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A review of research progress of heterotrophic nitrification and aerobic denitrification microorganisms (HNADMs)

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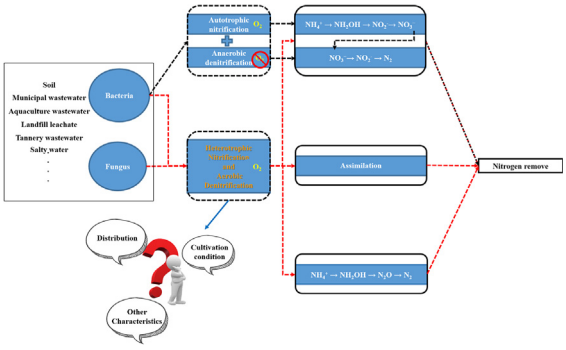
HIGHLIGHTS

- Heterotrophic nitrification and aerobic denitrification microorganism is reviewed.
- Cultivation condition impact on the microorganism is discussed.
- Nitrogen and phosphorus metabolism mechanism of the microorganism is proposed.
- The EPS and QS of the microorganism are summarized.

GRAPHICAL ABSTRACT



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ABSTRACT

Traditional nitrogen removal relies on the autotrophic nitrification and anaerobic denitrification process. In the system, autotrophic microorganisms achieve nitrification under aerobic condition and heterotrophic microorganisms complete the denitrification in anaerobic condition. As the two types of microorganisms have different tolerance on oxygen concentration, nitrification and denitrification are normally set in two compartments for high nitrogen removal. Therefore, large land occupying is required. In fact, there is a special type of microorganism called heterotrophic nitrification & aerobic denitrification microorganisms (HNADMs) which can oxidize ammonium nitrogen, and perform denitrification in the presence of oxygen. HNADMs have been reported in many environments. It was found that HNADMs could simultaneously achieve nitrification and denitrification. In addition, some HNADMs not only have the ability to remove nitrogen, but also have the ability to remove phosphorus. It suggests that HNADMs have great potential for pollution removal from wastewater. So far, individual work on single strain was carried out. Comprehensive summary of the HNADMs would provide a better picture for understanding and directing its application. In this paper, the studies related on HNADMs were reviewed. The nitrogen metabolism pathway of HNADMs was summarized. The impact of pH, DO, carbon source, and C/N on HNADMs growth and metabolism were discussed. In addition, the extracellular polymeric substance (EPS) production, quorum sensing (QS) secretion and P removal by HNADMs were displayed.

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Contents

1. Introduction

With the development of industry, agriculture and living standards, more and more nitrogen is discharged into natural water bodies. Excessive nitrogen releasing into water can cause eutrophication and affect ecology balance. Usually, the ammonia nitrogen removal in wastewater mainly includes ammonia oxidation process and denitrification process. The ammonia oxidation process of traditional wastewater treatment process mainly relies on autotrophic nitrifying bacteria (ANB). However, ANB grow slowly and are sensitive to the environment (Sun et al., 2016; Huang et al., 2017). The denitrification microorganisms in traditional process can only denitrify under anoxic condition (Luo et al., 2016). It suggests that there is limitation of their application in nitrogen removal treatment. In contrast to ANB and anoxic denitrifying bacteria, heterotrophic nitrification & aerobic denitrification microorganisms (HNADMs) can simultaneously accomplish nitrification and denitrification under aerobic conditions (Chen et al., 2016). When sufficient organic carbon sources are provided to HNADMs, they can grow quickly and form effective nitrification and denitrification capabilities (Chen et al., 2012). With more and more HNADMs were found in different environment, the coupling of heterotrophic nitrification and aerobic nitrite/nitrate denitrification under aerobic conditions has attracted great attention in recently years.

Many studies found that HNADMs could keep nitrification and denitrification under extreme conditions (low pH, salty wastewater, low temperature and so on) (Huang et al., 2020a, 2020b). At present, many heterotrophic nitrification & aerobic denitrification (HNAD) genera such as *Acinetobacter*, *Bacillus*, *Cuprobacter*, *Halomonas*, *Klebsiella Marinobacter*, *Pseudomonas*, and *Photobacterium*, have been isolated from various environments (Huang et al., 2020a, 2020b). However, only few studies on HNADMs used for nitrogen removal in reactors have been reported, and many characteristics and enrichment methods of HNADMs still unclear (Quartaroli et al., 2019).

This review is aimed to provide a comprehensive summary of the general characteristics of HNADMs and offering assistance to the subsequent research on HNADMs. The impact factors and metabolic pathways of HNADMs have been reviewed in this paper. In addition, the quorum sensing (QS) secretion and extracellular polymeric substance (EPS) production in HNADMs have been introduced. Finally, the phosphorus removal mechanism of HNADMs has been discussed.

2. The distribution of HNADMs

HNADMs widely distribute and have been found in many environment conditions (Chen et al., 2016). The HNADMs' genera and original isolation environment are shown in Table 1. HNADMs have a wide range of biological resource and environmental adaptability (Table 1). Autotrophic nitrifying microorganisms mainly exist in bacteria genera, while HNADMs exist in the genera of fungus and bacteria. HNAD fungus were often found in soil, and few were reported in water environment. HNAD bacteria are widely found in soil, sludge and wastewater.

Generally, ANB are gram-negative bacteria, but HNADMs include gram-positive bacteria and gram-negative bacteria (Zhang et al., 2012). There are many differently physiological characteristics between gram-positive and gram-negative bacteria (such as drug tolerance and mechanical strength). It means that HNADMs may have more complex physiological function than ANB. The potential function of HNADMs need more study. Some HNADMs genera have been reported to cause diseases such as *Klebsiella*, while some HNADMs genera can prevent diseases and improve water quality such as *Bacillus*. (Huang et al., 2017). The classification and function of HNADMs is still being improved and updated, and more efforts are required for better understanding on the microorganisms.

Although these isolated and identified HNADMs have been discovered from different environments, it does not mean that they perform heterotrophic nitrification-aerobic denitrification function in certain environments. In fact, environmental conditions have great impact on triggering the heterotrophic nitrification and aerobic denitrification function of HNADMs. To identify whether the function is proceeding, the expression of related functional genes and pollutant removal performance should be determined. The influence of environmental conditions on the performance of HNADMs is discussed in other section.

