Is Denitrifying Anaerobic Methane Oxidation-Centered Technologies a Solution for the Sustainable Operation of Wastewater Treatment Plants?

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ABSTRACT: With the world's increasing energy crisis, society is growingly considered that the operation of wastewater treatment plants (WWTPs) should be shifted in sustainable paradigms with low energy input, or energy-neutral, or even energy output. There is a lack of critical thinking on whether and how new paradigms can be implemented in WWTPs based on the conventional process. The denitrifying anaerobic methane oxidation (DAMO) process, which uses methane and nitrate (or nitrite) as electron donor and acceptor, respectively, has recently been discovered. Based on critical analyses of this process, DAMO-centered technologies can be considered as a solution for sustainable operation of WWTPs. In this review, a possible strategy with DAMO-centered technologies was outlined and illustrated how this applies for the existing WWTPs energy-saving and newly designed WWTPs energy-neutral (or even energy-producing) towards sustainable operations.

Keyword: denitrifying anaerobic methane oxidation, wastewater treatment, denitrification, Candidatas *'Methylomirabilis Oxyfera'*, Candidatus *'Methanoperedens nitroreducens'*

1. Introduction

As a response to the massive amounts of discharged wastewater caused by rapid urbanization and industrialization, wastewater treatment plants (WWTPs) play an important role in the protection of environment, especially natural water bodies, via a series of biomass mediated processes such as assimilation, dissimilation, nitrification, and denitrification (Xie et al., 2017; Chen et al., 2016). Treating wastewater to environmentally acceptable level is usually costly, requiring considerable energy input (electricity consumption for domestic wastewater treatment alone accounts for ~3% of global electricity consumption). In China, ￥0.6-0.8 (￥10 [10 yuan] = US$1.40) is generally needed to clean one cubic meter of domestic wastewater, which is much higher for industrial wastewater treatment. Due to the huge quantity of wastewater treated annually (more than 38 billion cubic meters in China alone (NREQ, 2014), any improvement in the operation of WWTPs is likely to significant economic and ecological outcomes.

With the growing global population, WWTPs are now faced with new challenges. The growth in the world's population inevitably produces an increasing amount of wastewater, posing risks in relation to overloading environmental capacity if the existing water-quality standards are maintained. To eliminate this threat, stricter water-quality standards are enforced by many countries, especially those countries, such as China, with vulnerable self-purifying natural water bodies. To meet the stricter quality standards, it is generally thought that treating wastewater will require more energy input. Nevertheless, large amounts of energy and resources in wastewater or municipal sludge including carbon, nitrogen, and phosphorus, are currently being squandered. The recovery of energy and resources from WWTPs could either offset part of the cost or make WWTPs energy-neutral or even energy-producing (Luo et al., 2011). This aspect is becoming even more important as the world's increasing population needs more energy and resources, with WWTPs increasingly considered as facilities for energy recovery rather than merely for waste removal (Wang et al., 2009). The paradox from a quickly increasing human population is pushing forward new improvements for future operation of WWTPs.

Efforts have been dedicated to designing new paradigms for WWTPs to recovery energy and resources as much as possible (Chen et al., 2014; Li et al., 2014), but enabling concurrent maximum energy recovery and desirable pollutant removal remain a huge challenge. Moreover, most of the proposed paradigms present completely new concepts with integrated advanced technologies (Li et al., 2008; Wang et al., 2008; Zhao et al., 2016; Zeng et al., 2011), however, the conventional "activated sludge process" is still (and will be) at the heart of municipal wastewater treatment technology both now and in the next few decades (Hülsen et al., 2016). This makes the proposed paradigms impracticable in the improving the numerous existing WWTPs which use the conventional "activated sludge process", leaving a gap between WWTPs' current and future operations. To date, there is a lack of critical thinking on whether and how new paradigms are to be implemented in WWTPs based on the conventional "activated sludge process".

The denitrifying anaerobic methane oxidation (DAMO) process, which does not require expensive electron donors such as acetate, methanol, and ethanol, has recently been found in both natural habitats and engineered systems (Raghoebarsing et al., 2006; Shi et al., 2013). In 2006 Raghoebarsing et al. (2006) cultivated an enrichment culture from a Dutch canal sediment and detected the concurrent consumption of methane, nitrite and nitrate as well as the emission of dinitrogen gas. Further investigation revealed that the amount of nitrite and nitrate consumed was equal to the dinitrogen gas produced, which, for the first time, demonstrated the existence of anaerobic methane oxidation (AMO) coupled to denitrification. Luesken et al. (2011b) found the DAMO process present in wastewater sludge enrichment cultures from ten selected WWTPs in Netherlands by using specific pmoA primers and fluorescence oligonucleotide probes. The following equations show the reactions of the DAMO process with nitrate (or nitrite) as the oxidant:

$$CH_4 + 4\ NO_3^- \longrightarrow CO_2 + 4\ NO_2^- + 2\ H_2O \qquad (\Delta G^o = -503\ kJ\ mol/CH_4) \qquad (1)$$

$$3\ CH_4 + 8\ NO_2^- + 8\ H^+ \longrightarrow 3CO_2 + 4\ N_2 + 10\ H_2O \qquad (\Delta G^o = -928\ kJ\ mol/CH_4) \qquad (2)$$

The findings on the DAMO process expand our understanding in terms of methane oxidation and may make an important contribution to the WWTPs' operation. It is generally considered that the two main issues faced by WWTPs are: 1) shortage of carbon sources in influent wastewater and 2) emission of greenhouse gas. Thus, if part of the methane produced from the anaerobic digestion of sludge is used to reduce nitrate or nitrite oxidized by denitrifiers, both the nitrogen level in effluent of WWTPs and the greenhouse gas emission from WWTPs will decrease. It is known that WWTPs are highly engineered systems, giving engineers opportunities to develop DAMO based strategies.

In this review, we summarize the critical outcomes arising from the research on the DAMO process. Based on critical analysis, we outline one possible strategy for applying the emerging DAMO-centered technologies to WWTPs, illustrate how this strategy would make the existing WWTPs energy-saving and newly designed WWTPs energy-neutral or even energy-producing, and discuss future efforts to be made for realizing such sustainable operations.

2. DAMO process: the emergence technology

2.1 Discovery of anaerobic methane oxidation process

Methane oxidization generally occurs in oxic environments as the break of methane's carbon-hydrogen (C-H) bonds of methane requires high-activation energy and aerobic methanotrophs can use oxygen as a highly reactive co-substrate to provide such high activation energy via a reaction catalyzed methane mono-oxygenase, however, several ions or ionic compounds could be used as electron acceptors for AMO (Figure1) (Bussmann 2005; Lopes et al., 2011).

