The New Developments Made in the Autotrophic and Heterotrophic Ammonia Oxidation

Mei Wang¹, Yurui Wu², Jiachao Zhu¹, Chenyi Wang², Yanbin Zhu¹, Qing Tian¹*¹

1 Department of Environmental Science and Engineering
DongHua University, 2999 Shanghai North people's Road, 201620 P.R. China
2 Shanghai Caoyang No.2 High School, 160 Meichuan Road, Putuo District, Shanghai, 200333 P.R. China
E-mail address: tq2004@dhu.edu.cn

Abstract. Nitrification is defined as conversion of the ammonium(NH₄⁺-N) to nitrate and it is generally divided into two categories: autotrophic nitrification and heterotrophic nitrification. With the metagenomes retrieved from the Nitrospira highly enriched biosamples, both the ammonium oxidation gens (full set of AMO and hydroxylamine dehydrogenase (HAO) genes) and nitrite oxidoreductase (NXR) coding genes necessary for nitrite oxidation were found in a completely nitrifying bacterium from the genus Nitrospira. Various species of Nitrospira (containing phylogenetically distinct ammonia monooxygenase and hydroxylamine dehydrogenase genes) had been found in many engineered systems and were proved to be comammox bacterium. Some anammox bacteria were found to use mono or dimethylamine and even methanol as electron donors, but the enzymes the employed in the special metabolism were not known. Many heterotrophic nitrifying bacteria have been isolated and characterized from soil, activated sludge, wastewater treatment system. A denitrification process was developed with poly(3-hydroxybutyrate) (PHB) granulesas source of carbon for denitrification. It is possible from metabolic foundations that the heterotrophic nitrifier can consume intracellular stored PHB as the alternative carbon source. This direction still waiting for further exploration.

1. Introduction
Nitrification, the oxidizing ammonia (NH₃) to nitrate, is a key process in nitrogen cycles in ecosystems. It is process that had been studied widely in natural and agricultural waters [1] (e.g. soil from farms or constructed wetlands) and been applied widely in the water and wastewater treatment processes [2]. In particular, the process of simultaneous nitrification-denitrification with complete or partial nitrification in order to achieve the most economic pattern for nitrogen removal [2]. E.g., the nitrification pathways in landfills leachate treatment process has aroused great attentions [3]. To date, studies on the artificial systems (such as aquaculture for the growing of fish and plants) in eliminating toxic ammonium and recycle nitrogen by plants via nitrate also made breakthrough [4]. The milestone breakthrough in recent research should attribute to complete oxidative ammonia into nitrate in one organism (name as complete ammoxidation or Comammox). A pure Comammox bacterium has been obtained from the genus Nitrospira [5].

In the conventional ammonia oxidation process, the biomass yield was low for the low energy yield during nitrification, and as autotrophic bacteria, nitrifiers only obtain carbon from autotrophic CO₂ fixation via the Calvin cycles. In this way, the autotrophic nitrifiers can only be predominant in the
low organic carbon environment. However, recent researches uncovered that many autotrophic nitrifiers and mixotrophic bacteria can propagate in the high carbon source contained environment and involve actively in the process of nitrification and phosphorous recycle and accumulation [6]. It is possible these bacteria survive via the mixotrophs growth passages and using organic and inorganic compounds as the alternative carbon and energy sources. Among the organic carbon sources, polyhydroxyalkanoate (PHA), a biopolymer naturally synthesized by many bacteria and intracellularly accumulated as granules has aroused attention in the study of nitrification. These intracellular stored carbon source were found to be important in denitrification [7]. This paper is to summarize the progress of nitrifying microorganisms’ researches in recent years. The culture conditions of synthesizing PHB by different microorganisms are introduced. Furthermore, the effect of varied factors on nitrifying microorganisms was also discussed and the outlook of future work was also proposed.

