

Nitrite Oxidation in Wastewater Treatment: Microbial Adaptation and Suppression Challenges

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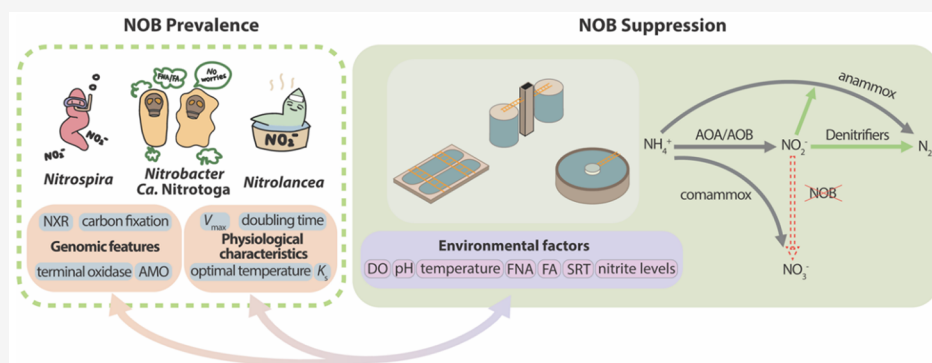
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ABSTRACT: Microbial nitrite oxidation is the primary pathway that generates nitrate in wastewater treatment systems and can be performed by a variety of microbes: namely, nitrite-oxidizing bacteria (NOB). Since NOB were first isolated 130 years ago, the understanding of the phylogenetical and physiological diversities of NOB has been gradually deepened. In recent endeavors of advanced biological nitrogen removal, NOB have been more considered as a troublesome disruptor, and strategies on NOB suppression often fail in practice after long-term operation due to the growth of specific NOB that are able to adapt to even harsh conditions. In line with a review of the history of currently known NOB genera, a phylogenetic tree is constructed to exhibit a wide range of NOB in different phyla. In addition, the growth behavior and metabolic performance of different NOB strains are summarized. These specific features of various NOB (e.g., high oxygen affinity of *Nitrospira*, tolerance to chemical inhibitors of *Nitrobacter* and *Candidatus Nitrotoga*, and preference to high temperature of *Nitrospira*) highlight the differentiation of the NOB ecological niche in biological nitrogen processes and potentially support their adaptation to different suppression strategies (e.g., low dissolved oxygen, chemical treatment, and high temperature). This review implicates the acquired physiological characteristics of NOB to their emergence from a genomic and ecological perspective and emphasizes the importance of understanding physiological characterization and genomic information in future wastewater treatment studies.

KEYWORDS: microbial nitrification, nitrite oxidation, nitrite-oxidizing bacteria (NOB), suppression, kinetics; short-cut nitrogen removal

1. INTRODUCTION

Microbial nitrite oxidation to nitrate is performed by a group of microorganisms, named after their major function as nitrite-oxidizing bacteria (NOB). As one of the primary producers of nitrate, NOB are widespread in natural environments, such as in soil,^{1,2} ocean,^{3,4} freshwater,⁵ and hot springs.^{6–8} Members of NOB are diverse, which have been found spanning 4 phyla and 12 genera (including candidate genera) with considerable functional and physiological diversities.

NOB also play an essential role in biological nitrogen removal processes within modern wastewater treatment plants (WWTPs).^{9–11} Biological nitrogen removal was initially proposed in the middle of the twentieth century in response to the growing issue of eutrophication. Until now, nitrification–denitrification has still been the most widely adopted nitrogen removal process. In this process, nitrite

generated from ammonia oxidation is subsequently oxidized to nitrate by NOB, which is then anoxically reduced back to nitrite and further to dinitrogen gas by denitrifying microorganisms. An obvious issue associated with the conventional nitrification–denitrification is the transformation between nitrite and nitrate, which leads to significant dissipation of energy (from nitrite to nitrate) and organic carbon (from nitrate to nitrite).¹²

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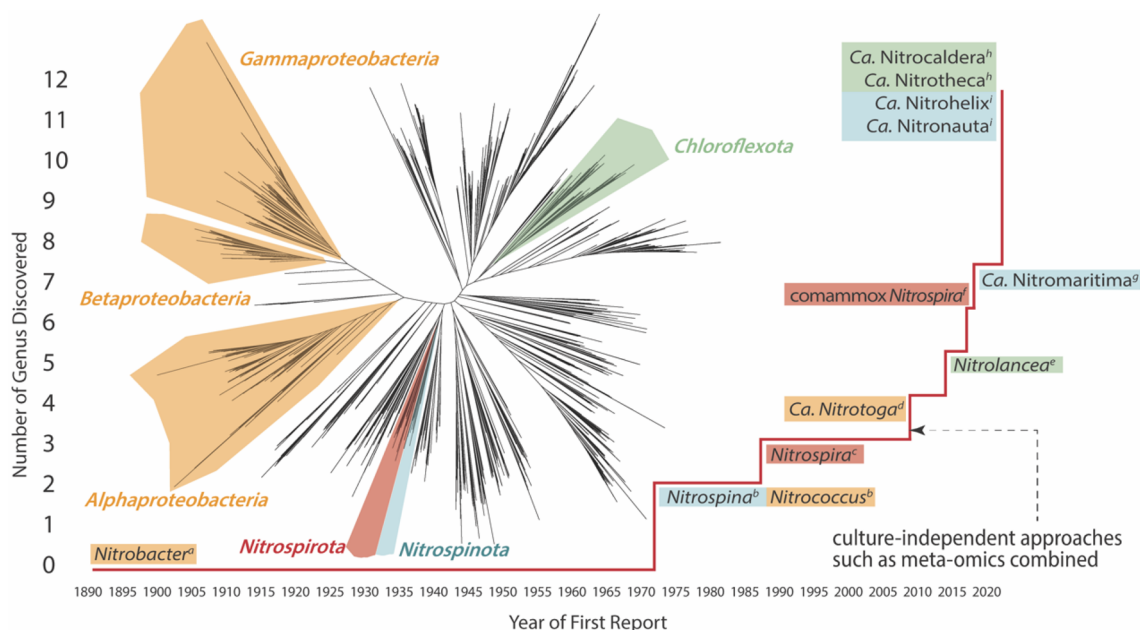


Figure 1. Historic discovery of NOB and complete ammonia oxidation (comammox) genera. The red line indicates the increase in the total number of NOB/comammox genera identified. The colored background of the genera names represents the phylum to which each NOB/comammox belongs to. The phylogenetic tree is a pruned tree from a previous “tree of life” study,²¹ and branches with colored contour depict the phylum. The discovery of NOB genera was first reported in the following studies: (a) ref 22, (b) ref 23, (c) ref 24, (d) ref 1, (e) ref 25, (f) refs 26 and 27, (g) ref 28 (h) ref 29, and (i) ref 30.