Anaerobic methane oxidation (AMO) was discovered for the first time in 1974 (Martens and Berner,

1974) in sediment geochemical profiles using sulfate as the electron acceptors with the controversy of responsible microbiologists. This discovery was only confirmed in 1994 by Hoehler et al. (1994) through a sediment incubation experiment with methanogens and sulfate reduced in laboratory condition. This process generally takes place in oxygen-free marine sediments, sulphate-methane transition zone, and the anaerobic methane oxidation zone (Chevalier et al., 2014; Hong et al., 2013; Liu and Wu, 2014; Sivan et al., 2014). The primary microorganisms responsible for methane oxidation are archaea while sulfate reducers are thought to be the contributors of sulfate reduction via a 'reverse methanogenesis' pathway (Niemann et al., 2006; Zhang et al., 2010). However, it has recently been found that the ANME-2 archaea can oxidize methane and reduce sulfate simultaneously without bacterial partners (Milucka et al., 2012). The process of AMO coupled to sulfate reduction is supposed to consume more than 90% of the produced methane in cold seep sediments and most of the methane in marine sediments (Hinrichs and Boetius, 2003; Orphan et al., 2001; Smemo and Yavitt, 2011). In addition, it was reported that AMO was also coupled with other electron acceptors, such as iron (Ⅲ), manganese (Ⅳ), hypochlorite (ClO_2^-) and perchlorate (ClO_4^-) (Beal et al., 2009; Miller et al., 2014; Zehnder and Brock, 1980). Many investigations have demonstrated that these processes were present in multitudinous environment (e.g. sea sediment, freshwater sediment, coastal sediment, and valley vent field and lake sediments) (Beal et al., 2009; Riedinger et al., 2014; Segarra et al., 2013; Sivan et al., 2014; Wankel et al., 2012), and their responsible microorganisms and mechanisms were also studied (Coates and Achenbach, 2004; Holmes et al., 2004; Luo et al., 2015; Miller et al., 2014; Rikken et al., 1996).

2.2 Discovery of DAMO process

A significant recent discovery is AMO is anaerobic oxidation of methane coupled to denitrification (i.e., DAMO). This phenomenon is first indicated in 1991by Smith et al. (1991) in a nitrite/nitrate polluted groundwater. Methane could be used as hydrogen donors for the in situ denitrification of groundwater, indicating the occurrence of DAMO. Modin et al. (2007) revealed the relevance of methanotrophs and denitrifies in aerobic methane oxidation denitrification. However, it has been a lack of direct evidence supporting the existence of DAMO for a long period, because this bio-reaction is thought to occur closely to the oxic/anoxic interface in sediments. Islas-Lima et al. (2004) presented experimental evidence that methane and nitrate were consumed concurrently in a batch test using methane as the sole carbon source under anoxic denitrifying conditions, but the responsible microorganisms were not illuminated. Subsequently, Raghoebarsing et al. (2006) successfully enriched and identified the microorganisms responsible for the DAMO process from a canal named Twentekanal in the Netherlands, which provided insights into this biological process. Although the DAMO process is a recently discovered phenomenon, it has attracted much attention in the past few years, because the DAMO process simultaneously consume redundant methane and nitrate/nitrite present in the environments which plays a significant role in the global C (carbon) and N (Nitrogen) cycling.

2.3 Microorganisms in DAMO process

The two types of microorganisms mainly responsible for the DAMO process that have been reported to date are Candidatus *'Methylomirabilis oxyfera'* bacterium (*M. oxyfera*) and Candidatus *'Methanoperedens nitroreducens'* (*M. nitroreducens*) (Ettwig et al., 2008; Haroon et al., 2013). The characteristics of the two different microbes are summarized in Table 1. Generally, the AMO process is considered to be implemented by the methanotrophic archaea while reducing the electron acceptors is postulated to be executed by other types of microorganisms. For example, the methanotrophic archaea and sulfate-reducing bacteria were demonstrated to be syntrophic partners responsible for the AMO coupled to sulfate reduction process in marine sediments (Boetius et al., 2000; Hinrichs et al., 1999; Knittel et al., 2005). Raghoebarsing et al. (2006) enriched a microbial consortium containing one unique bacterium (80%) and a methanotrophic archaea

(10%) in the presence of methane, nitrite and nitrate using seed sludge taken from a canal sediment in the Netherlands, but the ratio of archaea to bacteria (approximately 1:8) in this consortium was different to that in the sulfate-dependent AMO culture (approximately 1:2). Thus, the bacterium and the methanotrophic archaea are assumed to be jointly responsible for the DAMO process.

It is demonstrated that one type of bacteria, i.e., *M. oxyfera*, could independently fulfill the DAMO process using nitrite as the electron acceptor via a series of experiments with powerful tools applied, including sequencing the key gene of methanotrophic and methanogenic archaea, *mcrA* and monitoring the subgroup by quantitative PCR (Ettwig et al., 2008; Ettwig et al., 2009; Ettwig et al., 2010). Candidatus '*Methylomirabilis oxyfera'*, which belongs to the uncultured NC10 phylum, is a gram-negative atypical polygon-shaped bacterium. This bacterium contains a diameter of 0.25-0.5 Lm and a length of 0.8-1.1 Lm (Ettwig et al., 2010; Ettwig et al., 2008) due to the special exoskeleton-like protein surface layer ultrastructure (He et al., 2014; Shen et al., 2014; Wu et al., 2012). Moreover, it is repeated that mreB and ftsZ genes of the *M. oxyfera* genome have important impact on the shape of cell (Margolin, 2009; Young, 2003). The *M. oxyfera*-like bacteria are assigned to two main groups, namely group A and group B, though the enriched experiments are tested with different sources of inoculum and temperature condition (Deutzmann et al., 2011). To date, most of the *M. oxyfera*-like bacteria have been verified to belong to the group A (Luesken et al., 2011a). *M. oxyfera* contain a unique fatty acid, the monounsaturated 10-methylhexadecenoic acid with a double bond at the △7 position ($10MeC_{16:1\triangle 7}$), which comprises up to 10% of the total fatty acid. This characteristic provides useful information to detect their existences in the culture (Kool et al., 2012). Metagenomic sequencing analysis has shown that the complete genome of *M. oxyfera* was assembled into a 2.7-Mb circular single chromosome. The cells of *M. oxyfera* lacked intra cytoplasmic membrane which is a common property for other bacteria (Wu et al., 2012). In addition, it has been confirmed that the enzyme of particulate methane monooxygenase, which is responsible for methane oxidation process, located on the surface of the cytoplasmic membrane of *M. oxyfera* bacteria (Wu et al., 2012). Further investigations with ^{13}C-labelling experiments and gene analysis showed that the primary C1-assimilatory pathway of the *'M. oxyfera'* was the Calvin-Benson-Bassham cycle (Rasigraf et al., 2014). The incomplete serine cycle was also found to be occurred in the *'M. oxyfera'*. Periplasmic PQQ-dependent methanol dehydrogenase, methylene-H4F dehydrogenase, and methylene-H4MPT dehydrogenase were determined. However, enzyme 6-hexulosephosphate synthase, malyl-CoAlyase, and phosphoserine aminotransferase were non-detectable, although they are also the key enzymes for the formaldehyde detoxification and the synthesis of intermediates (Wu et al., 2011b). The comprehensive information on *'M. oxyfera'* was reviewed elsewhere (Shen et al., 2015).