2. Autotrophic Nitrifying Bacteria

2.1. Complete Nitrification

Conventional nitrogen removal is a two-step process involving two groups of microbes: i) oxidize ammonia to nitrite by the ammonia oxidizing microorganisms (i.e., ammonia oxidizing archaea, AOA and ammonia oxidizing bacteria, AOB); ii), nitrite is oxidized to nitrate by the nitrite oxidizing bacteria (nitrite oxidizing bacteria, NOB). Two separate processes of nitrification lead to the coexistence of AOB with NOB. However, Costa et al [8] proposed that the ammonia is oxidized to nitrate by a microorganism completely is viable based on the kinetic theory of optimal metabolic processes. In 1999, Zheng Ping et al [9] proposed that the nitrification substrate (ammonia) is more conducive to supporting the growth of one kind of microorganism than two kinds of microorganisms (ammonia oxidizing microorganisms and nitrite oxidizing bacteria) theoretically because of lower energy. With the metagenomes retrieved from the *Nitrospira* highly enriched biosamples, both the ammonium oxidation gens (full set of AMO and hydroxylamine dehydrogenase (HAO) genes) and nitrite oxidoreductase (NXR) coding genes necessary for nitrite oxidation were found in a completely nitrifying bacterium from the genus *Nitrospira*. Daims H. et al. revealed that complete ammonia oxidation (Comammox) bacteria were successfully enriched in deep oil wells [5] and the pure culture were obtained in growing biofilms with oligotrophic conditions in an aquaculture system [10]. Meanwhile, Van Kessel, MAHJ [10] and Daims [5] reported three different cultivated bacteria and an uncultivated bacterium [11] that carry out complete oxidation of ammonia to nitrate. From then on, various species of *Nitrospira* (containing phylogenetically distinct ammonia monooxygenase and hydroxylamine dehydrogenase genes) had been found in many engineered systems including the drinking water systems [12] and were proved to be comammox bacterium. These findings fundamentally set up a new concept of nitrification. Up to date, the enormous potential of the completely nitrifying *Nitrospira* in the nature and engineered system still need to be deplored.

2.2. Anaerobic Ammonium Oxidation (ANAMMOX) Bacteria and Their Mixotrophic Growth

Most nitrifying bacteria need to survive low ammonium presence environments, some of the situation comes from the electron loss in the cyclic electron flow in the nitrification and simultaneous denitrification process. As a result, some nitrifiers can utilize the alternative external organics as the electron donors to replace ammonium. E.g. the new strain Pseudomonas sp. C27 that performs both autotrophic and heterotrophic denitrification in mixotrophic DSR medium was isolated by Chen Chuan [13]. Feray and Montuelle [14] revealed that several nitrifying strains were able to grow under mixotrophic conditions in response to environmental changes. These microorganisms were found to be prevalent in biofilms in wastewater treatment system. Another example is anaerobic ammonium oxidation (anammox) bacteria. Anammox was discovered in the early 1990s in a denitrifying fluidized bed reactor in the Netherlands [15]. Anaerobic ammonia-oxidizing bacteria (anammox) oxidize ammonium using nitrite as an electron acceptor and producing nitrogen under anoxic conditions and are considered to have tremendous potential in the treatment of rich-ammonia wastewater. These novel bacteria have a highly unusual physiology, in that they live by consuming ammonia in the absence of oxygen. Some anammox bacteria were found to use mono or
dimethylamine and even methanol [16] as electron donors, but the enzymes the employed in the special metabolism were still waiting for uncover.

3. Heterotrophic Nitrifying Bacteria
Heterotrophic nitrification process implies heterotrophic microorganisms oxidize ammonia nitrogen to nitrite nitrogen and nitrate nitrogen under aerobic conditions. Most heterotrophic nitrifying bacteria have a phenomenal ability to reduce nitrate to N₂ or N₂O. In general, compared with autotrophic nitrifying microorganisms, heterotrophic nitrifying microorganisms gain several advantages. E.g., higher buffering capacity to organic loads, less oxygen demand and higher growth rates. In addition, these heterotrophic microorganisms presented higher growth rates.