In recent years, increasing attention has been paid to upgrading traditional WWTPs with the aim of reducing operational costs and carbon footprints. These advanced biological nitrogen removal processes are usually associated with the fate of nitrite. Unlike the traditional nitrification–denitrification pattern, researchers are endeavoring to develop a more energy-efficient and more sustainable flow of nitrogen in WWTPs, that is, to directly reduce nitrite to nitrogen gas, bypassing nitrate. For example, partial nitrification and anammox (PN/A, or deammonification) and nitrite shunt are the two most widely known state-of-the-art shortcut nitrogen removal techniques, which could significantly save energy and organic carbon sources compared to the traditional nitrification–denitrification process.^{13,14} With that, nitrite accumulation becomes a critical prerequisite, while as an oxidizer of nitrite, NOB are considered as the enemy to the shortcut nitrogen removal systems. Indeed, the NOB suppression can be achieved easily in high-strength wastewater due to the in situ formed free ammonia (FA) and free nitrous acid (FNA). However, in mainstream systems where the nitrogen is low (40–60 mg N/L), stable NOB suppression is a known challenge, which is a hot research topic and also the focus of the present review. To suppress the NOB in low-strength wastewater, various control strategies have been proposed. For example, lowering the dissolved oxygen (DO) concentration is a very typical approach, as it manipulates the competition between AOB and NOB.¹⁵ Shortening the solid retention time (SRT) is another strategy to wash out NOB due to the higher growth rates of AOB compared to those of NOB.¹⁶ Moreover, the *ex situ* treatment of mainstream sludge has been proven effective as well. About 20–30% of mainstream sludge is centrifuged and treated by different methods on a daily basis, e.g., FA and FNA, which is shown capable of selectively suppressing NOB in the mainstream treatment.¹⁷ However, most of these strategies have only been shown to inhibit NOB

in a relatively short period, and the long-term operation under practically relevant conditions usually results in the emergence and adaptation of specific nitrite oxidizers.¹⁸ This is intrinsically because of distinct physiological characteristics of diverse nitrite oxidizers, which may pose a fundamental challenge to next-generation shortcut nitrogen removal processes.

Previous reviews focused either more on the fundamentals of NOB or summarizing approaches to suppress NOB from practical engineering aspects. For instance, Daims et al.¹⁹ delved into the biological mechanisms and ecological interaction of NOB, while Cao et al.²⁰ discussed key microbes and interactions in PN/A and suggested critical factors for the process control, including operational parameters and bioreactor selection. Additionally, Wang et al.¹² models from a kinetic perspective for sidestream and mainstream PN/A processes and identifies suitable operational windows. This review aims to build a bridge between these two aspects. Specifically, the integration of perspectives from microbiologists and wastewater engineers will be beneficial to shed light on the question of why NOB suppression in engineered wastewater systems is difficult. Moreover, the review will facilitate the development of more practical solutions for NOB suppression in municipal WWTPs based on the most recent fundamental knowledge, thus underpinning the paradigm shift to carbon-neutral and energy-positive wastewater management in the future.

2. HISTORY OF NOB DISCOVERY

The advancement in cultivation technology, molecular methods, and bioinformatics has broadened the diversity of NOB. To date, all known chemolithoautotrophic nitrite oxidizers are bacteria spanning 4 phyla including Pseudomonadota (also known as Proteobacteria), Nitrospirota (also known as Nitrospirae), Nitrospinota (also known as Nitrospinae), and Chloroflexota (also known as Chloroflexi), which

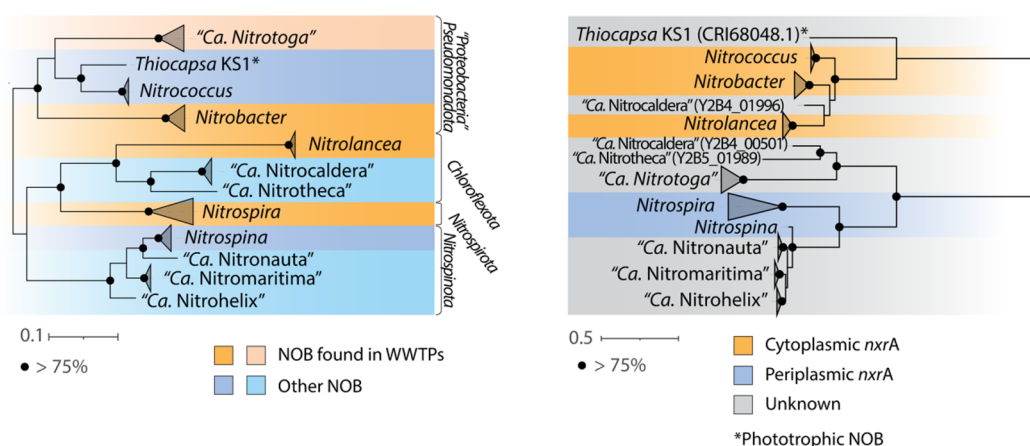


Figure 2. Phylogenetic tree of currently known NOB and their *nxrA*. (a, left) 16S rRNA phylogenomic tree of NOB. Colored backgrounds indicate the NOB found in WWTPs. Light blue and light orange indicate not validly published nomenclature, or no isolates were reported so far. (b, right) Phylogenetic tree of NOB *nxrA*. Colored backgrounds indicate the types of *nxrA*. Black dots at the branch nodes indicate bootstrap values (based on 1000 iterations) >75%. On both sides, an asterisk indicates phototrophic NOB.

consist of 7 genera and 5 candidate genera (without isolates obtained) (Figure 1).

Nitrobacter (*Nb.*) *winogradsky* was the first NOB isolate obtained by serial dilution, which is a significant milestone in the history of NOB discovery.³¹ *Nb. winogradsky* grows aerobically with nitrite as an electron donor and can also alternatively live on simple organic compounds (e.g., pyruvate, formate, acetate, and yeast extract-peptone).^{32–34} After about a century, *Nitrococcus* and *Nitrospina* were identified in marine ecosystems in 1971, with two strains isolated from surface samples of South Pacific waters and South Atlantic waters, respectively.²³ Unlike *Nitrobacter*, they were both obligate chemoautotrophic NOB, as no growth was observed in organic media. Until 1986, a new genus of NOB *Nitrospira* was discovered, and *Nitrospira* (*Ns.*) *marina* was isolated from a water sample at a depth of 206 m from the Gulf of Maine in the Atlantic Ocean.²⁴ In the following more than a century, until now, the genus *Nitrospira* has been found to be the most ubiquitous NOB in natural and engineered ecosystems. A phylogenetic analysis of 16S rRNA genes indicates that *Nitrospira* can be classified into at least six sublineages, exhibiting a greater phylogenetic diversity than other NOB genera.¹⁹ Within sublineage II, a number of *Nitrospira* species including the representative isolate *Ns. inopinata* were shown to be able to perform complete ammonia oxidation (comammox).^{26,27} This discovery is a breakthrough that fundamentally changed the previous perception by demonstrating that complete nitrification can be carried out by a single microorganism rather than multiple microorganisms in concert. Additionally, *Nitrospira* belonging to a distinct novel phylum Nitrospirota was recently discovered based on an in-depth evaluation of the draft genome of *Nitrospira* (*Nn.*) *gracilis*.³⁵ It is considered to be an important NOB population in marine environments.^{30,36,37}

The knowledge of NOB diversity keeps evolving, and more novel NOB have come to light based on the improvement of culture-independent approaches (e.g., meta-omics). The novel NOB genus *Candidatus* (*Ca.*) *Nitrotoga* belonging to β -Proteobacteria was discovered in 2007 based on the enrichment from permafrost-affected soil in Siberian Arctic.¹ Members of the genus *Ca. Nitrotoga* can generally adapt to the cold environment (<10 °C) except for *Ca. Nitrotoga* (*Nt.*)

fabula.³⁸ In contrast, the genus *Nitrolancea* positioning in the Chloroflexota phylum seems to be thermophilic, which was initially discovered in a nitrifying bioreactor at an elevated temperature of 37 °C.²⁵ More recently, two draft genomes of putative thermophilic NOB were retrieved from a metagenome yielded from the Yellowstone Hot Spring enrichment, provisionally named “*Ca. Nitrocaldera robusta*” and “*Ca. Nitrotheca patiens*”.⁸ Both candidate genera belong to the phylum Chloroflexota and expectedly prefer a thermophilic lifestyle. In the Nitrospinae phylum, two new marine genera, “*Ca. Nitrohelix vancouverensis*” and “*Ca. Nitronauta litoralis*” were recovered by using cell sorting, activity screening, and incubation.³⁰ These studies exemplify an efficient protocol enabling physiological investigation rather than traditional cultivation. A recent work using metagenome-assembled genomes and single-cell amplified genomes also proposed a provisional genus “*Candidatus Nitromaritima*” in a deep-branching lineage under Nitrospirota.²⁸ These results collectively indicate that applications of culture-independent approaches will likely further enlarge the pool of NOB than we expected before.