Table 1
The distribution and genus of HNADMs.

Phylum	Genus	Isolation sources	References
Fungus	*Basidiomycetes*	Soil	(Zhang et al., 2020a, 2020b)
	Aspergillus	Soil	(Zhang et al., 2020a, 2020b)
	Penicillium	Soil	(Wang and Yu, 2010)
	Mortierella	Soil	(Zhang et al., 2020a, 2020b)
	Trichoderma	Soil	(Zhang et al., 2020a, 2020b)
	Verticillium	Soil	(Zhang et al., 2020a, 2020b)
	Penicillium tropicum	Surface water	(Yao et al., 2020)
Bacteria	*Arthrobacter*	Soil & sewage	(Verstraete and Alexander, 1972; Zhang et al., 2020a, 2020b)
	Paracoccus	Soil & sea sludge	(Moir et al., 1996; Yang et al., 2008)
	Pseudomonas	Waste water treatment system	(He et al., 2016)
	Klebsiella pneumoniae	Tannery wastewater	(Padhi et al., 2013)
	Bacillus	Soil& wastewater	(Zhang et al., 2012)
	Acinetobacter	Municipal sludge	(Yao et al., 2013; Yang et al., 2015)
	Rhodococuus	Swine wastewater	(Chen et al., 2009)
	Agrobacterium	Landfill leachate	(Chen and Ni, 2012)
	Aeromonas	Activated sludge	(Chen et al., 2014)
	Microvirgula	Activated sludge	(Zhu et al., 2012)
	Alcaligenes faecalis	Sewage sludge	(Joo et al., 2007)
	Diaphorobacter	Wastewater	(Khardenavis et al., 2007)

In addition, it is worth noting that some microorganisms can perform aerobic denitrification, but they have not been found to perform heterotrophic nitrification (Verstraete and Alexander, 1972; Patureau et al., 2000; Zhao et al., 2010a, 2010b). This study only discusses microorganisms with heterotrophic nitrification and aerobic denitrification.

3. Nitrogen removal mechanism by HNADMs

Inorganic nitrogen can be transformed into organic nitrogen and gaseous nitrogen (N_2, N_2O) by the assimilation and dissimilation of HNADMs (Huang et al., 2020a, 2020b). The nitrification and denitrification pathway of the HNADMs metabolic is shown in Fig. 1. HNADMs has a complex metabolic pathway (Fig. 1). The state between organic nitrogen, inorganic nitrogen and gaseous nitrogen transforms according to oxidation, reduction and assimilation.

3.1. Nitrogen dissimilation in HNADMs

3.1.1. HNAD nitrate-nitrite reduction nitrogen removal pathway

Wehrfritz et al. (1993) proposed a HNAD coupling model in 1993, and the classic model has been widely accepted (Wehrfritz et al., 1993; Zhao et al., 2012). It shows that the heterotrophic nitrification pathway is as following:

$$NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-.$$

NH_4^+ is first catalyzed by ammonia monooxygenase (AMO), which oxidizes the ammonia nitrogen into hydroxylamine. Hydroxylamine oxidoreductase (HAO) catalyzes hydroxylamine to nitrite, which is then converted into nitrate via the catalysis of the nitrite oxidoreductase enzyme (Duan et al., 2015). In addition, pyruvic oxime dioxygenase (POD) is another enzyme catalyzing the formation of nitrite from hydroxylamine. Hydroxylamine is converted to pyruvic oxime by a non-enzymatic reaction with pyruvate, which is then oxidized to nitrite and pyruvate by POD (Tsujino et al., 2017). In the study of Zhang et al., the HNADMs strain (*Pseudomonas bauzanensis* DN13-1) can convert hydroxylamine to nitrite, but HAO and POD have never been identified, so it is speculated that the DN13-1 strain may have other nitrification pathways (Zhang et al., 2020a, 2020b). This shows that the current research on key enzymes and metabolic pathways in the nitrification process of HNADMs is insufficient, and further confirmation studies are highly needed.

In HNADMs, the nitrate-nitrite reduction nitrogen removal pathway is similar to that of heterotrophic denitrification (Zhang et al., 2012). The aerobic denitrification pathway is (Wehrfritz et al., 1993): $\rightarrow NO_3^- \rightarrow NO_2^- \rightarrow N_2$.

In this nitrogen metabolic pathway, the reduction of nitrate and nitrite are the two key steps of denitrification. The first step reduces nitrate to nitrite, which depends on nitrate reductases. Nitrate reductases was classified into three distinct types, periplasmic nitrate reductase (Nap), respiratory nitrate reductase (Nar) and assimilatory nitrate reductase (Nas) (Sparacino-Watkins et al., 2014). Chen et al. (2012) reported that Nap plays important role in converting nitrate to nitrite under aerobic conditions, due to that Nap was expressed under aerobic conditions (Chen et al., 2021). In the study of Zhang et al., the relative expression of Nas was detected during aerobic denitrification process, indicated that Nas may play significant roles in aerobic denitrification (Zhang et al., 2020a, 2020b).

Nitrite reduction is the second step, in which copper-containing nitrite reductase (NirK) or cytochrome *cd*1 nitrite reductase (NirS) catalyzes the reduction of nitrite to nitric oxide (Li et al., 2015). Some studies have reported that cd1 nitrite reductase (cd1-NirS) is a homodimerase, which is responsible for the aerobic conversion of nitrite to nitric oxide (Zhang et al., 2020a, 2020b).

