



Review

Integrating comammox into anammox-dominated one-stage mainstream partial nitrification/anammox: nitrifier niche regulation and nitrite partitioning

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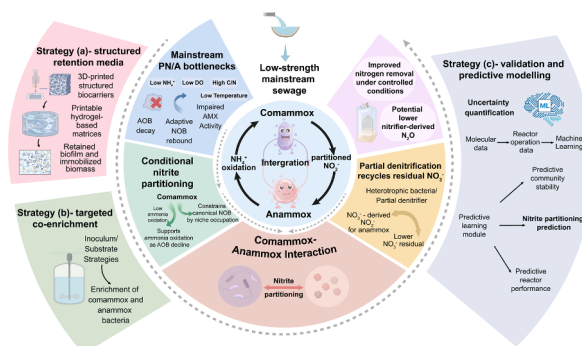
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HIGHLIGHTS

- Synergy of Comammox and Anammox bacteria offers a low-emission route for sustainable mainstream nitrogen removal.
- Comammox supports ammonia oxidation and nitrite availability under mainstream conditions to alleviate AOB decay, adaptive NOB rebound, and impaired anammox in one-stage PN/A.
- Stable ecological displacement may replace transient inhibition as a new strategy for NOB control.
- Retention media, co-enrichment, and machine learning-driven modeling support future process development to investigate comammox and anammox dynamics.

GRAPHICAL ABSTRACT



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ABSTRACT

Mainstream partial nitrification/anammox (PN/A) provides a low-carbon process pathway for municipal nitrogen removal. However, one-stage PN/A deployment remains constrained by unstable ammonia-oxidizing bacteria (AOB)-mediated nitrification, adaptive resurgence of nitrite-oxidizing bacteria (NOB), and inadequate nitrite availability for anammox under variable influent conditions. This review evaluates complete ammonia-oxidizing (comammox) bacteria as an ecological strategy to enhance mainstream PN/A stability by reconfiguring nitrifier competition and nitrite partitioning. Comammox *Nitrospira* exhibit high ammonia affinity, persistence under low dissolved oxygen, and preferential retention under long solids retention times. These traits enable sustained ammonia oxidation under conditions that often compromise canonical AOB. Although comammox possess nitrite-oxidizing capacity encoded by nitrite oxidoreductase, reactor observations indicate that ammonia oxidation by comammox exceed their subsequent nitrite oxidation. This kinetic imbalance enables transient nitrite accumulation and facilitates anammox nitrite uptake when biomass spatial organization promotes nitrite transfer to anammox before *Nitrospira* reoxidation. Conditions that favour comammox also diminish the

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competitive advantage of many canonical NOB, reframing NOB control from transient chemical inhibition toward ecological constraint through comammox niche occupation. The resulting comammox-anammox interaction is governed by nitrite partitioning among ammonia oxidation, nitrite oxidation and anammox uptake. Endogenous partial denitrification further recycles residual nitrate to nitrite when nitrate accumulation limits nitrogen removal. Current evidence supports comammox integration as a promising, boundary-defined strategy for stabilizing anammox-dominated one-stage mainstream PN/A. Future efforts should focus on validating nitrogen transformation pathways, improving the retention of functional biomass, demonstrating long-term reactor-scale performance, and developing predictive modelling that accounts for uncertainty.

1. Introduction

Nitrogen pollution from wastewater discharge remains a major environmental pressure on receiving waters. Beyond nitrogen loading, wastewater treatment is a substantial contributor to greenhouse gas (GHG) emissions, accounting for approximately 3–7% of global emissions (Song et al., 2024). A key driver of this emission burden is nitrous oxide (N₂O), a by-product of conventional nitrification-denitrification, which has a 100-year global warming potential 273 times that of CO₂ (Forster et al., 2021; Espenberg, 2024). These pressures underscore the need to develop energy-efficient and low-emission nitrogen removal technologies.