According to the 16S rRNA phylogeny (Figure 2a), NOB is distributed in at least four deep branches. The phylogenetic distance between NOB groups is large, with a 16S rRNA sequence identity of 62.4–92.2% between genera. The phylogeny of the key marker gene *nxrA* (alpha subunit of nitrite oxidoreductase) of NOB shows a congruent pattern (Figure 2b), which can be divided into three main clusters and likely correlated with the different features, for example, energy efficiency (see more discussions in Section 4.1) and the locale in the cell membrane. Specifically, the cytoplasmic nitrite oxidoreductase (NXR) is represented by *Nitrobacter* and *Nitrolancea*, and the periplasmic NXR is represented by *Nitrospira* and *Nitrospina*. The recent identification of *Ca. Nitrotoga* has revealed novel sub-branches of periplasm NXR (Figure 2b), which is associated with a unique soluble NXR periplasmic holoenzyme.³⁹ Analogous to the 16S rRNA phylogeny, the three branches of *nxrA* are divergent from each other, indicating at least three evolution origins.^{39,40}

The metabolic versatility of NOB has been revealed previously, showing their additional capability of performing for example urea hydrolysis and using formate,⁴¹ hydrogen,⁴²

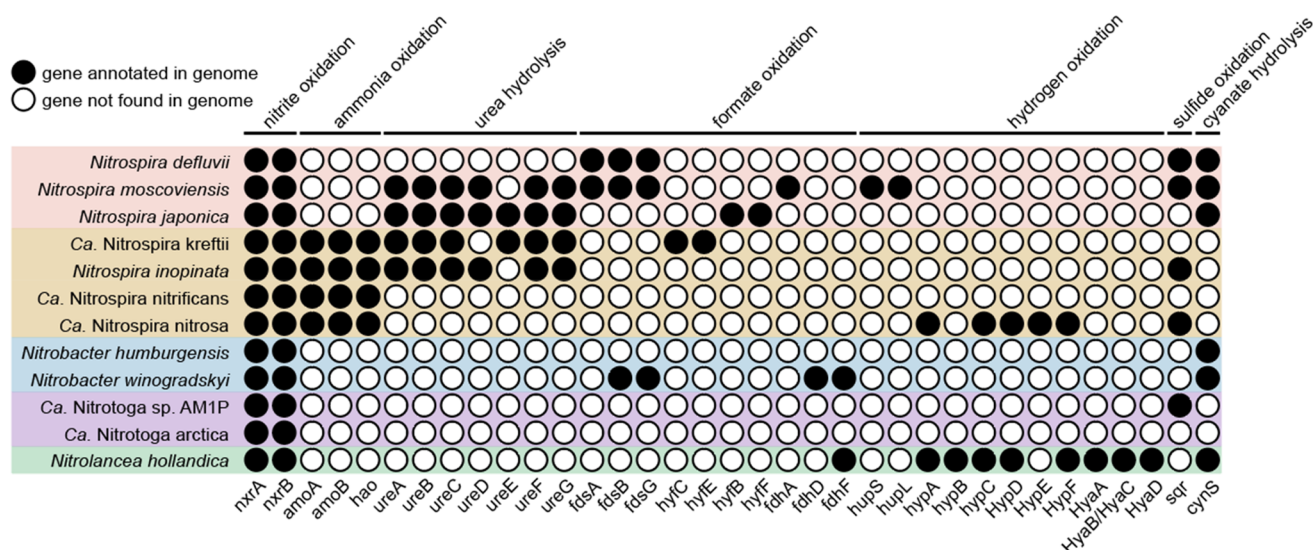


Figure 3. Functional genes in NOB *Nitrospira*, comammox *Nitrospira*, *Nitrobacter*, *Ca. Nitrotoga*, and *Nitrolancea* based on mapped reads to the annotated ORFs. Twelve representative genomes that contained at least one of the related genes are displayed. Metagenemarks⁴⁵ was used to predict the genes of each genome, and functional annotation referred to KEGG,⁴⁶ COG,⁴⁷ and Uniprot⁴⁸ databases. Notably, due to differences in MAG quality and limitations of databases we used, it is possible that some genomes missed functional genes. As gene expression is regulated by various complex factors, such as environmental conditions, nutritional status, and intra- and extracellular signals, gene annotation only represents the potential for functional expression of these genes and cannot ensure that they will be expressed in the actual environment, which requires further investigation.

Table 1. Physiological Features of 22 Isolated/Enriched NOB Cultures, Including Optimum Temperature, Mean (\pm SD) Half-Saturation Constants (K_m), and Maximum Nitrite Oxidation Activities (V_{max})^a

organism name	sample source	optimum temp (°C)	K_m (μ M NO_2^-)	V_{max} (μ mol NO_2^- /(mg protein h))	source
<i>Nitrospira moscoviensis</i>	corroded iron pipe of a heating system	39	9 \pm 3	18 \pm 1	56, 85
<i>Nitrospira defluvii</i>	enrichment from activated sludge sample	32	9 \pm 3	48 \pm 2	56, 84, 86
<i>Nitrospira</i> sp. NJ1	WWTP	31	10 \pm 2	31 \pm 5	87
<i>Nitrospira</i> sp. ND1	WWTP	ND	6 \pm 1	45 \pm 7	88
<i>Nitrospira</i> sp. Ecomares 2.1	moving-bed filter of an aquaculture system	28–30	54.0 \pm 11.9	21.4 \pm 1.2	89
<i>Nitrospira</i> sp. BS10	WWTP	28	27 \pm 11	20 \pm 2	56
<i>Nitrospira inopinata</i>	hot water pipe from an oil exploration well	37	372 \pm 55	16.9	56, 69
<i>Nitrobacter hamburgensis</i> X14	soil	ND	544 \pm 55	64 \pm 1	56, 90, 91
<i>Nitrobacter winogradskyi</i>	soil	32	309 \pm 92	78 \pm 5	34, 56, 91, 92
<i>Nitrobacter vulgaris</i>	sewage	ND	49 \pm 11	164 \pm 9	84, 93
<i>Nitrobacter</i> sp. Nb-311A	surface waters of the Atlantic Ocean	ND	27.6 \pm 6.7	95.2 \pm 7.0	89
<i>Ca. Nitrotoga</i> sp. AM1	sand of eelgrass zone	16	24.7 \pm 9.8	ND	55
<i>Ca. Nitrotoga arctica</i>	permafrost soil	10/13–19	58 \pm 28	26 \pm 3	56, 80
<i>Ca. Nitrotoga</i> sp. HAM-1	WWTP	ND	ND	ND	83
<i>Ca. Nitrotoga fabula</i> KNB	WWTP	24–28	89.3 \pm 3.9	27.6 \pm 8.4	38
<i>Ca. Nitrotoga</i> sp. HW29	recirculation aquaculture system	22	ND	ND	94
<i>Nitrospina watsonii</i> 347	Black Sea, 100m depth	28	18.7 \pm 2.1	36.9 \pm 2.2	23, 89, 95
<i>Nitrococcus mobilis</i>	surface water of the South Pacific Ocean	25–30	119.7 \pm 34.0	141.0 \pm 10.6	23, 89
<i>Nitrolancea hollandica</i>	laboratory-scale bioreactor	40	1000	NA	25,96
<i>Ca. Nitrohelix vancouverensis</i>	sandy coastal surface sediment samples	ND	8.7 \pm 2.5	ND	30
<i>Ca. Nitronauta litoralis</i>	sandy coastal surface sediment samples	ND	16.2 \pm 1.6	ND	30
<i>Nitrospina gracilis</i> 3/211	surface water of the Atlantic Ocean	25–30	20.1 \pm 2.1	41.4 \pm 9.4	23