It was reported that the DAMO process used nitrite prior to nitrate as the electron acceptor in a short-term investigation, however, the methanogenic archaea disappeared in the absence of nitrate after long-term operation, indicating that the methanogenic archaea is not capable of reducing nitrite (Ettwig et al., 2008; Ettwig et al., 2009). By investigating the genome sequence the methanogenic archaea was also hypothesized to play an important role in denitrification (Haroon et al., 2013; Hatamoto et al., 2014). It is thought that the methanotrophic archaea executes the bio-reaction of nitrate to nitrite in the DAMO process while *M. oxyfera* accomplishes the remaining step of nitrite to nitrogen gas. Haroon et al. found a novel archaeal lineage related to ANME-2d that could independently complete AMO using nitrate as the terminal electron acceptor in a bioreactor by ^{13}C- and ^{15}N- labelling experiments, during which reducing nitrate to nitrite was implemented by the methanogenic archaea while reducing nitrite to nitrogen gas was executed by an anaerobic ammonium-oxidizing bacterium. On the basis of this finding, they defined this new microorganism as Candidatus *'Methanoperedens nitroreducens'* (Haroon et al., 2013). By combining metagenomics, single-cell genomic, transcriptomic analyses, and ^{13}C- and ^{15}C-lableing experiments, it was found that *M. nitroreducens* oxidized methane via a reverse methanogenesis pathway (Hatamoto et al., 2014). As yet, little information is obtained with regard to the properties and characteristics of *M. nitroreducens* and further studies are required in future.

2.4 Potential mechanisms of DAMO process

To date, the potential mechanisms of DAMO process have been proposed via two different pathways, namely the new pathway of 'inter-aerobic denitrification' carried out by *M. oxyfera* and the general pathway of 'reverse methanogenesis' executed by *M. nitroreducens* with one syntrophic relationship (Figure 2) (Ettwig et al., 2010; Haroon et al., 2013).

Raghoebarsing et al. (2006) enriched a microbial consortium capable of fulfilling the DAMO process that mainly consisted of *M. oxyfera* and *M. nitroreducens*. They hypothesized that the enriched consortium carried out the DAMO process via 'reverse mehtanogenesis'. It was thought that *M. nitroreducens* finished the DAMO process via reverse methanogenesis in association with the denitrifying bacterial partner, *M. oxyfera*, which is analogous to the process of AMO-sulfate reduction. However, it was subsequently found that the DAMO process could be accomplished by the bacterial *M. oxyfera* without archaea (Ettwig et al., 2008; Ettwig et al., 2009). Thauer and Shima (2008) demonstrated that methyl-coenzyme M reductase did not involve in *M. oxyfera* and the pathway of reverse methanogenesis was also suppressed in *M. oxyfera* through the bromoethane sulfonate inhibition experiment. It was assumed that *M. oxyfera* might implement a new way for the DAMO process, as the activation of methane must occur either in the presence of oxygen or via reversed methanogens anaerobically (Hanson and Hanson, 1996; Modin et al., 2007; Shima and Thauer, 2005). Etting et al. further studied the complete genomes of the *M. oxyfera* and supposed this way as the 'inter-aerobic denitrification'. In this pathway proposed, *M. oxyfera* first reduce nitrite to nitric oxide and then bio-convert nitric oxide to nitrogen and oxygen. The oxygen produced is further utilized for methane oxidation via the canonical aerobic pathway by the same microorganism, *M. oxyfera*. Although some key enzymes of anaerobic alkane activation including methyl-coenzyme M. reductase and alkane-activating glycol radical enzyme are absent in *M. oxyfera*, the particulate methane monooxygenase which catalyzes the first step of the methane oxidation in aerobic condition is detected, suggesting that *M. oxyfera* contains the complete pathway to oxidize methane aerobically (Luesken et al., 2011c). Deutzmann and Schink (2011) demonstrated that *M. oxyfera* contained two sequence clusters which are closely affiliated to the pmoA genes, the key gene of the particulate methane monooxygenase, supporting this assumption. Recently researchers have successfully developed a new pmoA gene-based PCR primer set which shows a high specificity in identifying *M. oxyfera*. This newly designed PCR primer is capable of not only amplifying the currently known *M.oxyfera* but also contributing to the diversity and distribution of DAMO microorganisms in ecosystems (Han and Gu, 2013).

In the 'inter-aerobic denitrification' pathway, methane is first oxidized to methanol by the particulate methane monooxygenase (Luesken et al., 2011c), which is represented by a single and phylogenetically divergent membrane-bound form in *M. oxyfera* (Ettwig et al., 2010; Wu et al., 2011b). The oxygen required in this step is provided by *M. oxyfera*-based nitrite reduction, as shown in Figure 2A. Methanol is then bio-converted to formaldehyde by the periplasmic PQQ-dependent MDH (methanol dehydrogenase). Although three sets of genes encoding MDH paralogues and all the proteins necessary for the catalytic function are found in *M. oxyfera*, the most genes required for PQQ biosynthesis (*pqq*ABCDEF) are absent (expect for *pqq*E and *pqq*F) (Wu et al., 2011b). To date, the biosynthesis pathway and the role of PQQ in *M. oxyfera* are unclear and need to be further investigated. The formaldehyde produced is further oxidized to formate by the methylene-H_4F dehydrogenase or methylene-H_4MPT dehydrogenase. This step is postulated to serve as an incomplete serine cycle for formaldehyde detoxification and the synthesis of intermediates for complete methane oxidation (Shen et al., 2015). Finally, carbon dioxide is generated by formate dehydrogenase and enters the Calvin-Benson-Bassham cycle to be fixed and provided as carbon source for the growth of *M. oxyfera* (Rasigraf et al., 2014).