Traditional nitrification-denitrification systems remain the standard process for biological nitrogen removal in wastewater treatment. However, this approach is energy- and carbon-intensive, requiring substantial aeration input for complete nitrification together with supplemental organic carbon to support heterotrophic denitrification (Kosgey et al., 2022). As one alternative, the partial nitrification/anammox (PN/A) process couples ammonia-oxidizing bacteria (AOB)-mediated partial nitrification with anammox-driven ammonium removal to reduce aeration demand by up to 63% without external carbon addition (Ren et al., 2022; Wang et al., 2022). In sidestream applications treating high-strength sidestream wastewater, PN/A has achieved stable nitrogen removal across multiple full-scale applications. However, under mainstream conditions, low ammonium availability and greater influent variability create operational vulnerabilities that are less pronounced in sidestream reactors. These process instabilities highlight the need to understand the microbial interactions and ecological selection pressures that govern community function, as well as to develop intervention strategies compatible with the operating constraints of mainstream PN/A (Duan et al., 2019).

The constraint becomes evident in the conventional AOB-dominated PN/A configuration. Under the ammonium- and oxygen-limited conditions of mainstream operation, AOB-mediated ammonia oxidation is progressively constrained, leading to AOB activity loss or washout before a stable nitrite supply to anammox is established (Choi et al., 2021; Geets et al., 2006). Weakened nitrification reduces nitrite availability to anammox and facilitates the resurgence of canonical nitrite-oxidizing bacteria (NOB). Once NOB activity recovers, nitrite is diverted from anammox-mediated nitrogen removal toward NOB-mediated nitrate formation (Wang et al., 2022). Anammox bacteria are also difficult to retain under mainstream conditions, given their slow growth and sensitivity to short solids retention time (SRT). Thus, mainstream PN/A instability is reflected in AOB activity loss, adaptive NOB recovery and impaired anammox retention. Under these conditions, AOB-dominated mainstream PN/A systems have also exhibited N₂O emission up to 4.1% of total nitrogen load, which conflicts with low-carbon objectives (Duan et al., 2017; Okabe et al., 2011a). Improving mainstream PN/A stability requires the enrichment or selection of nitrifying microorganisms with kinetic and ecological properties distinct from canonical AOB.

Complete ammonia oxidizing (comammox) bacteria, first identified through the discovery of *Candidatus Nitrospira inopinata*, *Nitrospira nitrosa*, and *Nitrospira nitrificans*, represent additional nitrifying guild that contribute to ammonia oxidation under low-strength mainstream

PN/A conditions (Daims et al., 2015; Van Kessel et al., 2015). Cultured representatives exhibit low apparent half-saturation constants for ammonia, indicating high ammonia affinity (Koch et al., 2019). Reactor studies have also reported their contributions to ammonia oxidation under prolonged SRT (> 15 d) and low dissolved oxygen (DO < 1 mg/L) conditions, where canonical AOB persistence is constrained (Gottshall et al., 2021; Shao and Wu, 2021; Vilardi et al., 2023). Because comammox *Nitrospira* are complete nitrifiers, ammonia oxidation is followed by nitrite oxidation to nitrate catalyzed by nitrite oxidoreductase (NXR). This creates competition for nitrite with anammox, which also depends on nitrite as an electron acceptor. Comammox bacteria become relevant to one-stage mainstream PN/A only when ammonia oxidation exceeds subsequent nitrite oxidation, allowing part of the transient nitrite intermediate to become available for anammox before complete oxidation to nitrate (Guo et al., 2024; Mehrani, 2020). Together, these kinetic and metabolic traits position comammox as a candidate functional group for redesigning mainstream PN/A systems. Their value lies not in the absence of nitrite oxidation, but in the rate imbalance between ammonia and nitrite oxidation that permits nitrite partitioning toward anammox within structured biomass. While their long-term reactor performance and operational control capability remain insufficiently validated.