3.1.2. HNAD-hydroxylamine reduction pathway for nitrogen removal

Although the heterotrophic nitrification-aerobic nitrite/nitrate denitrification coupling pathway has been widely accepted, some HNADMs may not follow this pathway. In the study on nitrogen removal with HNADMs, intermediate product detection and enzyme activity measurement are often used to speculate the metabolic

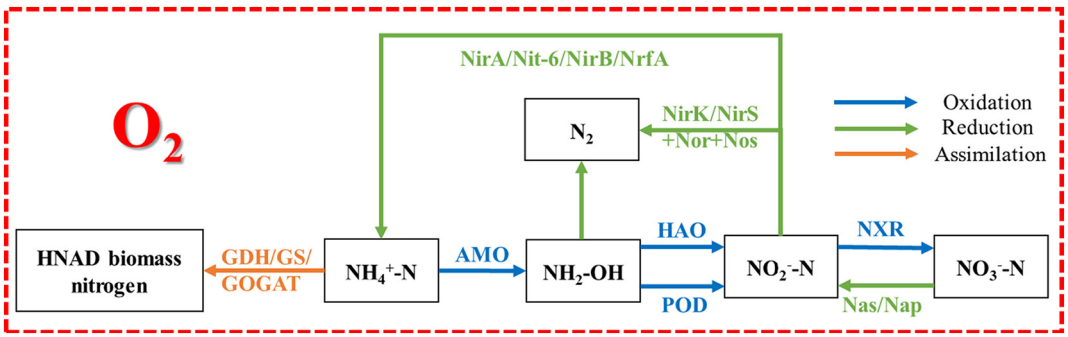

Fig. 1. Nitrogen metabolism pathways of HAND microorganisms.

pathways. Intermediate products of nitrate and nitrite were little or not detected with *Alcaligenes faecalis* No. 4 and *Alcaligenes faecalisNR* for nitrogen removal, and the enzyme activity related to product formation was extremely low (Shoda, 2017; Zhao et al., 2012). It was speculated that the HNAD denitrification pathway was through the conversion of hydroxylamine to nitrogen ($NH_4^+ \rightarrow NH_2OH \rightarrow N_2O \rightarrow N_2$) for removing ammonia nitrogen.

In other studies, it was found that some bacteria have the ability to perform heterotrophic nitrification and produce nitrate, nitrite and N_2 (Jin et al., 2019; Zhao et al., 2010a, 2010b). However, when these bacteria were added to a culture medium with nitrite or nitrate as the sole nitrogen source under aerobic conditions, the reduction of nitrate and nitrite were not found (Zhao et al., 2010a, 2010b). It indicates that these bacteria cannot use nitrite and nitrate for aerobic denitrification. When hydroxylamine is the sole nitrogen source, nitrogen gas is produced during the cultivation process. The metabolic pathway of these strains may be hydroxylamine reduction denitrification pathway (Joo et al., 2005; Zhao et al., 2012; Ren et al., 2014). The nitrogen removal pathway is:

Nitrification: $NH_4{}^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-$
Denitrification: $NH_2OH \rightarrow N_2O \rightarrow N_2$

The hydroxylamine pathway can greatly simplify the denitrification pathway, which is of great significance to nitrogen recycling and treatment. However, nitrate and nitrous nitrogen produced by the nitrification process still require other methods to improve the total nitrogen removal, and hydroxylamine oxidation may be the rate-limiting step in the heterotrophic nitrification-aerobic denitrification process (Chen and Ni, 2011).

3.1.3. Incomplete nitrogen removal pathway

Generally, the aerobic denitrification process includes four specific reductases: nitrate reductase (Nap), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos). However, due to the lack of specific genes encoding Nir, Nor, Nos or reductase damage, denitrify ability is not completely developed for some HNADMs (Duan et al., 2015; Wehrfritz et al., 1993). Incompletely denitrify ability may lead some phenomenon such as the accumulation of nitrite and nitrate (Xie et al., 2021). In the study of Zhao et al. (2010a, 2010b), it was found that TN can be removed through the hydroxylamine pathway, and there is hydroxylamine oxidase (HAO) but not nitrite reductase in the metabolic pathway. It shows that hydroxylamine produces nitrite through HAO, and nitrite cannot be removed, which eventually leads to the accumulation of nitrite. (Zhao et al., 2010a, 2010b). In addition, environmental factors will also affect gene expression and enzyme activity, leading to incomplete metabolic processes. For example, high DO and low carbon source will limite nitrite reductase and cause nitrite accumulation (Sun et al., 2015).

So far, due to the limitation of the number of tested species, it is still difficult to summarize the biochemical mechanism. Therefore, it is necessary to conduct further research on a wider range of species.

3.1.4. Organic nitrogen removal pathway in HNADMs

HNADMs not only can use inorganic nitrogen but also can use organic nitrogen as nitrogen resource. In the study of He et al. (2016), tryptone was used as organic nitrogen to evaluate the denitrification of strain Y-11. The strain Y-11 could easily convert the organic nitrogen into biomass nitrogen and inorganic nitrogen but hardly convert it into nitrogenous gas (He et al., 2016).

3.2. Nitrogen assimilation in HNADMs

Compared with ANB, nitrogen assimilation plays an important role in the HNAD reaction during the nitrogen removal process with HNADMs. As shown in Table 2, some studies have found that the ratio of nitrogen used in assimilation took up to 40% of the total nitrogen removal of the process (Joo et al., 2005; Xia et al., 2020). *Pseudomonas stutzeri* T13 strain has been found to have the function of HNAD (Sun et al., 2015). It shows that O_2 is not the key factor of nitrogen assimilation of *Pseudomonas stutzeri* T13 strain, which can assimilation nitrogen under aerobic and anaerobic conditions. Nitrogen can be used as electron donor (NH_4^+) or electron acceptor (NO_3^-) to be assimilated (Joo et al., 2005; Ren et al., 2014; Zhao et al., 2012).

Table 2
Nitrogen removal in HNADMs.