For the nitrite reduction pathway, it was reported that most genes of the typical denitrification pathway were present in *M. oxyfera*, including the genes for the reduction of nitrate to nitrite (*nar*GHJI, *nap*AB), nitrite to NO (*nir*SJFD/DH/L), and NO to N_2O (*nor*Z), but nitrous oxide reductase, which performs the last step in the denitrification pathway (i.e., N_2O to N_2), was absent (Ettwig et al., 2010; Luesken et al., 2011c). However, previous studies showed that the predominant intermediate of *M. oxyfera*-based denitrification was NO rather than N_2O (Ettwig et al., 2008; Ettwig et al., 2009). The produced NO is thought to be converted into O_2 and N_2 by one unknown NO dismutase, which is similar to the process of chlorite dismutation producing Cl^- and O_2 (Miller et al., 2014). This hypothesis was further verified by Etting et al. (2010) via an isotopic labeling experiment. They found that the intracellularly produced O_2 was partly (75%) used for methane activation, and the remainder was used for normal respiration. *M. oxyfera* contains the membrane-bound *bo*-type terminal oxidase (Wu et al., 2011a). Most of the terminal oxidases act as proton pumps. A proton-pumping NADH dehydrogenase, which can derive from the methylene-H_4MP, methylene-H_4P dehydrogenase, and format dehydrogenase reactions, is present in the genome of *M. oxyfera*. This discovery indirectly proved that the *M. oxyfera* could couple the oxidation of NADH to the reduction of quinone with the concomitant export of protons. The re-oxidation of the quinol and proton translocation was realized by the *bc*$_1$ complex. The reduced cytochrome *c* may serve as the electron donor in the nitrite reduction process by *cd*$_1$ Nir. ATP synthesis utilizes the proton motive force with F1Fo ATP synthase (Wu et al., 2011a).

Apart from bacterial *M. oxyfera*, a novel archaeon, *M. nitroreducens*, was discovered to be able to reduce nitrate to nitrite using electrons derived from methane oxidation (Cui et al., 2014). All *mcr* subunit genes (*mcr*ABCDG) and F420-dependent 5,10-methenylte-trahydromethanopterin reductase were detected in *M. nitroreducens* (Cui et al., 2014) suggesting that the derived *M. nitroreducens* catalyzes methane oxidation via reverse methanogenesis (Figure 2B). The electrons produced in the reverse methanogenesis process are provided for both the *M. nitroreducens*-based and other denitrifying partners-based denitrification, similar to the processes involved in the consortia of sulphate-reducing bacteria and methanogenic archaea (Deusner et al., 2014). In addition, it is supposed that *M. nitroreducens* might be able to produce acetate due to a full reductive acetyl-CoA (carbon fixation) pathway and acetyl-CoA synthetase discovered in *M. nitroreducens* (Cui et al., 2014). However, *M. nitroreducens* was found to contain genes for nitrate reduction (*nar*G and *nar*H), and the genes for subsequent steps in denitrification were not determined, which may be the primary reason for *M. nitroreducens* being disable to further reduce nitrite. Thus, the reduction of nitrite is performed by other syntrophic relationships in anaerobic conditions.

2.5 Application of DAMO process

Previous studies demonstrated that DAMO microbes could be successfully enriched in reactors fed with effluent discharged from WWTPs (Kampman et al., 2012; Shi et al., 2013), indicating that DAMO could be applied in wastewater treatment. As DAMO microorganisms grow slowly (He et al., 2013), the proper reactor configuration is an effective way to retain sufficient biomass. Although the sequencing batch reactor/sequencing fed-batch reactor and the continuously stirred tank reactor were the earliest used to cultivate the DAMO microbes (Ettwig et al., 2009; He et al., 2013; Kampman et al., 2012; Strous et al., 1998), it was found that a magnetically stirred gas lift reactor (MSGLR) was the most useful tool among these three type reactors, because MSGLR enhances the mass transfer of gas-liquid phases and the mixing of liquid-solid phases (Hu et al., 2014). However, compared to suspended cultures, the membrane biofilm reactor (MBfR) could retain microorganisms with very slow growth kinetics, and biomass could be naturally accumulated in the biofilm at different depths (LaRowe et al., 2014; Modin et al., 2008). The biofilm is accumulated on an active surface as biofilm grows on the outside of a gas-transfer membrane and the substrate, which is consumed by the bacteria in the biofilm, diffuses through the wall of the membrane (Rittmann, 2007). Based on this principle, Shi et al. (2013) demonstrated, for the first time, that nitrogen removal could be achieved by co-culturing anammox bacteria and DAMO microorganisms in a MBfR. Subsequently, it was developed a mathematical model to describe this process and identified the role of DAMO microbes and anammox bacteria in the co-culture in a MBfR (Kampman et al., 2012). DAMO archaea plays a role in converting nitrate to nitrite with methane as the electron donor while DAMO and anammox bacteria conjointly completed the nitrite reduction with methane and ammonium as the electron donors, respectively. In addition, Hu et al. (2015) also investigated the interactions between DAMO microbes and anammox bacteria in two MBfRs and obtained the same conclusion.

Recently, a new concept, which incorporates DAMO process into a UASB-digester system and a nitritation reactor to treat sewage under low-temperature (10-20℃), has been designed and tested (Kampman et al., 2012). Moreover, ammonium and methane are major end-products of anaerobic digestion, and anammox bacteria and DAMO bacteria are autotrophic which both convert ammonium and nitrite directly into N_2 in the absence of oxygen (Hu et al., 2013). Luesken et al. (2011a) successfully co-enriched and tested the DAMO and anammox bacteria in a SBR. In the test, ammonium supply was an important element, and equal amounts of DAMO and anammox bacteria was established via the analysis of FISH and 16S rRNA and pmoA gene clone libraries after a 161-day operation. Zhu et al. (2011) also enriched anammox and DAMO bacteria using medium only containing methane, ammonium, and nitrite in a laboratory-scale SBR with the seed sludge taken from a full-scale anammox bioreactor. Moreover, Winkler et al. (2015) identified the distribution of co-cultured anammox bacteria and DAMO bacteria in a single granule using a mathematical model. They found that the anammox bacteria grew on the outside of the granule whereas DAMO bacteria were located inside the granule. The feasibility of co-cultivation of anammox and DAMO bacteria fed with nitrite and ammonium in different reactors has recently become a research hotspot. This combination process is being considered as an environmentally friendly and cost-efficient nitrogen-removal process.

