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# Mass transfer affects reactor performance, microbial morphology, and community succession in the methane-dependent denitrification and anaerobic ammonium oxidation co-culture

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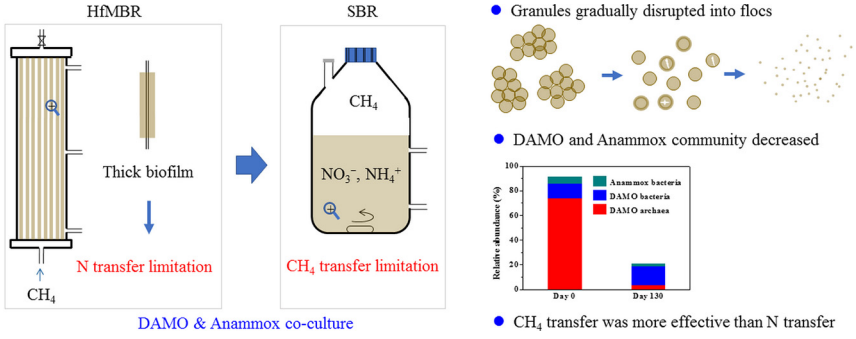
## HIGHLIGHTS

- $CH_4$ transfer is more crucial than N transfer in the DAMO & Anammox co-culture.
- Mass transfer limitation causes DAMO-Anammox granules disruption and bad settling.
- Mass transfer affects N removal and DAMO archaea and Anammox bacteria activity.
- $CH_4$ limitation affects microbial community and decreases DAMO archaea by 94.6%.
- Mass transfer impacts interactions between DAMO and Anammox microorganisms.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Denitrifying anaerobic methane oxidation (DAMO) combining anaerobic ammonium oxidation (Anammox) process is a novel nitrogen removal technology. However, the roles of methane transfer (gas phase) and nitrogen transfer (liquid phase) in the heterogeneous process remain unclear. In this study, granular DAMO and Anammox co-cultures were inoculated from a hollow-fiber membrane bioreactor into a sequence batch reactor (SBR). Since the methane transfer became limited in SBR, the nitrate removal rate first decreased and then increased to 10 mg/(L·day), while the ammonium removal rate did not recover and was around 2 mg/(L·day). The activity of DAMO archaea and Anammox bacteria decreased noticeably. Furthermore, granular aggregates dispersed into small granules and ultimately became flocs with poor settleability in SBR. The content of extracellular polymeric substances decreased, especially that of proteins and humics. DAMO archaea decreased by 94.6% and Anammox bacteria decreased by 72%. In summary, the limitation of methane transfer affected DAMO and Anammox processes more notably than nitrogen transfer, resulting in lower nitrogen removal, granule disruption, and microbial community succession.



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## 1. Introduction

Denitrifying anaerobic methane oxidation (DAMO) microorganisms utilize $CH_4$ as their sole electron donor in the denitrifying process, in which $NO_3^-$ or $NO_2^-$ is reduced to $N_2$ and $CH_4$ is oxidized to $CO_2$ (Alrashed et al., 2018; He et al., 2018b; Martinez-Cruz et al., 2017). Anaerobic ammonium oxidation (Anammox) bacteria consume $NO_2^-$ and $NH_4^+$ and generate $N_2$ without an organic carbon source. This process also produces a small amount of $NO_3^-$ (Kuenen, 2008), which can be utilized by the DAMO process, thus achieving complete nitrogen removal (Shi et al., 2013; Xie et al., 2017). Compared to traditional biological nitrogen removal technology, DAMO and Anammox processes release no nitrous oxide and consume methane (Ettwig et al., 2010; He et al., 2018a), and thus greatly contribute to the decrease of greenhouse gas emissions (Cheng et al., 2018; Short et al., 2017; Stazi and Tomei, 2018). Consequently, as a novel anaerobic biotreatment technology, DAMO combined with Anammox is regarded as a tool for the sustainable operation of wastewater treatment plants and will be applied in the future (Wang et al., 2017).

As the typical heterogeneous bioreactions in the DAMO process, the mass transfer is generally considered to be a rate-limiting step that can notably impact the DAMO and Annamox processes because methane (poor solubility, 3.5 mg per 100 mL water) must first dissolve in the liquid before it can be transferred to the DAMO cells (He et al., 2013). Mass transfer limitation of methane results in a low nitrogen removal rate of about 20 mg/(L·day) of the DAMO and Anammox co-culture system (Hu et al., 2015). Some strategies have been developed to solve this problem, such as methane partial pressures ($P_{CH4}$) increase, non-aqueous phase addition, and hollow fiber membrane bioreactor (HfMBR) utilization (Cai et al., 2018; Fu et al., 2017). For example, Ding et al. reported that the nitrate consumption rate increased 1.8-fold as $P_{CH4}$ increased from 0.1 MPa to 0.4 MPa (Ding et al., 2014). In HfMBR, microorganisms are attached on the surface of fibers and form a biofilm, where methane directly transfers from the gas phase to DAMO cells via the hollow fibers. In this way, the mass transfer process is shortened and the limitation of methane transfer is relieved. Therefore, HfMBR is considered as an effective method to promote $CH_4$ utilization in DAMO (Fu et al., 2017; Xie et al., 2017).