^aND means not determined.

sulfide,⁴ and cyanate^{43,44} as substrates for their growth (Figure 3). Such highly functional diversity supports the survival of NOB in different natural and engineered systems but adds difficulty to suppressing the nitrite oxidation in wastewater treatment using a single strategy. Moreover, these diverse

functions are widely possessed by different NOB, while the species within the same genus may show different functions. As the nitrifying community in wastewater-engineered systems is mostly revealed by 16S rRNA amplicon sequencing, the resolution is inadequate to show the microbial community

structure at the species level. In specific circumstances, especially when the NOB control failed and the adaptation of NOB is observed, using advanced metagenomic sequencing will be conducive to providing more accurate information in high resolution.

Both phylogenetic trees based on 16S rRNA and the *nxrA* gene are rooted at midpoints. In the 16S rRNA gene tree (left), there are 4 major branches in accordance to phyla level, namely Pseudomonadota (Proteobacteria), Chloroflexota, Nitrospiraota, and Nitrospinota. In contrast, the *nxrA* phylogenetic tree is mainly divided into three major branches. These include cytoplasmic *nxrA* represented by *Nitrococcus*, *Nitrobacter*, and *Nitrolancea*, periplasmic *nxrA* represented by *Nitrospira* and *Nitrospina*, and a middle branch mainly represented by *Ca. Nitrotoga* yet to be clarified. The major branches of the trees are composed of highly divergent sequences, suggesting significant evolutionary divergence between lineages. Upon closer inspection, the topology of the branching patterns has similarities between the trees. For example, in both trees, *Nitrospira*, *Nitrospina*, and other Nitrospinota-affiliated genera still cluster together (although *Nitrospira* seems more independent), with only minor differences in their branching patterns or sequence divergence. Moreover, *Nitrococcus*, *Nitrobacter*, and *Thiocapsa* also remain relatively close to each other. These clades likely represent closely related lineages that have undergone relatively recent evolutionary diversification. However, the overall patterns of evolutionary relationships among the 16S rRNA and *nxrA* genes are not necessarily alike; therefore, it is necessary to choose the appropriate gene when using genetic analysis to interpret NOB evolutionary relationships.

3. PRIMARY NOB IN GLOBAL FULL-SCALE WWTPS

Nitrification converts ammonia (NH_3) to nitrite (NO_2^-) and then to nitrate (NO_3^-), which is generally carried out by ammonia-oxidizing bacteria (AOB)/ammonia-oxidizing archaea (AOA), NOB, and comammox bacteria in wastewater treatment systems. NOB *Nitrobacter* cells were frequently detected together with *Nitrospira*, which were believed to dominate nitrification in wastewater treatment systems.^{49,50} In recent years, with more accessible sequencing and quantification technologies, there has been an increasing amount of evidence highlighting the prevalence of *Nitrospira* in activated sludge of WWTPs.⁵¹ A systematic global-sampling effort of ~1200 activated sludge samples from 269 WWTPs demonstrated that *Nitrospira* were the dominant NOB in most examined WWTPs.⁵² Similarly, other studies also showed that sublineages I and II of the genus *Nitrospira* were ubiquitously detected in WWTPs.^{50,53} It is therefore tempting to assume that the operational conditions in conventional activated sludge processes are favorable to *Nitrospira* in comparison to those of other NOB genera. The dominance of *Nitrospira* is likely due to the physiological features and metabolic versatility of this genus, as elaborated below.

3.1. Physiological Features. Table 1 summarizes the key physiological features of the most kinetically characterized NOB, including the optimum temperature, half-saturation nitrite constant (K_m), and maximum nitrite oxidation rate (V_{\max}). These characterization studies unanimously reveal that the nitrite affinity constant of *Nitrospira* ($K_m = 6\text{--}54 \mu\text{M NO}_2^-$, excluding comammox *Nitrospira*) is much lower than the counterpart of other NOBs found in wastewater treatment. Since the *in situ* nitrite concentration is generally below $50 \mu\text{M}$

NO_2^- in widely installed continuous flow activated sludge processes, *Nitrospira* may have a greater resilience and adaptive ability over other NOB genera. For example, *Nitrospira* outcompeted *Nitrobacter* when the nitrite concentration was controlled below $3 \text{ mg NO}_2^- \text{-N/L}$.⁵⁴ However, it should be noted that the sequencing batch reactor (SBR), a configuration widely applied in small-/medium-scale WWTPs, often transiently accumulates nitrite in the aerobic period. The increased nitrite concentration can provide opportunities for the growth of other NOB such as *Ca. Nitrotoga*, which have a slightly higher K_m value in the range $24\text{--}86.5 \mu\text{M NO}_2^-$.^{38,55,56} By using 16S rRNA gene specific PCR and FISH, Lückner et al. verified the presence of *Nitrotoga*-like bacteria in 11 of 15 full-scale SBRs surveyed, in which the nitrite level could reach as high as 0.48 mg/L NO_2^- within a typical cycle.¹⁰

Nitrospira generally has a slow growth rate. For example, the generation times for two *Nitrospira* representatives, *Ns. moscoviensis* (sublineage II) and *Ns. defluvi* (sublineage I), were determined to be 32 and 37 h, respectively,⁵⁶ which were longer than the doubling time of 13 h for *Nb. vulgaris* and 26 h for *Nb. winogradskyi*. This indicates that the SRT applied in many WWTPs (~15 days) is important to the retention of *Nitrospira*. In agreement with this hypothesis, a study adopting different SRTs reported that *Nitrospira* greatly outnumbered *Nitrobacter* at a longer SRT of 40 days.⁵⁷ Attributed to the slow growth rate, biofilms provide a more suitable niche for the growth of *Nitrospira*. In a full-scale hybrid biofilm and activated sludge reactor, the metagenomic approach revealed that the biofilm had significantly higher abundances of *Nitrospira* compared to the suspended sludge.⁵⁸ In addition to the canonical *Nitrospira*, researchers highlighted that the comammox *Nitrospira* also prefer to grow in biofilms with long SRT. For example, comammox *Nitrospira* were found dominating the biofilm in a rotating biological contactor in WWTP,⁵⁹ and a survey on 14 full-scale nitrogen removal systems also revealed that the long SRT (>10 days) and attached growth phase were significantly correlated to the prevailing comammox *Nitrospira*.⁶⁰ Our recent studies also showed the prevalence of comammox *Nitrospira* in nitrification biofilms attached to sponge and plastic carriers.^{61,62}

Although oxygen is also essential to NOB growth, the oxygen affinity was rarely quantified due to the mass transfer resistance among cell aggregates. In engineered systems, researchers usually use apparent half-saturation constants for oxygen (K_O) to describe the oxygen affinity of NOB, which typically ranges from 0.06 to 1.0 $\text{mg O}_2/\text{L}$ for NOB in WWTPs.^{63–67} Since DO concentrations in nitrifying tanks are usually in the range of 1–3 $\text{mg O}_2/\text{L}$, which is higher than most of the apparent K_O values reported, the oxygen may not be the factor affecting the competition between *Nitrospira* and other NOB. However, this is only applicable to the NOB community in flocs, while in biofilms, the DO level may play an important role because of the higher apparent K_O due to mass transfer resistance.⁶⁸ From the above discussion, the low *in situ* nitrite concentration and the long SRT are likely to jointly contribute to the dominance of *Nitrospira* in full-scale WWTPs.