3. DAMO-centered technologies: a promising paradigm for sustainable WWTP's operation

3.1 The paradigm of DAMO-centered technologies

Based on the analysis above, we think that the existing WWTPs could save a large amount of energy and that new WWTPs could even produce an excess of energy if the emerging DAMO technology could be scaled up. DAMO process may also help engineers to address some of the key issues faced by WWTPs, e.g., 1) shortage of carbon sources in influent wastewater and 2) emission of greenhouse gas. We assume that combining DAMO technology with other available technologies including high-rate activated sludge, deammonification, anaerobic digestion, and struvite precipitation partially or completely, can be a solution for sustainable operation of both the currently existing and new designed WWTPs. The combined strategy proposed is shown in Figure 3.

3.2 The way to apply DAMO-centered technologies for the existing WWTPs

In existing WWTPs, wastewater would first be treated by the current “activated sludge” process, in which most of organic matter, nitrogen, and phosphorus in wastewater are removed. The treated effluent contains relatively high levels of nitrate (usually > 10 mg/L, depending on the available organic carbon present in wastewater) bio-converted from ammonium. The remaining nitrate would be further reduced to nitrogen gas in the DAMO reactor with methane as the electron donor before the effluent would be finally discharged or reused. Methane would be generated from the anaerobic digestion of sewage sludge. It has been shown that the DAMO organisms would enriched in a reactor fed with the effluent from the “activated sludge” process (Kampman et al., 2012).

The sewage sludge produced could be used as both a source of energy and a resource to not only generate methane for DAMO reactor but also recoup a part of cost. Firstly, the sewage sludge is digested in an anaerobic digester, where the volume of sludge is reduced, pathogens are killed, and methane is produced (Wang et al., 2015). A small fraction of the methane produced is supplied to the DAMO reactor while the remainder is utilized to generate electricity. According to the estimation, the methane supplied to the DAMO reactor accounted for only ~10% of methane produced from sludge digestion. The recirculated digestion liquid generally contains 1000-1500 mg/L of ammonium and 200-400 mg/L of phosphorus that are released from cell disintegration, which contribute to 10-20% of total nitrogen and 20-40% of total phosphorus in WWTPs, respectively. By addition of magnesium and simultaneous pH adjustment or an MgO slurry, both the ammonium and phosphorus present in the digestion liquid could be effectively recovered by a valuable fertilizer, struvite. This is very important as the main source of phosphorus, phosphate rock, is a limited and non-renewable resource and is threatened by its exhaustion in next 100-250 years (Association, 2000). The recovery of phosphorus not only offsets a portion of WWTP costs but also satisfies world demand for phosphate rock (estimated to be 15-20%). Moreover, the recovery of struvite further reduces the nitrogen and phosphorus loading, benefitting to obtain a cleaner effluent.

3.3 The way to apply DAMO-centered technologies for the newly designed WWTPs

In the future new WWTP concept, the recovery of energy and resource should be maximized, while the effluent pollutant concentrations should be minimized. Based on these principles, the mainstream deammonification based process is considered to be the most promising technology by highly-respected leaders in the field (Kartal et al., 2010; McCarty et al., 2011;Wang et al., 2012; Luo et al., 2014), though some other encouraging technologies such as the anaerobic membrane bioreactor, microbial fuel cells, and microbial electrochemical cells are also proposed (Li et al., 2015). Mainstream deammonification includes upfront separation of organic carbon, partial nitritation, and anaerobic ammonium oxidation (anammox) (Cho et al., 2011; Liu et al., 2016;Wang et al., 2016b; Zhang et al., 2014). Despite deammonification from anaerobic sludge digestion liquor (i.e., side stream) containing high-levels of ammonium (1.0-1.5 g/L) having been implemented on a full-scale (> 100 installations worldwide) (Lackner et al., 2014), the application of stable autotrophic nitrogen removal from domestic wastewater (i.e., mainstream deammonification) is currently in its infancy (Xu et al., 2015).

The three key barriers hindering the application of mainstream deammonification are: i) phosphorus removal is not considered, posing the threat of phosphorus pollution of the environment; ii) stable partial nitritation is difficult to be established in the mainstream bioreactor, resulting in nitrite-limiting conditions for the anammox conversion; and iii) it is difficult to achieve high-levels of nitrogen removal, as nitrate (11% of total nitrogen) is an end product of the anammox reaction, and in addition, residual ammonium and/or nitrite may remain in effluent when the ideal molar ratio of 1.32 to 1 between nitrite to ammonium is not produced by the partial nitritation process (equations 3 and 4). To date, the maximal nitrogen removal efficiency reported is only ~70%, which does not meet the wastewater discharge standard in most areas (for example, the Queensland Environmental Protection Agency in Australia now specifies a total nitrogen concentration in effluent of 3 mg/L). All these barriers could be removed with the new emerging technologies being tested (or the recent discoveries) if they could be scaled up.

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O \qquad \text{(Nitritation)} \qquad (3)$$

$$NH_4^+ + 1.32NO_2^- \rightarrow 1.02N_2 + 0.26NO_3^- + 2H_2O \qquad \text{(Anammox)} \qquad (4)$$

The first technology in relation to new discoveries is the high-rate activated sludge process. In fact, this technology was exploited for organic carbon recovery (removing > 80% organic carbon) by biomass assimilation and/or accumulation in the 1970s. Nevertheless, recent investigations showed that controlling solids retention times (SRT) at 2.0-2.5 days, > 90% phosphorus could be removed in this process by novel polyphosphate accumulating organisms, clade *Comamonadaceae* (Chen et al., 2013; Ge et al., 2015; Wang et al., 2013). The second technology is the strategy that effectively suppresses the growth of nitrite-oxidizing bacteria treating domestic wastewater by controlling either SRT, or dissolved oxygen, or several combined parameters. By combining free nitrous acid-based sludge treatment and oxygen limitation (0.3-0.8 mg/L), stable nitritation (~80%) could be achieved in a mainstream reactor (Wang et al., 2016a). The third technology is the anammox-DAMO co-cultured membrane biofilm reactor. This membrane biofilm reactor enables the enrichment of both anammox and DAMO microorganisms, thereby enhancing the total nitrogen removal. Methane is delivered from the interior of hollow fibers and the anammox -DAMO biofilm grows on the outer walls of the fiber. Shi et al (2013) enriched 20-30% of the DAMO bacteria, 20-30% of the DAMO archaea, and 20-30% of the anammox bacteria and achieved a nitrate and an ammonium removal of ~190 mg N/(L·d) and ~60 mg N/(L·d), respectively, after 24 months of operation.