Strain	N gas	Biomass N	References
Agrobacterium sp. LAD9	50.1%	40.8%	(Chen and Ni, 2012)
Rhodococcus sp. CPZ24	48%	24%	(Chen et al., 2012)
Halophilic Vibrio diabolicus SF16	53.98	35.83	(Duan et al., 2015)
Bacillus sp. LY	45.9	40.5	(Zhao et al., 2010a, 2010b)
Providencia rettgeri strain YL	44.5	49.7	(Chen and Ni, 2012)
Alcaligenes faecalis C16	44–60	28–45	(Liu et al., 2015)
Acinetobacter calcoaceticus HNR	40.2	52.1	(Duan et al., 2015)
Pseudomonas putida strain NP5	34.53	61.79	(Zhang et al., 2012)

In *Klebsiella* sp., genes related to traditional nitrification and denitrification pathways are incomplete, and genes related to nitrification (AMO and HAO genes) and denitrification (Nir and Nos genes) are not found (Jin et al., 2019). It believes that NH_4^+ assimilation by *Klebsiella* sp. was through the NADP-glutamate dehydrogenase pathway. In the nitrogen assimilation pathway of HNADMs, nitrate (from organic nitrogen) is converted into nitrite by nitrate reductase, and then nitrite is converted into ammonia by nitrite reductase. Finally, glutamate dehydrogenase converts ammonia to glutamate for cell growth (Pal et al., 2015).

HNADMs are often found in soil and can degrade organic nitrogen, but some bacteria show high assimilation in inorganic nitrogen environment (Pal et al., 2015). Assimilation may be beneficial to improving the adaptability of microorganisms to high ammonia nitrogen. Enzymes that may be involved in the assimilation of ammonia during assimilation are glutamate dehydrogenase, glutamine synthetase, and glutamate synthase (Jin et al., 2019).

HNADMs have diverse nitrogen metabolic pathways, which can simultaneously use different carbon sources to transform hydroxylamine, ammonium, nitrite and nitrate progressively into gaseous nitrogen (N_2 and N_2O) by nitrification and denitrification, or transform nitrogen into organic nitrogen by integrating with N-assimilation (Zhao et al., 2012).

In addition, HNAD microflora are more effective for nitrogen removal from wastewater with low organic carbon than single strain, due to the co-existence and cooperative interaction between microorganisms with different functions of nitrogen metabolic enzymes (Zhang et al., 2019a, 2019b). For example, the conjunction and interactions of *Bacillus subtilis*, *Pseudomonas stutzeri*, and *Rhodococcus* sp. mainly improved the removal of total nitrogen and organic carbon from wastewater with rich nitrate (Zhang et al., 2019a, 2019b). Moreover, microflora may have higher biomass production ability and stability than single strain in the process of wastewater treatment (Liu et al., 2017; Yang et al., 2017).In different natural ecosystems, the coexistence patterns of microorganisms that contribute to the conversion of effective nitrogen are different.

4. The effect of culture conditions on HNADMs

At present, the researches mainly focus on the study of individual purified strains. Some strains showed good denitrification under batch experimental conditions, and the reactor study to improve the treatment efficiency was studied (Wang et al., 2020; Jia et al., 2020). However, they have not yet reached to application level, which is mainly due to the lack of effective bacterial resources and the uncertainty of the metabolism characteristics of HNADMs strains. To provide support for the construction of large-scale application systems, more HNAD

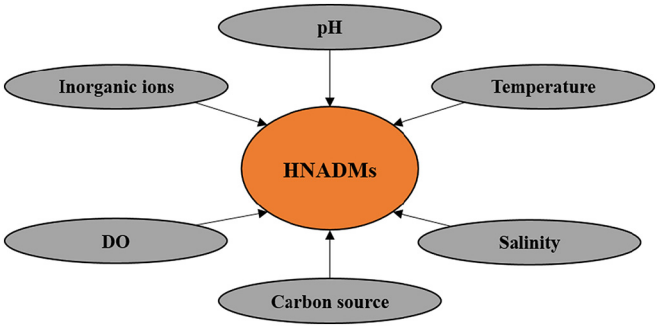

Fig. 2. HNADMs from different conditions.

strains are needed to be found and the influencing factors on growth and metabolism should be explored. Studies have revealed that carbon source, pH, C/N ratio, dissolved oxygen (DO), temperature, mental ions and salinity are the major factors affecting on HNADMs (Shoda, 2017) (Tan et al., 2020; Zhu et al., 2020). These factors were discussed as following: (Fig. 2)

4.1. The pH effect on HNADMs

In general, biological nitrogen removal systems are sensitive to the pH. Weakly alkaline conditions promotes nitrification, and low pH condition inhibits ANB (Zhang et al., 2020a, 2020b).It could be due to the pH value affecting the free ammonia and ammonia monooxygenase in the medium (Zhang et al., 2012). Generally, pH is highly correlated with the intensity of nitrification. Low pH causes the decrease of the heterotrophic nitrification rate. Compared with ANB, some heterotrophic nitrifying microorganisms have better acid resistance ability. Generally, fungi and acidophilic bacteria are considered to be potential heterotrophic nitrification microorganisms (Li et al., 2018). Many researchers reported that fungi could have better acid resistance and heterotrophic nitrification properties than bacteria, and they usually play an important role in oxidizing organic nitrogen and ammonium in soil (Liu et al., 2017; Yang et al., 2017).

Some studies have found that different HNADMs have different acid tolerance ability. Some HNADMs can normally grow between the pH 3 and 9 (Table 3). However, the pH tolerance range of HNADM varies as many strains can further expand the pH tolerance range after adaptation (Zeng et al., 2020). HNADMs have wide range of acid adaptability, which makes them have good application potential in acid wastewater treatment. Although some HNADMs can tolerate a wide range of pH values, neutral or weakly alkaline environments are generally suitable for growth of most of the HNADMs. The optimal pH range for most of HNADMs is 6–9 (Table 3), and extremely high or low pH values may reduce their growth and metabolic (Nancharaiah et al., 2018).

It is interesting that the optimum pH range for nitrogen removal efficiency and microbial growth of HNADMs may different. For example, Strain *Bacillus* MS 30 has the highest ammonia oxidation efficiency between pH 7.5 and 8, but the optimum pH for growth is 6.0–6.5 (Zhang et al., 2012).

Table 3
The pH range for HNADMs.