3.2. Metabolic Versatility. Recent findings have suggested that the prevalence of *Nitrospira* in wastewater treatment systems could also be related to their metabolic versatility. First, some *Nitrospira* members are capable of utilizing ammonia as an energy source (i.e., comammox *Nitrospira*), which is the main form of nitrogen in raw wastewater. In

Table 2. Dominant NOB under Different Control Strategies

strategy	reactor type	NOB	DO (mg O ₂ /L)	SRT (days)	harsh treatment	ref
oxygen-based control	UMABR	<i>Nitrospira</i>	0.6 ± 0.1	infinite		110
oxygen-based control	MBBR	<i>Nitrospira</i>	0.15–0.18	uncontrolled		111
oxygen-based control	MBBR	<i>Nitrospira</i> and <i>Nitrobacter</i>	<0.03	uncontrolled		112
oxygen-based control	SBR	<i>Nitrospira</i>	0.2–6.5	uncontrolled to 14.4 ± 2		64
FNA-based sidestream treatment	SBR	<i>Ca. Nitrotoga</i>			>1 mg HNO ₂ -N/L FNA	113
FA-based sludge treatment	SBR	<i>Ca. Nitrotoga</i>	1.5–2.0	15	354 ± 58 mg N/L FA	114
FNA-based sidestream treatment	anoxic/oxic (An/O) reactor	<i>Ca. Nitrotoga</i>		15	1.87 mg N/L FNA	115
<i>ex situ</i> control with harsh treatments	SBR	<i>Nitrospira</i> and <i>Nitrobacter</i>	1.5–2.0	15	4.23 mg N/L FNA and sequentially 210 mg N/L FA	103

particular, comammox *Nitrospira* exhibit a higher affinity for ammonia compared to most other canonical ammonia oxidizers.⁶⁹ Field investigations have shown that comammox *Nitrospira* are present in nearly all wastewater treatment processes.^{70–72} Due to the high affinity for ammonia, the systems with comammox *Nitrospira* may have higher ammonia removal efficiencies compared to nitrogen removal systems where they are absent.⁶⁰ Still, more studies should be dedicated to further quantifying the contribution of comammox *Nitrospira* to ammonia and nitrite removal in WWTPs.

Second, *Nitrospira* members have versatile metabolic pathways, such as the metabolism of urea, cyanate, and hydrogen. Among all, the degradation of urea is more relevant to wastewater treatment, as over 80% of ammonia nitrogen in domestic wastewater is from urine.⁷³ It has been demonstrated that *Ns. moscoviensis* belonging to *Nitrospira* sublineage II can cleave urea to ammonia and CO₂,⁴¹ and the produced ammonia can be further supplied to ammonia oxidizers. Indeed, the operon encoding the functional urease (*ure*) exists in the genomes of many other species of *Nitrospira* such as *Ns. sp. BS10*⁷⁴ and *Ns. sp. ND1*,⁷⁵ illustrating the ecological importance of urea to *Nitrospira*. Additionally and intriguingly, three novel comammox species were selectively enriched in a urine-fed reactor.⁷⁶ Similar to other comammox, *Ns. inopinata*, *Ca. Ns. nitrosa*, and *Ca. Ns. nitrificans*, all these comammox *Nitrospira* genomes contained a complete urea utilization pathway. The slow release of ammonia from urea hydrolysis might contribute to the prevalence of comammox *Nitrospira* in the urine-treatment reactor. Although some AOB and AOA also have ureolytic activities,^{77–79} the successful enrichment from the urine-fed reactor indicates that urea is an important factor to the selection of comammox *Nitrospira*, which requires further investigations.

3.3. Impact of Seasonal Temperature Variation. From a global perspective, seasonal temperature variation is an important factor shaping the microbial communities in full-scale WWTPs. In particular, some full-scale WWTPs are located in the temperate zone, which generally exhibits annual average temperatures lower than those of tropical and subtropical regions. Various *Ca. Nitrotoga* strains were discovered from the surface layer of permafrost soil with an extreme temperature range (−48 to 18 °C) and permanently frozen sediments.⁸⁰ Despite the extremely low temperature where they were found, the optimal temperatures of seven *Ca. Nitrotoga* strains were above 10 °C, and five among them were above 20 °C. The result suggested that *Ca. Nitrotoga* are psychrotolerant rather than psychrophilic, which might adapt

to the low temperature when the soil froze more than 3000 years ago and survive as a frozen “living fossil”.⁸⁰ Because of the psychrotolerant feature, *Ca. Nitrotoga* may become the primary NOB in wastewater-engineered systems with low temperatures. For example, *Ca. Nitrotoga* were observed to be the transiently dominant NOB in cold seasons at some WWTPs.^{11,81} An investigation recognized *Ca. Nitrotoga* as the key NOB in full-scale WWTPs, with *Ca. Nitrotoga* detected in WWTPs between 7 and 16 °C.¹⁰ In this study, *Ca. Nitrotoga* was the only detectable NOB in two full-scale WWTPs from Germany at temperatures of 16 and 9 °C, respectively. A recent study showed *Ca. Nitrotoga* co-occurred with *Nitrospira* only in an SBR reactor operated at low temperatures (4–14 °C), while it was almost not detected in a similar reactor but at elevated temperatures (22–34 °C). This together indicates that *Ca. Nitrotoga* may have a specific temperature preference and highlights the importance of operating temperature as a factor in the selection of NOB communities in wastewater treatment processes.⁸² Indeed, the optimal temperature for the growth in the *Ca. Nitrotoga* genus is obviously lower than that of *Nitrospira* (Table 1), which supports the competitive advantages of *Ca. Nitrotoga* over *Nitrospira* at low temperatures.⁸³ In a coculture of *Ns. defluvi* and *Ca. Nt. sp. BS*, the microbial community shifted at 17 °C, where *Ca. Nt. sp. BS* became the dominant NOB.⁸⁴ Laboratory bioreactor and coinoculation studies also confirmed that temperature is a deciding factor affecting niche occupation of *Nitrotoga*-like bacteria in activated sludge.^{1,82}

4. NOB SUPPRESSION AND ADAPTATION IN ADVANCED NITROGEN REMOVAL PROCESSES

In traditional wastewater treatment, aeration for nitrification consumes significant energy for the oxidation of nitrite to nitrate, while the reduction of nitrate to nitrite requires organics for heterotrophic denitrification or potentially through endogenous denitrification.⁹⁷ Therefore, increasing attention has been drawn to advanced nitrogen removal via the nitrite pathway, which is a cost-effective alternative and also known as shortcut biological nitrogen removal. The shortcut nitrogen removal technologies mainly include the nitrite shunt and PN/A (or deammonification), which can be achieved in both two-stage (i.e., oxidation and reduction processes in two bioreactors) and one-stage (i.e., oxidation and reduction processes in one bioreactor) configurations. In theory, ammonia is first converted to nitrite, which will be removed by heterotrophic denitrifiers in the nitrite shunt and by anammox bacteria in the PN/A process. Compared with

