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The content of trace element iron is a key factor for competition between anaerobic ammonium oxidation and methane-dependent denitrification processes

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HIGHLIGHTS

- Effect of trace element iron content is first investigated in Anammox-DAMO co-culture systems.
- Proper contents of trace element iron stimulate the activity and growth of Anammox/DAMO microbes.
- Anammox bacteria outcompete DAMO bacteria in the long-term experiments.
- Enhanced enrichment of DAMO archaea by regulating the contents of trace element iron.

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ABSTRACT

Coupling of anaerobic ammonium oxidation (Anammox) with denitrifying anaerobic methane oxidation (DAMO) is a sustainable pathway for nitrogen removal and reducing methane emissions from wastewater treatment processes. However, studies on the competitive relation between Anammox bacteria and DAMO bacteria are limited. Here, we investigated the effects of variations in the contents of trace element iron on Anammox and DAMO microorganisms. The short-term results indicated that optimal concentrations of iron, which obviously stimulated the activity of Amammox bacteria, DAMO bacteria and DAMO archaea, were 80, 20, and 80 μM, respectively. The activity of Amammox bacteria increased more significant than DAMO bacteria with increasing contents of trace element iron. After long-term incubation with high content of trace element iron of 160 μM in the medium, *Candidatus* Brocadia (Amammox bacteria) outcompeted *Candidatus* Methylomirabilis oxyfera (DAMO bacteria), and ANME-2d (DAMO archaea) remarkably increased in number and dominated the co-culture systems (64.5%). Meanwhile, with further addition of iron, the removal rate of ammonium and nitrate increased by 13.6 and 9.2 times, respectively, when compared with that noted in the control. As far as we know, this study is the first to explore the important role of trace element iron contents in the competition between Anammox bacteria and DAMO bacteria and further enrichment of DAMO archaea by regulating the contents of trace element iron.

1. Introduction

Denitrifying anaerobic methane oxidation (DAMO) is a microbial process that methane is oxidized for denitrification under anaerobic conditions (Raghoebarsing et al., 2006). Ettwig et al. (2010) previously confirmed that a nitrite-dependent DAMO

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bacterium, *Candidatus* Methylomirabilis oxyfera, independently mediated anaerobic methane oxidation coupling nitrite reduction (Eq. (1)). Furthermore, nitrate-dependent DAMO archaea, *Candidatus* Methanoperedens nitroreducens that belong to ANME-2d lineage, can reduce nitrate to nitrite with methane (Eq. (2)) (Haroon et al., 2013). Among them, about 10% of nitrite can be further reduced to ammonium by DAMO archaea when nitrate is limited (Ettwig et al., 2016).

Anaerobic ammonium oxidation (Anammox) is an economical bioprocess for reducing nitrogen pollution from wastewater, which has been successfully implemented at large scales (Joss et al., 2009). The anammox bacteria, such as *Candidatus* Brocadia, convert ammonium and nitrite to nitrogen gas and about 15% nitrate (Eq. (3)) (Jetten et al., 1998; Schmid et al., 2005). However, nitrite rarely occurs in municipal and industrial wastewater, therefore a partial nitrification process is always combined with Anammox (van der Star et al., 2007). Ammonium and dissolved methane are always coexisted in anaerobic digester effluents, but the current treatment processes focus on the nitrogen removal rather than methane emission to the atmosphere as greenhouse gas (Abma et al., 2010). Coupling DAMO with Anammox is an ideal solution to limit methane emission while achieving nitrogen removal (Luesken et al., 2011). Nitrate is by-product from Anammox process and leads to incomplete nitrogen removal. DAMO archaea can reduce this part of nitrate to nitrite, and DAMO bacteria and Anammox bacteria can further reduce nitrite to nitrogen gas simultaneously. With DAMO microorganisms coupled with Anammox, the main end products are dinitrogen gas and carbon dioxide, and the theoretical stoichiometry is listed as follows:

$$NO_2^- + 3/8CH_4 + H^+ \rightarrow 1/2N_2 + 3/8CO_2 + 5/4H_2O \quad (1)$$

$$NO_3^- + 1/4CH_4 \rightarrow NO_2^- + 1/4CO_2 + 1/2H_2O \quad (2)$$

$$NO_2^- + 1/1.32NH_4^+ \rightarrow 1.02/1.32N_2 + 0.26/1.32NO_3^- \quad (3)$$

Recently, co-cultures of Anammox, DAMO microorganisms have been enriched experimentally (Luesken et al., 2011). Besides, molecular biology studies have proved that the co-cultures of Anammox, DAMO microorganisms also coexist in paddy fields, lake sediments, forestry soils, wetlands, etc (Zhu et al., 2010; Wang et al., 2012; Yang et al., 2012a; Shen et al., 2014; Meng et al., 2016). Both Anammox bacteria and DAMO bacteria compete for nitrite (Luesken et al., 2011; Hu et al., 2015), but the influence factors of competition have been seldom studied so far.