3.1. Isolation of Heterotrophic Nitrifying Bacteria
In the last twenty years, many heterotrophic nitrifying bacteria have been isolated and characterized from soil, activated sludge, wastewater treatment system. It is shown that heterotrophic nitrifying bacteria widely exist in all kinds of natural environments. More recent studies have highlighted the existence of heterotrophic nitrifiers such as Pseudomonas, Acinetobacter, Bacillus and Alcaligenes [17]. Since, the heterotrophic nitrification process implies heterotrophic microorganisms can oxidize ammonia nitrogen to nitrite nitrogen and nitrate nitrogen under aerobic conditions. It is possible, the nitrogen removal can occur under aerobic conditions with the process of simultaneous nitrification and denitrification. A denitrification process was developed with poly(3-hydroxybutyrate) (PHB) and copolymers with 3-hydroxyvalerate (PHBV) granules, as source of carbon for denitrification. Vock et al. [7] discovered that activated sludge could store a carbon source, by transforming organic carbon from wastewater to the form of PHA stored in bacteria cells. Wenjie Wan [18] reported that denitrifying phosphorous-accumulating bacterium(DPAB). Enterobacter cloacae HW-15 presented high heterotrophic nitrification–aerobic denitrification capacity. Barak and van Rijn [19] revealed that P. denitrificans was able to reduce nitrate With PHB and without an external carbon source. Since the autotrophic nitrifier (including the AOB and NOB and anammox bacteria) can use a broad range of inorganic and organic compounds as the energy and carbon sources. It is possible from metabolic fundations that the heterotrophic nitrifier can consume intracellular stored PHB as the alternative carbon source. Although, this concept is still hypothetical without experimental evidence. However, from the review of the recently published reference demonstrate this is fascinating direction that still waiting for further exploration.

3.2. Factors Affecting Heterotrophic Nitrification
The insufficient DO might be responsible for the low substrate removal, Huang [20] et al report that Acinetobacter sp. Y16 only showed an ammonium removal efficiency of 20.4% in static cultivation. However, an extremely high DO level may also inhibit the substrate removal, as described by Zhao [21], A. faecalis strain NR. performed well in removing ammonium and TOC with DO concentration of 1.89-7.71 mg/L.

As we know, the C/N ratio plays an important role in the Heterotrophic nitrification and aerobic denitrification process. C/N ratio is the main control factor to obtain efficient denitrification, the removal efficiency would be comparatively low when the carbon source was insufficient, or the C/N ratio was low. Heterotrophic microbes often require high concentrations of organic carbon to assimilate ammonium, but few have rapid ammonium removal rates within wide C/N ratios [22], [23]. Temperature and pH are all the important environmental factors as for microorganisms. Mike S.M. Jetten showed that anammox biomass was positively affected by the digester effluent. The pH (7.0-8.5) and temperature (30-37°C) is the best for the process.

Many researchers screened heterotrophic nitrification-aerobic denitrifying bacteria, and in the exploration of the growth conditions in the optimal pH range. Duan et al. [24] have respectively reported two strains that have good nitrogen removal performance at alkaline conditions but low nitrogen removal rates at neutral conditions. Aeromonas sp. HN-02 grow at pH range of 6-9 [25]. Chen [12] et.al found that optimal pH is 9.0 as for heterotrophic denitrifying strain Pseudomonas sp. C27. Previous studies revealed that Heterotrophic nitrifiers have a broader range of pH generally [26].
As described by Yan G [27], when the temperature elevated from 37°C to 50°C, the removal efficiency of nitrate decreased 53% for heterotrophic nitrification-aerobic denitrification strain Halomonas Camposalis ha3.

Carbon source is essential for bacterial growth, so it was considered to be an important factor influencing heterotrophic nitrification ability. Sodium succinate, citrate and acetate can be directly utilized by microorganisms because their molecular structures are low-molecular-weight. Therefore, microorganisms can quickly obtain energy by using those compounds and achieve higher efficiency of nitrogen removal. Zhang Shusong [28] isolated new strain of D. polyhydroxybutyrativorans SL-205 that can utilize nitrate and nitrite for denitrification and ammonium for nitrification using poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) as the sole carbon source under aerobic conditions from a denitrification reactor.

4. Conclusions

More and more studies reported that comammox are all autotrophic microorganisms that coexists with AOA and AOB in the soil, and utilize ammonia-nitrogen as the nitrogen source and carbon dioxide as the sole carbon Source. However, the relationship of heterotrophic nitrifying bacteria and comammox should be further investigated. Heterotrophic nitrifying bacteria can synthesize PHB, but its contribution to the biological nutrient removal (BNR) system is unknown. With the continuous popularization of new detection methods and the decrease of cost, it is easier to obtain new isolated species and their metabolic pathways that change their growth pattern in the nature and engineered environments. In this way, people have more opportunities to find out metabolic pathways of new species that change their lifestyle with the change of environment and being actively pursued for engineering application.

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Acknowledgments.
This study was financially supported by National Natural Science Foundation of China (21777024), National Natural Science Foundation of China (51478099) and Natural Science Foundation of Shanghai, China (16ZR1402000), and Shanghai Adolescence Science Innovation Practice Funds.