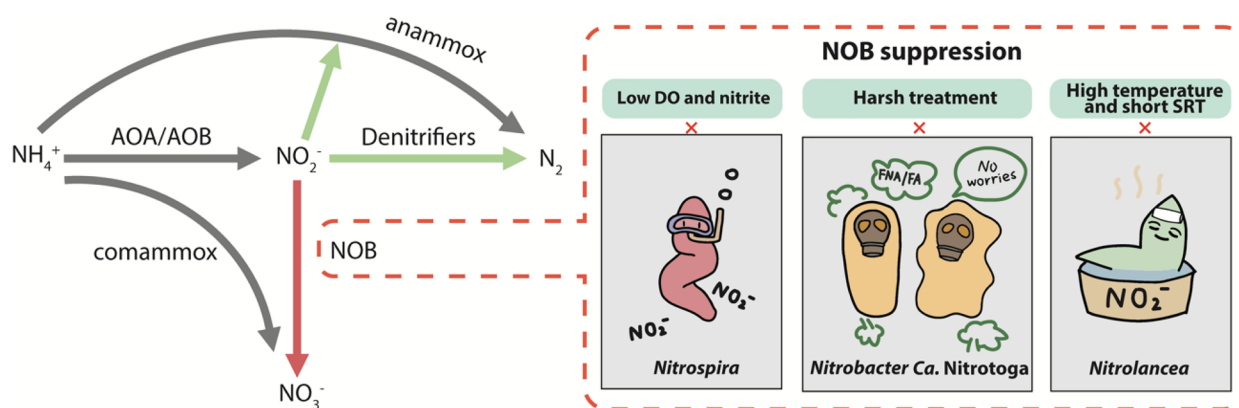


Figure 4. Conceptual diagram describing the adaptation of NOB to different treatment processes in the shortcut nitrogen removal processes.

conventional nitrification and denitrification, the nitrite shunt process reduces the aeration requirement by 25%, organic carbon consumption by 40%, and sludge production by up to 55%,⁹⁸ while these benefits are further enhanced in the PN/A process achieving the reduction of aeration, organic carbon consumption, and sludge production by nearly 60%, 100%, and 80%, respectively.^{99,100}

Despite these remarkable benefits, a critical challenge associated with the success of nitrite shunt and PN/A is the suppression of NOB. Generally, strategies to minimize NOB activity and achieve PN are developed based on three principles: (1) different kinetics of AOB and NOB, (2) different resilience of AOB and NOB to harsh treatment, and (3) the competition for nitrite by other microorganisms (e.g., anammox bacteria) (Table 2). Accordingly, the suppression of NOB can be realized by *in situ* control and *ex situ* treatment. For the *in situ* control, strategies adopting low DO, high residual ammonium, short SRT, and combining anammox bacteria to compete for nitrite are proposed as favorable strategies to selectively inhibit NOB. Apart from that, recently, a novel approach was developed by forming an *in situ* acidic condition in promoting the stable nitrite accumulation, or partial nitrification.^{101,102} Regarding the *ex situ* control, biocidal treatments using such as FA,¹⁰³ FNA,¹⁰⁴ sulfide,¹⁰⁵ ultrasound,^{106,107} and light irradiation¹⁰⁸ were found to be efficient for the selective inhibition of NOB. Both *in situ* control and *ex situ* treatment can be practical. While the *in situ* strategies are generally easier to implement, the *ex situ* treatment most times requires additional capital costs for a separate treatment tank. However, the previous economic analysis showed that the capital cost of the *ex situ* treatment by FNA was only approximately 5% of the total reduced cost by replacing nitrification-denitrification with the PN/A process.¹⁰⁹

Nonetheless, the stable NOB suppression in long-term operation remains challenging under mainstream conditions with low ammonium concentration and seasonally varying temperatures. From a kinetic point of view, the development of NOB control strategies can be found in a recent review.¹² Herein, a microbial perspective of NOB suppression and adaptation to different treatment processes is detailed, since different types of NOB (i.e., *Nitrospira*, *Nitrobacter*, *Nitrolancea*, and *Ca. Nitrotoga*) appear to be adapted to different control strategies, leading to the failure of nitrogen removal via the nitrite pathway (Figure 4).

4.1. *Nitrospira* Surviving in Low-DO and Low-Nitrite Systems.

Low-DO control is undoubtedly the most

recognized strategy for NOB inhibition and is widely applied in shortcut nitrogen removal processes. In comparison to the two-stage configuration, the one-stage shortcut nitrogen removal processes require more strict DO control strategies because the residual oxygen may pose inhibitory effects to anaerobic microbes such as anammox bacteria and denitrifiers growing in the same niche. Therefore, the supplied oxygen in theory should be sufficient only to support the ammonia oxidation while limiting the subsequent nitrite oxidation by NOB and any potential inhibitory impacts on anammox bacteria or denitrifiers. In previous reports, measures such as transient anoxia, DO-based aeration, and intermittent aeration have been proven to be effective in achieving shortcut nitrogen removal performance.^{116–118}

However, *Nitrospira* has stubbornly appeared in many one-stage shortcut nitrogen removal processes, likely because the *Nitrospira* sublineage I, as one of the most abundant *Nitrospira* branches in WWTPs, has an even higher affinity for oxygen (0.09 ± 0.02 mg O₂/L) than most of AOB.⁶⁴ For example, Liu and Wang⁵⁷ showed that *Nitrospira* gradually became predominant, leading to the failure of nitrification in a long-term reactor operated at low DO (≤ 0.5 mg O₂/L). The complete nitrification was achieved at a low DO of 0.3 ± 0.14 mg O₂/L with *Nitrospira* abundance of up to 2.64×10^6 cells/mL, Liu et al.¹¹⁹ also detected *Nitrospira* under extremely hypoxic conditions (0.02–0.10 O₂ mg/L) in a one-stage PN/A process.

The different oxygen affinities of the NOB are likely related to electron transfer and terminal oxidases. Specifically, aerobic respiration relies on the respiratory chain, where electrons generated during nitrite oxidation flow from NXR to cytochrome *c* and then to the terminal oxidase.³⁸ Three distinct types of terminal oxidase have been well-studied and present in the respiratory chain of different NOB.^{120–122} The *aa*₃-type heme-copper oxidase (A-class HCO) is commonly found in *Nitrobacter*,^{123,124} which shows a lower affinity for oxygen. *Nitrospira* contain a putative cytochrome *bd*-like terminal oxidase, which could also receive electrons derived from nitrite or low-potential donors like organic carbon.^{125,126} The *cb*₃-type terminal oxidase, a member of the C-class HCO possessing an extremely high affinity for oxygen,³⁸ is used by NOB species to adapt to O₂ concentrations at nanomolar concentration, e.g., *Nn. gracilis*³⁵ and *Ca. Nt. fabula*.³⁹ This thus supports the occurrence of *Ca. Nitrotoga* and *Nitrospira* in a low-oxygen nitrifying bioreactor.¹²⁷

Previous studies also reported the dominance of comammox *Nitrospira* in a mainstream nitrifying moving bed biofilm reactor (MBBR)¹²⁸ and nitrogen removal system based on an anammox process,¹²⁹ both of which were under low-DO conditions. However, a recent full-scale survey suggested that the presence of comammox is not significantly related to DO levels.⁶⁰ In addition, comammox *Nitrospira* were successfully enriched from an MBBR with a sufficient DO supply,⁶² and the apparent K_O value of this comammox-dominated culture was determined to be 2.8 ± 0.4 mg O_2/L , indicating that this cluster of comammox *Nitrospira* may not have a strong affinity for oxygen. However, the study has its limitations on the mixed culture. Moreover, the efficiency of oxygen mass transfer in biofilms can also affect the measurement of the apparent affinity for oxygen.