Iron is an indispensable metal element for the growth of almost all the microorganisms (Martin and Fitzwater, 1988; Glass and Orphan, 2012). It is a cofactor of some important protein classes, such as heme, Fe-S protein, etc. (Ayala-Castro et al., 2008; Hopkinson et al., 2008), and is an essential element for all organisms. Moreover, iron has been demonstrated to apparently affect the metabolism of Anammox bacteria (Zhang et al., 2012; Zhao et al., 2014). In most of the Anammox systems, the content of trace element iron in the medium is set as 30 μM, based on the Anammox medium developed by van de Graaf et al. (1996). Nevertheless, studies on the importance of trace element iron in the DAMO process have been scarce. He et al. (2015) reported that the activity of DAMO bacteria increased with further addition of trace element iron. It must be noted that the contents of trace element iron in the DAMO archaea culture and DAMO bacteria culture were significantly different. The culture medium of DAMO archaea always contains about 40 μM iron (Haroon et al., 2013), while the culture medium of *Candidatus* Methylomirabilis contains about 3.75 μM iron (Ettwig et al., 2009), and the iron-rich environments in the early earth may result in more iron requirements for archaea. To the best of our knowledge, investigations on this difference in the contents of trace element iron utilization by DAMO microorganisms are limited. Besides, the contents of trace element iron used in Anammox, DAMO co-culture systems have also been diverse. For instance, previous studies have used 3.75 and 40 μM iron, respectively (Shi et al., 2013; Ding et al., 2014). This obvious difference of trace element iron contents and its effects cannot be ignored in Anammox, DAMO co-culture systems.

The aims of the present study were to: 1) clarify the effects on the contents of trace element iron on Anammox, DAMO co-culture systems; 2) identify the optimal contents of trace element iron; 3) explore the development of DAMO archaea; and 4) investigate the role of trace element iron on the competitive relation between Anammox bacteria and DAMO bacteria. As far as we know, these purposes are rarely explored by other studies. Short- and long-term experiments were performed with enriched Anammox, DAMO co-culture sludge with different contents of trace element iron. The activities and communities of Anammox bacteria, DAMO bacteria and DAMO archaea were determined.

2. Materials and methods

2.1. Inoculum and medium

A hollow-fiber member biofilm reactor (HfMBR) containing DAMO archaea, DAMO bacteria and Anammox bacteria was used for inoculum collection (Ding et al., 2017). The HfMBR had been in operation for more than 2 years and verified the involvement of anaerobic methane oxidation using $^{13}CH_4$ isotopic experiment (Ding et al., 2017). When the inoculum was collected, 60.5 mg N/L of nitrate and 23.1 mg N/L of ammonium were removed in the HfMBR daily. The inoculum used for follow-up examination was homogeneously mixed and rinsed thrice with mineral salts medium (named routine medium) in an anaerobic chamber. The initial biomass concentration of inoculum was determined using standard methods (Rice et al., 2012) and was found to be about 0.15 g mixed-liquor volatile suspended solids (MLVSS)/L. The routine medium was the same as the one used in the HfMBR (Ding et al., 2017). The contents of trace element iron in the routine medium was adjusted as needed according to the experiments performed by adding extra 80 mM Fe(II)-EDTA stock solution. To avoid precipitation, the Fe(II)-EDTA stock solution was prepared when it was used. The O_2 in the medium was removed by sparging N_2/CO_2 (95:5, v/v), and the initial pH of the medium was controlled in 7.3–7.6. Since iron was used as a trace element, its concentration and state changes were negligible during the tests.

2.2. Short-term experiments

At first, 100-mL serum vials with working volume of 40 mL were sparged with CH_4/CO_2 (95:5, v/v) or N_2/CO_2 (95:5, v/v) for 15 min and sealed with butyl rubber stoppers immediately. Subsequently, 10 mL of the washed inoculum was added into each vial, and the initial biomass concentration of Anammox, DAMO culture was 0.03 g VSS/L. The short-term effects of trace element iron contents on the DAMO bacteria, Anammox bacteria, and DAMO microorganisms with or without Anammox bacteria were respectively assessed (Table 1). The activities of DAMO bacteria, Anammox bacteria and DAMO archaea were reflected by each consumption rate of nitrogen sources. To measure the DAMO bacteria activity, NO_2^--N stock solution was added only to the CH_4-filled vials. To