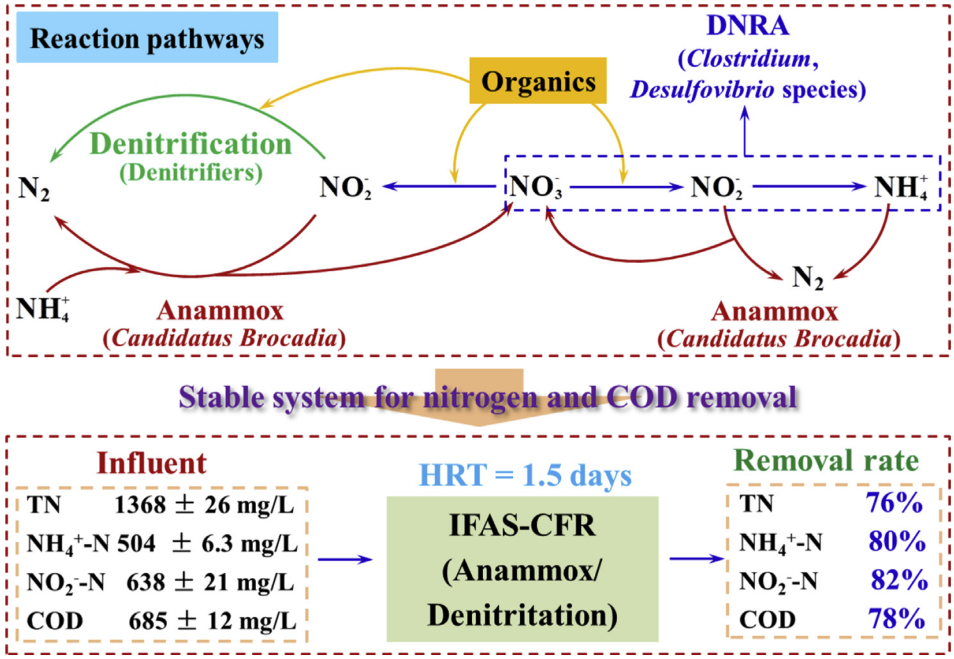

Fig. 6. Proposed mechanisms for simultaneous removal of nitrogen and organics, as well as the synthesis of their removal efficiency in the integrated fixed film activated sludge-continuous flow reactor (IFAS-CFR) for anaerobically digested blackwater treatment.

the process (Fig. 2(d)), which is lower than the theoretical value of 1.32 (Kuenen, 2008). The ratio of NO_3^--N production to NH_4^+-N consumption (R_p) was 0.19, also lower than the theoretical value of 0.26 (Kuenen, 2008), indicating a synergistic combination of anammox and denitrification due to organic substances in the ADB (Chen et al., 2019) or a DARN process (Tiedje, 1988). The detected *Clostridium* and *Desulfovibrio* species in this study also approved this process. An increase in the ADB feed load from stages III to IV led to a decrease in average rate of NO_3^--N consumption to NH_4^+-N removal (R_p) from 1.21 to 1.18, indicating that denitrification, or NO_3^--N removal, was improved by increasing the ADB feed load (Du et al., 2016).

Increased feed loadings improved the growth of biomass in both biofilm and suspended sludge, leading to an improved removal of nitrogen and organic matter (Fig. 4). Denitrifiers compete with anammox bacteria for electron acceptors, and the relatively low growth rate of anammox bacteria make them less competitive than denitrifiers (Kumar and Lin, 2010; Du et al., 2014). Therefore, *Candidatus Brocadia* decreased and denitrifiers increased in suspensions under high COD loadings (Fig. 5). The abundance of denitrifiers was positively correlated with denitrification rates (Gao et al., 2017), and the increased denitrifiers in both biofilm and suspensions improved organic and inorganic nitrogen removal (Fig. 4). Total nitrogen (contains inorganic and organic) removal was higher in biofilm than in suspended biomass, however, COD removal was greater in suspended biomass than in biofilm.

4. Conclusions

Denitrification-anammox treatment of anaerobically digested blackwater (ADB) with a low C/N ratio removed nitrogen and organic matter with average NH_4^+-N, NO_2^--N, TN, and COD removal efficiencies of 80%, 82%, 76%, and 78%, respectively. Increasing the feed load of nitrogen and COD reduced the removal efficiency of NH_4^+-N and NO_2^--N, however, the nitrogen removal rate recovered and stabilized. Anammox and denitrification contributed 44–48% and 52–56% to TN removal, respectively. Both protein- and humic acid-like matter was removed, however, protein-like substances were preferentially biodegraded an under high organic feed load. Increasing the feed load promoted the sustained growth of the anammox bacteria - *Candidatus Brocadia* in biofilm, leading to increased nitrogen removal. Denitrifiers *Pseudomonas*, *Thermotonus*, *Phodanobacter*, and *Caulobacter* gradually increased in both biofilm and suspensions, leading to an increased removal of both TN and COD. The higher proportion of denitrifiers in suspensions than in biofilm resulted in a higher COD removal in suspensions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.125101.

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