Strain	pH tolerance range	Optimum pH	References
Penicillium sp. M25-22	5–8.5	7.5	(Wang and Yu, 2010)
Stenotrophomonas sp. MSNA-1	3–10	7.5	(Zeng et al., 2020)
Acinetobacter sp. JR1	4–11	4.5	(Yang et al., 2019a, 2019b)
Acinetobacter junii YB	4–11	7.5	(And and D.P., 2000)
Marinobacter strain NNA5	6–10	7.5	(Liu et al., 2016)
Marinobacter sp.F6	7.5–9	7.5	(Zhang et al., 2012)
Bacillus strain N31	6.5–9	8.0	(Huang et al., 2017)

4.2. Carbon source effect on HNADMs

Contrary to autotrophic nitrification, the presence of organic carbon in heterotrophic nitrification process significantly enhances ammonium removal and cell growth (Joo et al., 2005; Ren et al., 2014). Carbon and nitrogen metabolism are the basic cellular metabolic pathways of all biological water treatment technologies (Chen and Ni, 2011). They are composed of catabolism and anabolism, which provide the necessary energy and nutrition for the metabolism and synthesis of bacteria (Nancharaiah et al., 2018).

The degradability, chemical structure and molecular weight of the carbon source may affect the microbial enzyme activity, metabolic system, its growth and nitrogen degradation (Zhang et al., 2014; Zhao et al., 2017). *Acinetobacter* Yii YB, which has HNAD capability, cannot carry out ammonium removal and grow in the absence of organic carbon, but the strain shows slight growth under the culture condition with organic carbon added (Yang et al., 2015). Organic matter and ammonium are simultaneously metabolized, which may be due to specific electron transfer and charge separation during heterotrophic nitrification.

4.2.1. Carbon source type effect on HNADMs

Generally, glucose, sucrose, acetate, citrate and succinate are commonly used carbon sources for cultivating HNADMs. There are obvious differences in the utilization of carbon sources by different microorganisms, and the denitrification ability of the same strain is different when the carbon sources is different (Yang et al., 2011; Ren et al., 2014). It can be seen that different microorganisms have their own optimal carbon sources (Table 4) as that different carbon sources have their own redox potential (Li et al., 2015). The nitrogen and denitrification removal efficiency of HNADMs is related to the redox potential of the carbon source (Li et al., 2015). When the type of carbon source is pyruvate, citric acid or acetic acid, a higher pH value will be produced after metabolism. When sucrose and glucose are used as the carbon source, the pH was decreased after microbial metabolism (Yang et al., 2011; Liu et al., 2015) . In fact, the pH change will further affect the metabolism and growth of HNADMs.

HNADMs not only can metabolize and grow in the environment with the presence of organic matter, but also can grow in the environment without organic matter. Researchers were surprised to find that some HNADMs also have autotrophic nitrification ability. When *Bacillus subtilis A1* was cultivated for a few days under 0 mg/L COD, part of the ammonia nitrogen is converted into organic nitrogen and inorganic nitrification products (NO_2-N, NO_3-N). NO_2-N and NO_3-N could be removed in the form of nitrogen by aerobic denitrification (Yang et al., 2011).

4.2.2. C/N effect on HNADMs

The growth of ANB does not require an external organic carbon source. In conventional sewage treatment plants, lower C/N ratio is beneficial to the ammonia oxidation process as high organic loading may be harmful to ANB. The autotrophic bacteria used in conventional nitrification is not suitable for treating wastewater with ammonium and organic matter because autotrophic bacteria are easily inhibited. Therefore, conventional nitrification is generally carried out after reducing C/N ratio or dilution (Itokawa et al., 2001; Carneiro Fidélis Silva et al., 2019).

Compared with ANB, HNADMs require a higher C/N, and an appropriate C/N ratio can significantly accelerate the nitrification rate during the nitrification process (Zhao et al., 2017; Carneiro Fidélis Silva et al., 2019). HNADMs have a wide C/N adaptation range (Table 5). The optimal C/N ratio for ammonia oxidation and denitrification of HNADMs was between 2 and 15. Increasing organic carbon concentration will significantly increase the treatment efficiency of heterotrophic bacteria. The optimal C/N for different bacteria varies. Most studies showed that the higher C/N is more beneficial to the removal of ammonium (Chen and

Table 4
Effect of carbon source on HNADMs.

Strain	Source type	Optimum carbon	References
Bacillus subtilis A1	Acetate, glucose, citrate, and succinate	Acetate	(Yang et al., 2011; Zhang et al., 2012)
Pseudomonas stutzeri YG-24	Citrate, succinate, acetate, glucose, sucrose	Citrate	(Li et al., 2015)
Pseudomonas stutzeri strain T1	Fructose, glucose, citrate, acetate, succinate	Citrate	(Guo et al., 2013)
Agrobacterium sp. LAD9	Glucose, sodium acetate, sodium succinate, sodium citrate, potassium sodium tartrate, sucrose	Acetate	(Guo et al., 2013)
Bacillus methylotrophicus strain L7	Sodium succinate, sodium pyruvate, sodium acetate, sodium citrate, potassium sodium tartrate, glucose	Sodium Succinate and Glucose	(Zhang et al., 2012)

Ni, 2011; Li et al., 2015). At lower C/N, it usually shows worse ammonia nitrogen removal capacity and denitrification capacity (Chen and Ni, 2011; Li et al., 2015; Ren et al., 2014). When C/N is further increased beyond the optimal value, it leads to a slight decrease in ammonia oxidation capacity and total nitrogen removal capacity (Chen and Ni, 2012). In addition, some studies have explored the effect of C/N on HNADMs in the reactor. In the study of Wang et al. (2020), mixed culture of *Delftia* sp. YH01 and *Acidovorax* sp. YH02 was introduced into the reactor to enhance the penetration of NO_3^--N. When the C/N was 8, 2000 mg/L of NO_3^--N was effectively removed. In the study of Jia et al. (2020), *Stenotrophomonas maltophilia* DQ01 was added to a moving bed biofilm reactor and studied the impact of different C/N ratios on nitrogen removal. A significant HNAD efficiency of 94.21% and total nitrogen removal rate of 94.43% were achieved at C/N of 7.5 (Jia et al., 2020).