However, until now, the resistance of liquid mass transfer in the combined DAMO and Anammox process was generally ignored. In the inner biofilm cross section of the HfMBR, the concentration gradient of methane (gas phase) and nitrogen (liquid phase) follow an opposite direction (Chen et al., 2015). The gas concentration gradually decreased by 97% in the biofilm from the membrane side to the bulk liquid side, while the substrates concentration in liquid gradually decreased by 83% from the bulk liquid side to the membrane side in biofilms with 750 μm thickness (Chen et al., 2015). In our previous study, DAMO and Anammox were inoculated into a HfMBR; however, we found that microorganisms formed thick biofilms consisting of granular aggregates with 500–1000 μm (Fu et al., 2017). Consequently, the nitrogen removal performance was only 14–49% of other reported HfMBR results (Cai et al., 2015; Shi et al., 2013). Han et al. (2012) reported that when the thickness of biofilms in activated sludge flocs exceeded 100 μm or granules were larger than 200 μm, the liquid-phase mass transfer was limited. Thus, the nitrogen transfer in the liquid solution should also lower the microbial activities in the biofilm of HfMBR. It is known that in a sequence batch reactor (SBR), the suspended microorganisms are uniformly dispersed in the liquid phase and the dissolved substrates ($NO_3^-$, $NO_2^-$, and $NH_4^+$) are transferred to cells via free diffusion, thus, the liquid-phase mass transfer is unlimited. When granules were inoculated into the SBR, the granules would be broken in the SBR due to malnutrition in the internal granules (Lettinga, 1995), which consequently promotes nitrogen transfer.

In summary, the roles of methane transfer (gas phase) and nitrogen transfer (liquid phase) in heterogeneous bioreactions of the DAMO and Anammox co-culture system remain unclear. Furthermore, the change of mass transfer effects on microbial community and interactions between these microorganisms should also be explored. Therefore, in this study, DAMO and Anammox microorganisms were detached from HfMBR and transferred into SBR for the long-term operation. The nitrogen removal rate, microbial activities, microbial morphology, settling performance, extracellular polymeric substances (EPS), and microbial community were analyzed. The obtained results will clarify the above contradiction and benefit DAMO application in future.

## 2. Material and methods

### 2.1. Inoculum and SBR operational conditions

The HfMBR containing DAMO microorganisms and Anammox bacteria (Fu et al., 2017), the work volume was 1260 mL and the concentration of volatile suspended solid (VSS) was 1.26 gVSS/L. A gas mixture of $CH_4$ and $CO_2$ (95%:5%, v/v) was continually supplied in HfMBR via the fibers and methane supply was ensured to be sufficient. After a 200-day operation, the membrane module of the HfMBR was dismantled in an anaerobic environmental chamber (BACTRON300 SHEL LAB, Sheldon Manufacturing, USA) to eliminate negative effects of oxygen gas.

The biofilm on the fiber surface was detached via rinsing with anaerobic mineral medium and transferred into an SBR (Fig. S1); subsequently, the operation was continued. The work volume of the SBR was 1200 mL and a gas mixture of $CH_4$ and $CO_2$ (95%:5%, v/v) was provided via the SBR headspace. The composition of the mineral medium used in the reactors was identical to that used in a previous study (Fu et al., 2015). The $NO_3^-$-N concentration was 80 mg/L and the $NH_4^+$-N concentration was 40 mg/L. The ratio of $NO_3^-$-N to $NH_4^+$-N was 2:1, which was consistent with the HfMBR conditions. The pH was 7.3–7.8 and the temperature was maintained at 35 °C. These conditions of the SBR were consistent with those of the HfMBR.

### 2.2. Settleability, reaction rate, morphology, and metabolic products analysis

A settling experiment was conducted to compare the settleability for the flocculating and granular microorganisms. The microorganisms were sampled from the SBR on day 0 and day 130, ensuring that the same amount of biomass was sampled, the detail procedures were described in Supporting information. The samples were transferred into 10-mL colorimetric tubes and the measurement was conducted at the interface between the supernatant and the settlement over time. The settling velocity (SV) and sludge volume index (SVI) were calculated using the Eq. (1) and Eq. (2), in which, $V_{total}$ is the initial sludge volume (mL), $V_{30}$ is the sludge volume after 30-min settling (mL), m is the dry weight of sludge (g).

$$SV(\%) = \frac{V_{30}}{V_{total}} \times 100 \quad (1)$$

$$SVI(mL/g) = \frac{V_{30}}{m} \quad (2)$$

The reaction rate was calculated with the concentration of $NO_3^-$-N, $NO_2^-$-N, and $NH_4^+$-N during the entire period of SBR operation. During the initial and final batch periods, $CH_4$ and $N_2$ were also measured to compare microbial activities. The gas was directly sampled from the headspace with a gas-tight glass syringe (SGE Analytical Science, Australia). $CH_4$ and $N_2$ were measured via a gas chromatography (Fuli 9790, China) equipped with a thermal conductivity detector and a TDX-01 column; the carrier gas was hydrogen (Ding et al., 2014). The analytic conditions consisted of a column oven temperature of 150 °C, an injector temperature of 170 °C, and a thermal conductivity detector temperature of 170 °C. The liquid was sampled via injection syringe, the samples were centrifuged (10,000 ×g, 5 min), and then