Another strategy to suppress NOB in one-stage nitrogen removal processes is a low *in situ* nitrite concentration. Ideally, nitrite produced by AOB in a one-stage nitrite shunt or PN/A is immediately consumed by denitrifiers and anammox bacteria, leading to a gradual washout of NOB due to failure in the competition for nitrite. However, the nitrite uptake competency depends on the nitrite affinities of NOB and other nitrite scavengers, which can vary between genera or even species levels. As shown in Figure 3b, the *nxr* genes can be divided into at least two major types, based on their subcellular locus at periplasm or cytoplasm.¹⁹ In particular, *Nitrospira* and *Nitrospina* harbor the periplasmic NXR,¹³⁰ while cytoplasmic NXR mainly occurs in *Nitrobacter*, *Nitrolancea*, and *Nitrococcus*. Theoretically, the periplasmic NXR energetically prevails over cytoplasmic NXR,^{131,132} as nitrite oxidation takes place outside the cytoplasm and the liberated protons directly contribute to the proton motive force (PMF). This could become the greater energetic advantage of NOB using periplasmic NXR than cytoplasmic NXR, as nitrite oxidation only yields low energy ($\Delta G^\circ = -74$ kJ/mol NO_2^-).^{19,130} In agreement with this fundamental knowledge, previous studies observed that the cytoplasmic group usually dominates over the periplasmic group in environments with a relatively higher level of nitrite, and *vice versa* for low concentration of nitrite.^{25,56} These two different types of NXR are likely associated with different affinities for nitrite, which therefore differentiate the growing niche of different NOB. Members of the *Nitrospira* with periplasmic NXR have a lower K_m for nitrite than other NOB populations or even anammox bacteria (Table 1). This may explain why *Nitrospira* have a stronger endurance to low levels of nitrite and can compete for nitrite against denitrifiers and anammox bacteria in the one-stage shortcut nitrogen removal process.

4.2. *Nitrobacter* and *Ca. Nitrotoga* Tolerating *Ex Situ* Harsh Treatment. In recent years, a number of studies have achieved NOB suppression based on FNA (HNO_2)/FA (NH_3) inhibitory and biocidal effects,¹³³ by sidestream sludge treatment,^{134–136} or *ex situ* treatment. Under acidic conditions, nitrite can form FNA ($HNO_2 \rightleftharpoons H^+ + NO_2^-$), which is an inhibitor of nitrite oxidizers,^{17,137} and free ammonia is the unionized form of ammonium that can form under alkaline conditions.¹³⁸ Generally, FNA causes biocidal effects likely due to the oxidative damage by various reactive nitrogen and oxygen species dissociated from FNA, which can lead to oxidative damage to cellular proteins, cell membrane, and nucleic acids.¹³³ Differently, the inhibitory effects of FA are attributed to the passive diffusion of FA molecules into cells, causing proton imbalance or potassium deficiency.^{103,104} All of

these strategies assume that NOB are more sensitive to harsh conditions than AOB, thus being selectively inhibited; *Nitrospira* is especially sensitive to these harsh conditions.

However, several strains of NOB show a certain robustness against these harsh treatments. As observed in the alternating treatment by FNA and FA, a clear shift in microbial community indicated that *Nitrobacter* may be tolerant to the FNA treatment.¹⁰³ FNA treatment often comes with a low pH, while to date, only a *Nitrobacter* strain has been reported to be acidophilic NOB that can oxidize nitrite at a pH as low as 3.5.² In the natural environment, *Nitrobacter* have been also detected in large numbers of acidic habitats such as acidic soil.² However, the mechanism of acidophilic *Nitrobacter* tolerating the acidic environment remains unknown.

Another recently recognized NOB, *Ca. Nitrotoga*, have been shown to have a high tolerance to both FNA and FA treatment as well.¹¹³ It was reported that FNA ranging from 0 to 1.37 mg HNO_2-N/L was unable to inactivate nitrite oxidation due to the emergence of *Ca. Nitrotoga* (from 0% to 4.51%).¹¹⁵ Likewise, in an activated sludge system regularly exposed to ~ 220 mg NH_3-N/L FA, the dominant NOB shifted from *Nitrospira* (from 61.12% to 2.18% of the nitrifiers) to *Ca. Nitrotoga* (from 4.6% to 85.25% of the nitrifiers).¹³⁴ The proliferation of *Ca. Nitrotoga* was also observed in a recent study, in which alternating FNA and FA treatment and low DO control were both applied.¹¹³ All of these results imply that *Ca. Nitrotoga* could be a critical challenge to the shortcut nitrogen removal that is achieved based on sidestream inactivation using FNA/FA. The mechanism for the tolerance of *Ca. Nitrotoga* to FNA (weakly acidic conditions) and FA (weakly alkaline conditions) is a fundamental question worth more exploration in the future.

As is known, the NOB suppression is easy in treating high-strength wastewater during which the entire system is continuously exposed to the *in situ* high FNA/FA. However, in the *ex situ* sludge treatment by FNA/FA to achieve NOB suppression in low-strength wastewater, only 20–30% of mainstream sludge is generally treated every day, which will provide a certain feasibility for NOB to adapt to the treatment in long-term operation, as observed in many aforementioned studies.^{102,109} Based on the learning from the NOB suppression in high-strength wastewater, researchers hypothesized that an *in situ* harsh treatment of mainstream sludge (e.g., *in situ* high FNA) might enable robust NOB suppression as the whole bulk of the sludge is continuously exposed to the harsh condition continuously. Drawing upon this hypothesis, a robust nitrification process and stable NOB suppression have been reported recently in an *in situ* acidic reactor, where the operating pH was controlled at 5–6.¹⁰¹ Of note, the low pH was not achieved by adding acids but was self-sustained by the microbial ammonia oxidation ($NH_4^+ + 1.5 O_2 \rightarrow NO_2^- + 2 H^+ + H_2O$), which produces protons. Operation using real sewage demonstrated stable suppression of NOB in the long term because AOB can produce nitrite and protons to form *in situ* FNA ($NO_2^- + H^+ \leftrightarrow HNO_2$) at a ppm level, as an inhibitor to NOB.^{139,140} As the entire system was exposed to high FNA continuously, the NOB suppression was very stable, similar to the principle of NOB suppression in high-strength wastewater treatment. Interestingly, the novel AOB *Ca. Nitrosoglobus* were enriched within these acidic systems, which exhibited extreme tolerance to acid and FNA,¹⁴¹ while no known NOB was able to survive under such harsh conditions (i.e., FNA > 1 mg HNO_2-N/L).¹⁴²

It is reasonable to speculate that *Nitrobacter* and *Ca. Nitrotoga* can be as phylogenetically complex as *Nitrospira*, with some branches being tolerant to the harsh treatment and some not. However, in comparison to *Nitrospira*, understanding the diversities of *Nitrobacter* and *Ca. Nitrotoga* is to be advanced, and the mechanisms behind their tolerance against FNA or FA are to be further explored. The above experimental evidence also suggests that it would be extremely challenging to suppress NOB growth using a single strategy.^{132,143}