4.3. Nitrogen source effect on HNADMs

Generally, HNADMs can effectively utilize ammonia nitrogen, but the utilization of nitrous nitrogen and nitrate nitrogen by HNADMs is normally different. For example, Zhao et al. found that the denitrification of nitrate and nitrite by *Bacillus* sp. LY had a difference and nitrite has a faster removal rate. The difference in metabolic rate may be caused by the different activities of nitrate reductase and nitrite reductase under aerobic conditions (Zhao et al., 2010a, 2010b). Yao et al. also found that under aerobic conditions, ammonia had the fastest degradation rate, and the denitrification rate of nitrite under aerobic conditions is higher than that of nitrate. (Yao et al., 2013). In addition, it is found that the type of nitrogen source could also affect the growth of HNADMs, and the order of the growth was: ammonia nitrogen > nitrous nitrogen > nitrate nitrogen (Yao et al., 2013). Some studies have found that when the content of nitrous nitrogen exceeded 50 mg/L, it would inhibit bacterial growth, nitrification and denitrification (Wan et al., 2011). However, it was observed that a HANDMs (*Pseudomonas tolaasii* Y-11) could still maintain stable growth and nitrogen removal effect when the nitrite reached 200 mg/L (He et al., 2016). Yao et al. also found that when the nitrite was 80 mg/L, it had no inhibitory effect on *Acinetobacter* sp. HA2 (Yao et al., 2013).

4.4. Dissolved oxygen effect on HNADMs

Dissolved oxygen (DO) is an important factor in the process of denitrification and ammonia oxidation. The presence of DO is not conducive to the growth and metabolism of anaerobic denitrifying bacteria. HNADMs can perform denitrification under aerobic conditions. The DO concentration impacts on HNADMs, but it is less than that on anaerobic denitrifying bacteria (Joo et al., 2005; Zhao et al., 2012; Ren et al., 2014). It can be seen that different HNADMs are most likely to have different optimal DO for denitrification (Table 6) (Robertson et al., 1989). In addition, studies found that a few of HNAD bacteria such as *Cupriavidus sp. S1*, *Acinetobacter sp. ND7* and *Alcaligenes faecalis No.* 4. have strong adaption ability to DO (Sun et al., 2016; Joo et al., 2005; Xia et al., 2020). With the increased of DO, the denitrification efficiency gradually decreases. There are also some exceptions. For instance, an isolated HNAD microorganism, *Alcaligenesfaecafis* strain TUD, still could perform nitrification and denitrification when the DO concentration was increased (Xia et al., 2020). The denitrification ability of *Pseudomonas stutzeri* YG-24 seems to be less impacted by DO (Li et al., 2015).

4.5. Temperature effect on HNADMs

Temperature is one of the key factors affecting the denitrification process. Both nitrification and denitrification processes are sensitive to temperature. Generally, nitrification and denitrification would be severely inhibited when environment temperature below 10 °C (Rodriguez-Caballero et al., 2012). The optimal temperature range of the most HNADMs is 25–37 °C (Table 7). For most of HNADMs, their metabolism will be significantly inhibited under high temperature and low temperature conditions (Liu et al., 2019). A study on an isolated HNADMs strain, *Providencia rettgeri* YL, showed that when the temperature was greater than 40 °C or less than 10 °C, the removal of ammonia nitrogen significantly decreased and the removal efficiency was less than 10% (Huang et al., 2013).

Although temperature change will significantly affect the activity of HNADMs, but they can still maintain part of the nitrification and denitrification capacity when the temperature is so high or so low. As found, an isolated HNADMs strain *Microbacter*-SFA13 could still carry out nitrification and denitrification in a 5 °C environment (Zhang et al., 2012). *Acinetobacter*-Y16 is also a low temperature resistant HNADMs strain (Huang et al., 2013). The discovery of HNADMs at different temperature indicates that HNADMs may have been playing an important role in the nitrogen cycle in different temperature environments. It reveals that HNADMs has great potential for the nitrogen removal from wastewater in the cold regions.

Table 5
Effect of C/N on HNADMs.

Strain	C/N	Optimum C/N	References
Alcaligenes faecalis No. 4	5–20	10	(Zhang et al., 2012)
Providencia rettgeri YL	10–30	10	(Taylor et al., 2009)
Pseudomonas stutzeri YG-24	2–10	8	(Li et al., 2015)
Marinobacter sp. F6	2.5–20	15	(Joo et al., 2005)
Acinetobacter junii YB	2–15	15	(Ren et al., 2014)

Table 6
Effect of DO on HNADMs.

Strain	Optimum DO (mg/L)	References
Acinetobacter sp. Y16	5.84	(Guo et al., 2013)
Bacillus methylotrophicus L7	4.82	(Guo et al., 2013)
Marinobacter sp. F6	6.75	(Zhang et al., 2012)
Pseudomonas sp. ADN-42	3.00	(Jin et al., 2015)
Cupriavidus sp. S1	4.37	(Sun et al., 2016)
Acinetobacter sp. T1	5.10	(Chen et al., 2018)
Pseudomonas putida ZN1	4.67	(Xia et al., 2020; Joo et al., 2005)

Table 7
Effect of temperature on HNADMs.

Strain	Temperature range (°C)	Optimum temperature (°C)	References
Pseudomonas stutzeri strain T1	5–30	30	(Guo et al., 2013)
Marinobacter F6	10–45	30	(Zheng et al., 2012)
Acinetobacter sp. Y16	2–35	20	(Huang et al., 2013)
Pseudomonas stutzeri YG-24	20–40	35	(Li et al., 2015)
Bacillus methylotrophicus strain L7	20–37	37	(Zhang et al., 2012)
Bacillus MS30	35–70	65	(Jin et al., 2015)

4.6. Inorganic ions effect on HNADMs

It can be seen that metals including Fe^{2+}, Fe^{3+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, and Mn^{2+} can enhance the removal of ammonia nitrogen, total nitrogen and TOC by HNADMs (Table 8) (Kim et al., 2005; Jin et al., 2015; Zhao et al., 2017). Mg^{2+} has been reported to have a great influence on the growth of HNADMs and the removal of ammonia nitrogen (Robertson et al., 1988). The promoting effect of ions on HNADMs may be due to their participation in the enzyme formation of HNADMs. For instance, AMO enzyme can catalyze the conversion of NH_4^+ to NH_2OH (And and D.P., 2000). It has been reported that the enzyme activity of AMO could be enhanced by addition of Mg^{2+}, Zn^{2+}, and Mn^{2+} (Li et al., 2015). The supplementation of Mg^{2+} could also enhance the assimilation of the strain (Zhao et al., 2017).