In new WWTPs with the above technologies integrated, the organic carbon and phosphorus in wastewater are first absorbed and stored, physically or biologically, by microorganisms in the high-rate activated sludge stage. The ammonium-rich supernatant is then subjected to the nitritation stage to partially convert ammonium to nitrite(Zhao et al., 2013). The nitritation effluent with appropriate ratio of ammonium and nitrite (also containing a small portion of nitrate), is further treated in the anammox-DAMO co-cultured membrane biofilm reactor to remove nitrogen. In this biofilm reactor, DAMO archaea reduces nitrate, both produced in the nitritation stage and Anammox stage, to nitrite, with methane as the electron donor. The nitrite produced is reduced to nitrogen gas by anammox and DAMO bacteria jointly using ammonium and methane as the electron donor, respectively. Lastly, depending on the purpose, the effluent of anammox-DAMO reactor is either discharged directly or further polished by membrane filtration processes to achieve high-quality water for reuse.

The organic carbon-rich activated sludge (accounting for >70% of total organic carbon) produced in the high-rate activated sludge reactor is passed to the anaerobic digester for the production of methane as either a renewable energy source (~50% of methane generated) or the electron donor (~50% of methane generated). The ammonium and phosphorus in the digestion liquid are recovered as struvite, as outlined above.

4. Benefits

With a rational integration of multiple technologies either recently developed or previously existing, both technical and economic benefits could be achieved. The combined technologies, and especially DAMO could address the main drawbacks that occur in current WWTPs and are likely to occur in future WWTPs with a minimal energy input. The conventional method applied in current WWTPs for the enhancement of nutrient removal is to add extra organic carbon that has been chemically synthesized (e.g., acetate and methanol). Due to influent variations in carbon, nitrogen, and phosphorus levels, it is very hard to control the dosage of additional carbon, resulting in either unacceptable nitrogen and phosphorus levels (shortage of additional carbon) or organic carbon pollution (excess of additional carbon). The excess of extra carbon addition also increases the operational cost. Although using fermentation liquid from sludge anaerobic fermentation as additional carbon does not require external energy input, shortage control and hazardous matters accumulation as well as fermentation liquid separation are also problems either un-resolved or untested. Applying DAMO-centered processes, as proposed in Figure 3, as a post-unit could overcome all these drawbacks. Methane, which could be produced in situ through anaerobic digestion of sludge, is the sole electron donor for DAMO microorganisms. The excess supply of methane do not cause risk in terms of effluent contamination.

In addition, the stuvite recovery significantly reduces the loading rate of total nitrogen (10-20%) and total phosphorus (20-40%), providing the benefit to obtain a cleaner effluent.

With an optimal ammonium to nitrite molar ratio (1:1.32) in effluent from the nitritation stage, the anammox process potentially removes 89% of the total nitrogen, with the residual 11% being nitrate produced by the anammox reaction. Due to the lack of strategies for effective suppression of nitrite oxidizing bacteria, the available nitrite is always a limiting factor for mainstream deammonification, decreasing the nitrogen removal efficiency. The proposed combined free nitrous acid-oxygen limitation strategy could achieve stable nitritation, providing a sufficient level of nitrite as required for anammox while DAMO would reduce nitrate, produced in both the nitritation stage and Anammox stage. In addition, co-cultured anammox-DAMO microorganisms could enhance nitrogen removal rate, as compared with the sole anammox bacteria. A 15-fold increase in the nitrogen removal rate was observed in an anammox-DAMO co-cultured membrane biofilm reactor.

It is estimated that the economic benefit of the operational concept with DAMO-centered technologies implemented, taking a WWTP with a 500 000 population equivalent (~100 000 cubic meters per day) as an example. In China, such a conventional “activated sludge” WWTP without sludge anaerobic digestion (these WWTPs account for >80% of total WWTPs in China) would consume ￥60 000 ~ ￥80 000 (depending on the available organic carbon, nitrogen, and phosphorus in the wastewater) per day for wastewater treatment and about ￥40 000 for the disposal of excess sludge. By incorporating DAMO-centered technologies into the activated sludge process, a WWTP of the same size would save about ￥20 000 in the cost of adding extra organic carbon, produce around 5 000 kWh of electrical energy, and also recover about three tons of struvite per day. Therefore, in a new WWTP paradigm, we estimate that each day it could save at least ￥50 000 for wastewater treatment, produce around 18 000 kWh of electrical energy, and recover about 3 tons of struvite as well. As thus, a WWTP operated with such new concept would save 68 000 kWh of electrical energy daily. It should be noted that the benefits presented here are indicative only, and in future work, the technical and economic merits require to be further refined at real-world situations in future.

5. Further perspective

Although many efforts have already been made, DAMO technology is still in its infancy, having been only tested on a bench-scale. In addition, both the biological phosphorus removal-based high-rate activated sludge process and mainstream deammonification have not been implemented in field situations. To make DAMO-centered technologies suitable for full-scale applications, scaling up these systems would be therefore inevitable. The biggest challenge for a real-world situation is how to concurrently scale up the system size of WWTPs while guaranteeing WWTPs' performance. All the DAMO reactors conducted before employed pure methane as the electron donor. However, besides the main composition, methane, the gas produced from the sludge digestion also contains some CO_2, hydrogen, and H_2S, and these compositions might affect the performances of DAMO. Further studies are required to be performed to examine their impacts.

The microorganisms in both DAMO and anammox are slow growing, thus the start-up period required in DAMO (DAMO-anammox) biofilm formation is long whereas the nitrogen removal rate is low, hindering the real-word application as well. Therefore, the second challenge is how to accelerate the biofilm formation process and enhance the reaction rates. By gaining a better understanding of the physiology and kinetics of DAMO and anammox organisms, optimal strategy (e.g., mathematical model-based strategy) will be obtained to address this challenge. In addition, efficient separation systems (e.g., membrane bioreactors) could be used for biomass retention, which also enhancing nitrogen removal rates. To date, DAMO microorganisms have not well understood, thus deeply understanding the microbial behaviors of DAMO microorganisms and developing strategies to apply DAMO in engineering are also needed.