4.3. Nitrolancea Proliferating with High Temperature and Short SRT. Due to the slow growth rate of NOB and different responses to temperature variation, short SRT and higher temperatures are proposed to suppress NOB activity. These strategies have been used in the classical SHARON (single reactor system for high activity ammonium removal over nitrite) process, one of the earliest processes designed to treat high-strength wastewater.¹³³ The SHARON reactors employed high temperatures (30–40 °C) and short SRT (~1.5 days). In spite of many successful demonstrations, the new NOB genus *Nitrolancea* has been identified in engineered systems with elevated ammonium or nitrite concentrations. *Nitrolancea* (*Nl.*) *hollandica* was first isolated from a bioreactor with 428 mM of ammonia to achieve partial nitrification but ended up with nitrite oxidation. In this case, only a small amount of *Nitrobacter* cells were detected, without other known NOB, indicating the major contribution from an unknown NOB. The obtained isolate *Nitrolancea* (*Nl.*) *hollandica* can tolerate a broad temperature range of 25–63 °C and nitrite up to 75 mM, with an optimum temperature of 40 °C.²⁵ More importantly, a maximal specific growth rate of 0.019 h⁻¹ was observed for *Nl. hollandica*,²⁵ which is several times higher than that of other known NOB, suggesting that to wash out *Nl. Hollandica*, an even shorter SRT may be needed. Recently, Spieck et al. obtained four more *Nitrolancea* strains from a centrate treatment reactor, all of which have similar physiological features including thermotolerance, the capability to grow at high nitrite concentrations, and a fast-growing pattern.⁹⁶ To date, limited studies have reported the presence of *Nitrolancea* in reactors; similar 16S rRNA sequences were found in a partial nitrification reactor¹⁴⁴ and FNA-treated wastewater.¹⁴⁵

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The discovery of a novel NOB is a key path to broaden our knowledge for understanding microbial nitrite oxidation in wastewater treatment. In recent years, novel NOB have come to light with differentiated physiology features and metabolic versatility, resulting in robust adaptability of the NOB to different environments. It can be foreseen that more unknown NOB species will be discovered in the future. A basic approach to study novel NOB in previous studies is based on cultivation/isolation and characterization, which is time-consuming and labor-intensive. In many studies focusing on wastewater treatment, 16S rRNA gene amplicon sequencing has been applied, but the resolution is too low to distinguish species or reveal their functions. Considering that activated sludge systems are significantly complex, *in situ* investigation of NOB in wastewater treatment processes can be an eclectic solution. Given the emergence of advanced detection methods, there should be a tendency for researchers to incorporate advanced detection techniques with traditional experimental approaches. For example, metagenomic sequencing is now intensely used to unravel functional NOB communities in

environmental samples, and this can be more explored in wastewater samples to identify new NOB. In particular, this approach can also be used in advanced nitrogen removal processes that require NOB suppression yet face challenges. Genomic features of these adapted NOB in shortcut nitrogen removal can be an important indicator of their physiological effects, like the nitrite and oxygen affinities, as mentioned above. In parallel, metatranscriptomics can be used to identify gene expressions in the coculture of NOB and other nitrifiers, which helps to reveal the synergy and other physiological effects with their partners compared to single culture and the NOB physiology, such as the mechanism of the tolerance to FNA and affinity for oxygen. Moreover, many previous studies have focused on the development of new control strategies while relatively missing the characterization of abundant NOB when the process performance fails. To better suppress NOB in such systems, more attention should be paid to profiling the physiological characteristics of the NOB, especially at species levels.

The discovery of a novel NOB also provides opportunities to suppress or promote nitrite oxidation in wastewater treatment. Taking the genus *Nitrospira* as an example, it is now gradually realized that the functional diversity within genera is non-negligible, exhibiting diverse physiological features. Among this genus, comammox *Nitrospira* feature an extremely high affinity to ammonia and may outcompete other ammonia-oxidizers under oligotrophic conditions.⁶⁹ While being detected in various wastewater treatment units, comammox *Nitrospira* was believed to damage the shortcut nitrogen removal via the nitrite pathway, as ammonia is oxidized straight away to nitrate by this unique single microorganism. However, more recent studies have indicated that ammonia oxidation can be interrupted at nitrite by comammox bacteria, which have been successfully coupled with anammox bacteria in different configurations.^{134,135} In two very recent reports, comammox *Nitrospira* were found to be the main ammonia-oxidizing community (accounting for 89.2 ± 7.9% in total prokaryotic *amoA* copies) in a PN/A process at low ammonium loading,¹³⁵ and in a symbiotic association of cocultivating comammox *Nitrospira* and anammox bacteria enabled a sustained nitrogen loss.¹³⁴ The new knowledge highlights a potentially positive role of comammox *Nitrospira* in shortcut nitrogen removal processes, in addition to producing less greenhouse gas N₂O and leading to low residual ammonium in the final effluent.^{136,146,147} More specifically, this review also elaborates that some members in the genera of *Ca. Nitrotoga*, *Nitrobacter*, and *Nitrolancea* can become important nitrite oxidizers under specific conditions, such as cold temperature, acidic pH, and thermophilic processes. With new insights into subdivided NOB species, there is thus a need to re-evaluate the regarding contribution to nitrite oxidation especially when corresponding strategies are being designed to suppress a specific NOB in advanced nitrogen removal processes. To resolve the puzzle of mechanisms that are not yet clear, cultivating and characterization of these new NOB would be the prerequisites, and new information needs to be bridged to detailed operation of engineering systems.

Finally, the review also highlighted the challenge of NOB suppression using a single strategy. This is primarily because while using a single strategy to suppress NOB, some specific NOB that are resistant to this applied strategy may gain a competitive edge over others, leading to the failure of suppression in long-term operation. Due to the high diversity

of NOB, it appears that there is no “one-size-fits-all” solution. However, it is also unlikely that a specific NOB strain can survive under all of the different strategies. For example, although *Nitrospira* can survive under low-DO and low-nitrite conditions, they are very sensitive to harsh treatment by FNA and FA. Adversely, *Nitrobacter* are relatively tolerant to FNA and FA treatment, while they do not usually survive under low-oxygen conditions. This knowledge suggests that the integration of multiple strategies may be necessary to effectively inhibit NOB in long-term operation, which can be applied either alternatively or concurrently. For instance, the alternation of *ex situ* FNA and FNA treatment on a monthly basis has shown to be more effective in maintaining NOB suppression than applying each of them individually;¹⁰³ a combined strategy including low DO, *ex situ* treatment by FA, and *in situ* competition by anammox bacteria has been applied to stably inhibit NOB growth, while without any of the three factors, NOB adaptation was observed.¹⁴⁸ In addition to the combined strategies, another potential option to suppress NOB is to achieve *in situ* harsh treatment, inspired by the learning from high-strength wastewater treatment. Due to the low nitrogen concentration, achieving high *in situ* FNA as that in high-strength wastewater needs to lower the pH to 5–6. Fortunately, this can be achieved by the newly discovered acidic-tolerant AOB “*Ca. Nitrosoglobus*”, which generates protons and nitrite forming *in situ* high FNA without any chemical input.¹³⁹ As the entire system is continuously exposed to the *in situ* high FNA, NOB adaptation is unlikely to be achieved. Notably, partial denitrification and anammox (PD/A) could be also a feasible alternative to skirt NOB suppression, which has been the challenging step in PN/A.¹⁴⁹ PD/A includes nitrification ($\text{NH}_4^+ \rightarrow \text{NO}_3^-$), partial denitrification ($\text{NO}_3^- \rightarrow \text{NO}_2^-$), and anammox and exhibits higher stability compared to PN/A,^{150,151} which can be a compromise if PN/A fails.

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Notes

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