Although some ions can enhance HNADMs' enzyme activity, it is also found the inhibition of Cu^{2+}, Zn^{2+}, and Cr^{6+} on HNADMs (Zhang et al., 2012; Sun et al., 2016). It suggests that the enhancement and inhibition of ions on HNADMs are strain depending. More researches are required for revealing the mechanism.

4.7. Salinity effect on HNADMs

High salinity can cause cell plasmolysis due to the dramatic increase osmotic pressure, microbial metabolism change, and enzyme activity inhibition (Duan et al., 2015). It is why that low treatment efficiency is observed in general biological wastewater treatment during treating saline wastewaters. Dinçer and Kargi reported that the biological activities of nitrification and denitrification were greatly reduced when the salt content exceeded 2% (Dinçer and Kargi, 1999). Salt reduces the transport of compounds between the culture medium and cells, changes the metabolism of microorganisms, leads to dehydration and cell lysis and directly interferes with the growth of microorganisms and the rate of ammonia oxidation (Shoda, 2017). In an autotrophic nitrification system, the salinity tolerance of AOB could reach 30 g/L NaCl or higher (Zhang et al., 2012). However, the tolerance of NOB was only 20 g/L NaCl, and it was completely inhibited when the salinity exceeded 20 g/L (Pan et al., 2020). Therefore, it is difficult to depend on the traditional ANB to treat saline wastewater.

Table 9
Effect of salinity on HNADMs.

Strain	Salinity (g/L)	References
Aeromonas sp. HN-02	20	(Chen et al., 2014)
Bacillus MS 30	25	(Jin et al., 2015)
Bacillus methylotrophicus strain L7	30	(Zhang et al., 2012)
Halophilic Vibrio diabolicus SF16	10–50	(Zhang et al., 2012)

The discovery of salt-tolerant ammonia oxidizing bacteria has become an option to treat saline wastewater. HNADMs may play an important role in the nitrogen cycle of salt-containing wastewater. It can be seen that some HNADMs can tolerant salinity to a wide range (Table 9). It is reported that an isolated HNADMs strain, *Pseudomonas mendocina*TJPUo4 could adapt to 4.5% salinity (He et al., 2019). TJPU04 has strong adaptability to high-salt environment and shows outstanding NH_4^+-N removal performance. Interestingly, the NH_4^+-N removal efficiency of TJPUo4 in 3% salinity medium was still higher than that in salt-free environment (0% salinity).

5. Other characteristics of HNADMs

5.1. The extracellular polymeric substance (EPS) of HNADMs

Microorganisms gather together to form aggregates that are conducive to synergy between cells, enhance gene expression and increase the resistance of bacteria to harsh external environments. Generally unfavorable environmental conditions may have a significant impact on the production and nature of EPS microbial aggregates (You et al., 2016). The resistance of HNADMs in different environments may be related to HNAD metabolism. At present, reports on HNADMs mainly study the influent factors on HNADMs growth, metabolism and nitrogen removal pathways. However, few studies have been conducted on the EPS production of HNADMs.

In wastewater, microorganisms consume organic molecules and produce EPS under aerobic/anaerobic conditions, and these EPS fill and decrease the space between microbial cells. EPS is composed of various organic substances, including polysaccharides (PS), proteins (PN), nucleic acids, lipids and humic acids. The quantity and characteristics of these biopolymers strongly affect the structure of the flocs and the properties of the microorganisms (surface charge, hydrophobicity). Therefore, characterizing EPS is important for understanding HNADMs. The strain *Klebsiella pneumoniae* CF-S9 showed self-aggregation and produced EPS (Padhi et al., 2013). It enriches biomass and has practical applications in wastewater treatment. Recent studies have indicated that bacterial EPS production is closely related to aggregation capacity and cell binding ability (Zhu et al., 2012). *Pseudomonas*, *Achromobacter*, and *Acinetobacter* were isolated from nutrient-poor ecosystems and it was observed that *Pseudomonas* sp. 3–7 showed outstanding EPS secretion and aggregation ability among all(Zhu et al., 2012). The EPS production is closely related to the ability of cells to aggregate. Aggregation ability and cell binding ability are important features in practical applications of bioremediation and wastewater treatment. Ren et al., found that *Acinetobacter junii* YB has good hydrophobicity

Table 8
Effect of inorganic ions on HNADMs.

Strain	Tested metal ions	Effect	References
Aeromonas sp. HN-02	Cu^{2+}, Zn^{2+}	Enchance	(Chen et al., 2014)
Bacillus strains	Ca^{2+}, Fe^{2+},Mg^{2+} Co^{2+}, Cu^{2+}, Zn^{2+} Mn^{2+} and Mo^{2+}	Enchance	(Zhang et al., 2011)
Pseudomonas putida strain NP5	Cu^{2+}, Zn^{2+}, Ni^{2+} and Cr^{6+}	Inhibition	(Yang et al., 2019a, 2019b)
Cupriavidus sp. S1	Zn^{2+}, Ni^{2+}, Cu^{2+}, Cr^{6+}	Inhibition	(Sun et al., 2016)
Pseudomonas putida ZN1	Zn^{2+}, Ni^{2+}, Cu^{2+}, Cr^{6+}	Inhibition	(Zhang et al., 2019a, 2019b)

and flocculation under different conditions (Ren et al., 2014). More research is needed on EPS production and self-flocculation capacity of HNADMs.