To provide suitable ratios for DAMO-anammox and scientific basis for methane supply, on-line monitoring and real-time process control must be executed. On-line measurements of ammonium, nitrite, and nitrate would not only control the nitrite to ammonium molar ratio in the nitritation reactor but would also avoid the shortage or excess supply of methane for the DAMO process, which is very important in practice. The shortage of methane supply would decrease nitrogen removal while excessive methane supply would waste energy, bring safety risks, and increase greenhouse gas emission. However, the construction investment might increase in such a system due to the fact that more complicate process, on-line monitoring and real-time process control are employed. This aspect requires to be assessed in future.

In addition, support from government, especially in the developing world, is necessary for the development of emerging technologies. Governments must issue regulatory frameworks that contains or enhances the costs of waste disposal, the carbon footprint, and greenhouse gas emission, thereby putting energy saving, energy-neutral or even energy-producing technologies into a priority position. Governments and wastewater facilities should also provide specific funds and appropriate infrastructure to promote the development of these technologies. As thus, endeavors from governors, scientists, and engineers make the DAMO-centered technologies high potential in practice, offering an enormous opportunity for the operations of our WWTPs to become sustainable.

6. Conclusion

This paper systematically summarized the advances of DAMO process, especially the recent outcomes regarding microorganisms, mechanisms and application potential of this process. These advances give us opportunity to both deeply understand and potentially apply this significant but recently focused phenomenon. In addition, the perspective of DAMO applying in an engineering way and future efforts to be made in future are also discussed. Based on the critical analysis, we deduce the DAMO process play important roles in future sustainable operation of WWTPS and calculate the benefits brought by the DAMO process application.

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Figure Captions

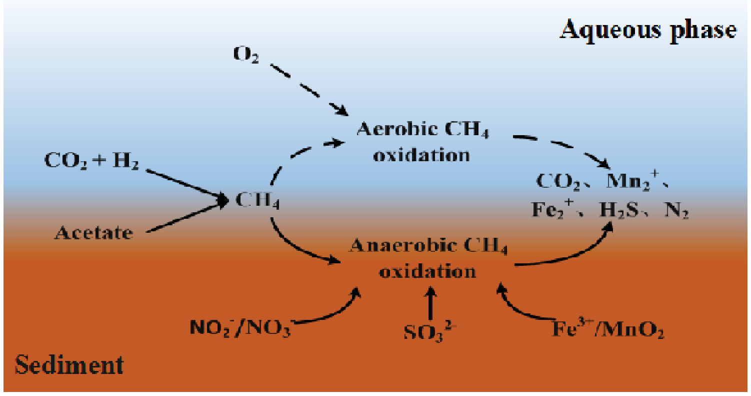
Figure 1. The main processes of methane oxidation with different elector acceptors in natural water sediments.

Figure 2. It was shown that the potential mechanisms of DAMO process via two different pathways, the pathway of 'inter-aerobic denitrification' dominated by M. oxyfera (A), adapted from Ettwig et al., 2010; Wu et al., 2011b; Rasigraf et al., 2014 and the general pathway of 'reverse methanogenesis' dominated by M. nitroreducens with one syntrophic relationship (B), adapted from Haroon et al., 2013; Cui et al., 2014.

Figure 3. The conceptual operation of WWTPs with DAMO-centered hybrid technologies for either saving energy or even producing energy. A: for the currently existing WWTPs; B: for the new designed WWTPs.

Table Caption

Table 1. The characteristics of two different microbes in the ANME-D process



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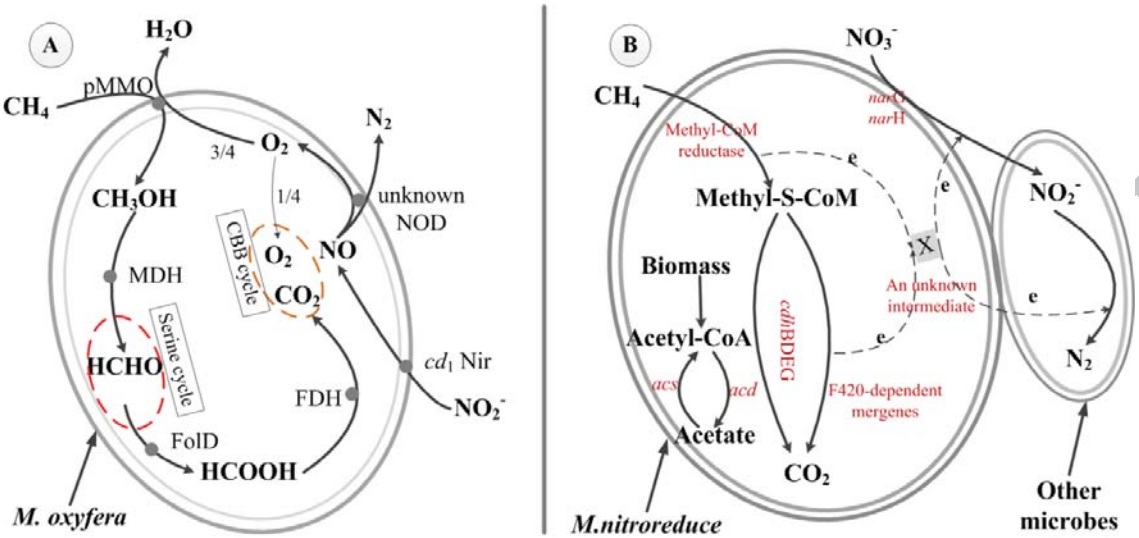

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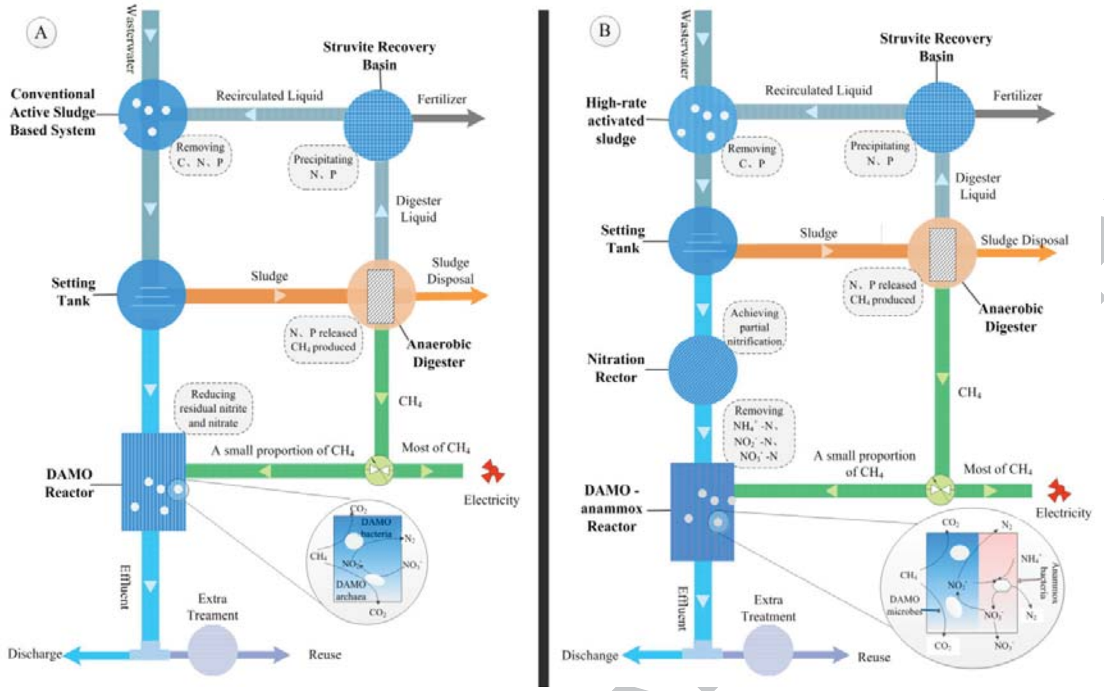

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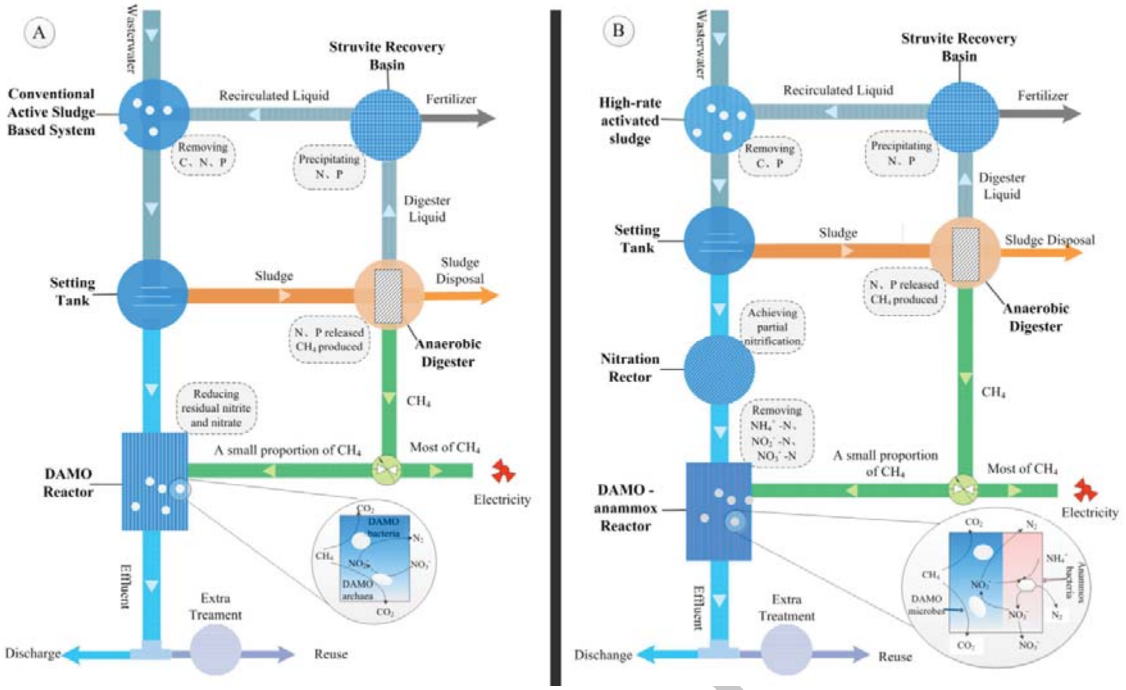
Table 1. The characteristics of two different microbes in the ANME-D process

	M.oxyfera	*M. nitroreducens*
Category	Bacteria	Archaea
Nomenclature	Candidatus Methylomirabilis oxyfera	Candidatus Methanoperedens nitroreducens
Phylum	CN10	ANME-2
Mechanism	Inter-aerobic pathway	Reverse methanogenesis pathway
Electorn acceptor	NO_2^-	NO_3^-
Formula	$3CH_4+8NO_2^{2-}+8H^+\rightarrow3CO_2+4N_2+10H_2O$	$CH_4+4NO_3^-\rightarrow CO_2+4NO_2^-+ 2H_2O$
Chemical energy	$\triangle G$=-765kJ mol^{-1} CH_4	$\triangle G$=-574kJ mol^{-1} CH_4
Shape	Polygonal	Irregular coccus
Related gene	*pmo* gene cluster	*mcr* gene cluster


A
Wastewater
Conventional Active Sludge Based System
Recirculated Liquid
Struvite Recovery Basin
Fertilizer
Removing C、N、P
Precipitating N、P
Digester Liquid
Setting Tank
Sludge
Sludge Disposal
N、P released CH_4 produced
Anaerobic Digester
CH_4
Reducing residual nitrite and nitrate
A small proportion of CH_4
Most of CH_4
DAMO Reactor
Electricity
CO_2
DAMO bacteria
N_2
CH_4
NO_2^-
NO_3^-
DAMO archaea
CO_2
Effluent
Extra Treament
Discharge
Reuse
B
Wastewater
High-rate activated sludge
Recirculated Liquid
Struvite Recovery Basin
Fertilizer
Removing C、P
Precipitating N、P
Digester Liquid
Setting Tank
Sludge
Sludge Disposal
N、P released CH_4 produced
Anaerobic Digester
Achieving partial nitrification
Nitration Rector
CH_4
Removing NH_4^+-N、NO_2^--N、NO_3^--N
A small proportion of CH_4
Most of CH_4
DAMO - anammox Reactor
Electricity
CO_2
N_2
NH_4^+
Anammox bacteria
CH_4
NO_2^-
DAMO microbes
NO_3^-
N_2
CO_2
NO_3^-
Effluent
Extra Treatment
Dischange
Reuse

Highlights

- Denitrifying anaerobic methane oxidation (DAMO) process is systematically summarized
- DAMO process is important to the carbon and nitrogen cycling
- DAMO-centered technologies may be a solution for sustainable operation of WWTPs