5.2. The quorum sensing (QS) of HNADMs

Some studies have described new forms of cell signals, identified as cell-to-cell correspondence signals, regulating the expression of most genes that regulate the behavior of cell populations (Abisado et al., 2018). Specifically, quorum sensing (QS) is the intercellular communication that different bacteria use to respond to fluctuations in cell population density (Goo et al., 2015). These bacteria can synthesize and spread different signaling molecules (called auto inducers) to coordinate their activities. At the same time, the QS system will change the expression level of related genes by detecting specific auto-inducers. (Huang et al., 2020a, 2020b). A few studies confirmed that quorum sensing also has an effect on the denitrification and growth of HNADMs.

At present, there are two main strategies for exploring the effects of quorum sensing on HNADMs: (1) investigating the effect of signal molecules on microbial activity by adding signal molecules; (2) comparing the differences in growth and metabolism between signal molecule encoding gene-deficient and normal microorganisms. Researchers used three signaling molecules to investigate their effects o on the growth and metabolism of a type of HNADMs (Wang et al., 2018). As a result, it was found that three kinds of N-acyl homoserine lactones (AHLs) at low concentration (10, 30 nmol/L) could promote the removal of ammonia nitrogen and cell growth. The promotion effect of the three AHL molecules on bacteria was: C8-HSL > C6-HSL > 3-oxo-C10 HSL. However, the removal of ammonia nitrogen was inhibited when the concentration of the signal molecule reached 50 nmol/L (Wang et al., 2018).

In addition, the external application of signal molecules will also affect the transcriptional expression of genes, biofilm formation and self-aggregation (Huang et al., 2013). Some studies have observed the effect of signal molecules on the molecular metabolism by constructing signal molecule-deficient microorganisms and comparing them with normal microorganisms (Ruan et al., 2020; Zhu et al., 2020). It showed that when HNADMs (*Acinetobacter*) were in the growth phase, the secretion of signaling molecules could promote the growth of bacteria, increase the growth rate of bacteria, and make the bacteria reach a stable period quickly. However, when the bacteria are in a stable period, the presence of signaling molecules will inhibit the number of bacteria and hinder the growth of bacteria, and the lack of AHL can promote the growth of bacteria. It shows that the ability of the crowd sensing system can control the growth of *P. aeruginosa* (You et al., 2016). The secretion of signaling molecules may enhance the strength of anaerobic denitrification, but will inhibit aerobic denitrification (Zhang et al., 2012; You et al., 2016).

In nature, both Gram-positive and Gram-negative bacteria transmit information through quorum sensing to regulate various life activities. Generally, gram-negative bacteria use AHLs as an auto inducer, and gram-positive bacteria use processed auto inducing peptides (AIPs) for communication (Yi et al., 2019). In fact, HNADMs include gram-positive bacteria and gram-negative bacteria, therefore the quorum sensing of HNADMs is more complicated.

6. P removal in HNADMs

Currently, there are two mainstream approaches in the removal of phosphorus: one is phosphorus accumulating organisms (PAOs) which were able to uptake soluble P into the cell in the form of intracellular polyphosphate in the aerobic phase; the other is that denitrifying PAO (dPAO) can store polyphosphates under anoxic conditions. The dPAO can obtain energy from the oxidation of an external carbon source. It is interesting to find that some HNADMs can remove phosphorus. It was reported that *Pseudomonas stutzeri* YG-24 (a type of HNADMs) has the function of removing phosphorus, which can simultaneously remove nitrogen and phosphorus in sewage treatment (Li et al., 2015). Rout et al. found that a strain of *Bacillus cereus* GS-5 with HNAD function has the ability to accumulate phosphorus (Rout et al., 2017). The bacteria can metabolize nitrate or nitrite without inhibiting the absorption of phosphate. However, the amount of phosphate removed by nitrite nitrogen is relatively small compared to nitrate nitrogen, and the toxicity of nitrite may be the cause to reduce the growth rate of microorganisms. Under optimal experimental conditions, this strain has excellent ability to remove nitrogen and phosphorus in synthetic wastewater and actual wastewater. *Enterobacter cloacae* HW-15 (Wan et al., 2017) and *Pseudomonas putida* NP5 (Yang et al., 2019a, 2019b) strain have the ability to simultaneously remove N and P. The 79.83% of P removed by NP5 was assimilated into the biomass by assimilation, and the rest of the P might be adsorbed by the EPS of NP5. Regarding the discovery of HNADMs with the ability to remove phosphorus, there are few studies on the process of phosphorus removal. The specific mechanism is still unclear, and more research in-depth and extensive is needed.

7. Conclusion and outlet

The microorganisms with HNAD function come from a wide range of sources, and they are mostly bacteria and fungi. Nitrogen can be removed by nitrification and denitrification at the same time. Assimilation is an important way of nitrogen removal by HNADMs. HNADMs can remove ammonia nitrogen up to 60% of the initial nitrogen concentration. In addition, there are two main metabolic pathways for nitrogen removal by HNADMs: (1) $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^- \rightarrow NO_2^- \rightarrow N_2$; (2) $NH_4^+ \rightarrow NH_2OH \rightarrow N_2O \rightarrow N_2$. Hydroxylamine plays an important role in both nitrogen removal pathways and may be a limiting step in the nitrogen removal process. The pH, types of carbon source, C/N, DO, temperature, ions and salinity have obvious impact on the metabolism of HNADMs. The response of different microorganisms to conditions is different. The EPS and QS of HNADMs may be important in the aggregation and information exchange of microorganisms during the growth of HNADMs. In addition to the removal of nitrogen, some HNADMs have phosphorus removal ability.

At present, most of the researches on HNADMs are about isolation of strain. Research in reactors or other practical integrated systems is still limited. It is mainly because of that it will increase the uncertainty of the reaction in reactors as the condition is difficult to control. Although some studies have explored the enhancement effect of HNADMs in reactors, great effort is still needed to be made in the influencing factors, the operation mode of the reactor and the long-term stable operation. Secondly, the functional genes and enzymes involved in the degradation pathways of heterotrophic nitrification and aerobic denitrification of nitrogen still need to be further explored. HANDMs are widely distributed and have great development potential in the future. The growth and metabolism characteristics of different HANDMs are significantly different, and the coordination and interaction with other microorganisms requires further exploration. In addition, the methods of detection and identification of HNADMs are required to be further improved and standardized, which is of great significance to the development and research of HNADMs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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