Previous reviews of mainstream one-stage PN/A have primarily focused on mitigating operational instability through control strategies designed to suppress canonical NOB activity and sustain anammox biomass retention, including DO regulation, free ammonia/free nitrous acid (FA/FNA) dosing, and SRT regulation (Wang et al., 2022, 2023; Shen et al., 2024). However, these strategies require narrow operational control that is difficult to maintain under the substrate and loading variability of mainstream influent. Although comammox bacteria have been detected in mainstream reactors, their role has often been described as an additional nitrifying guild with potential effects on microbial competition (Shaw et al., 2024; Lawson and Lückner, 2018; Guo et al., 2024). This review addresses the knowledge gap in anammox-dominated one-stage mainstream PN/A. It evaluates comammox enrichment as a niche-based intervention for stabilizing nitrite supply and constraining canonical NOB dominance, rather than as the incidental presence of an additional community member. Under the low-ammonium and low-DO conditions typical of mainstream operation, the kinetic and ecological traits of comammox bacteria provide basis for reshaping competition among AOB, canonical NOB, and anammox. This review reframes the control of canonical NOB from repeated operational suppression toward ecological restriction through niche occupation by comammox within the *Nitrospira* lineage. It develops a nitrite-partitioning framework that links comammox-mediated ammonia oxidation, reduced canonical NOB dominance, and sustained anammox activity. The framework clarifies the mechanisms and operational boundaries for integrating comammox into anammox-dominated one-stage mainstream PN/A systems.

2. Identifying bottlenecks in mainstream one-stage PN/A stability from system and microbiome perspectives

2.1. Low ammonium availability accelerates AOB decay and destabilizes AOB-mediated nitrification

Ammonia in mainstream influent is usually reported as total ammonia nitrogen (TAN, $\text{NH}_4^+\text{-N} + \text{NH}_3\text{-N}$), typically ranging from 20 to 60 mg-N/L (Duan et al., 2023; Zheng et al., 2022). The mainstream TAN concentration is far lower than that in sidestream wastewater, where AOB-based PN/A was originally developed, and limits the ammonia availability for sustaining AOB activity and persistence. Under near-neutral mainstream pH, only a small fraction of TAN is present as free NH_3 , the substrate directly used by ammonia monooxygenase (AMO). In biofilm-based systems, diffusion and reaction gradients further reduce NH_3 availability at AOB-active sites, while short hydraulic retention and dilution reduce substrate replenishment. These constraints are consistent with the decline in AOB *amoA* gene copy numbers observed in mainstream reactors (Sims et al., 2012; Wu et al.,

2021). Reported apparent free-ammonia affinity constants ($K_{m(app),\text{NH}_3}$) for cultured AOB fall within 30–150 $\mu\text{mol-N/L}$, with *Nitrosomonas* sp. Is79 exhibiting a $K_{m(app),\text{NH}_3}$ value of $45 \pm 4 \mu\text{M NH}_3$ (Prosser and Nicol, 2012). Local free NH_3 concentrations in mainstream biofilms fall within the same micromolar range as reported AOB ammonia-affinity constants even when TAN remains measurable in the bulk liquid. AOB-mediated nitrification in mainstream biofilms is more likely to be substrate-limited rather than substrate-saturated (Fig. 1).

Substrate-limited kinetics directly lead to a sustained loss of AOB activity and retention. The endogenous decay coefficient (b) rises from 0.10 d^{-1} at 10 mg-N/L to 0.30 d^{-1} at 2 mg-N/L as ammonium availability declines (Munz et al., 2011a). Reported decay rates for *Nitrosomonas europaea* under ammonium starvation exceed those observed under hypoxia, reaching 0.43 d^{-1} compared with 0.09 d^{-1} under oxygen limitation (Geets et al., 2006). Two physiological mechanisms underlie this accelerated AOB decay. Ammonia monooxygenase activity in the absence of ammonia generates reactive oxygen species, including peroxides and radicals, and the resulting detoxification demand diverts reducing equivalents away from biomass maintenance (Kim et al.,

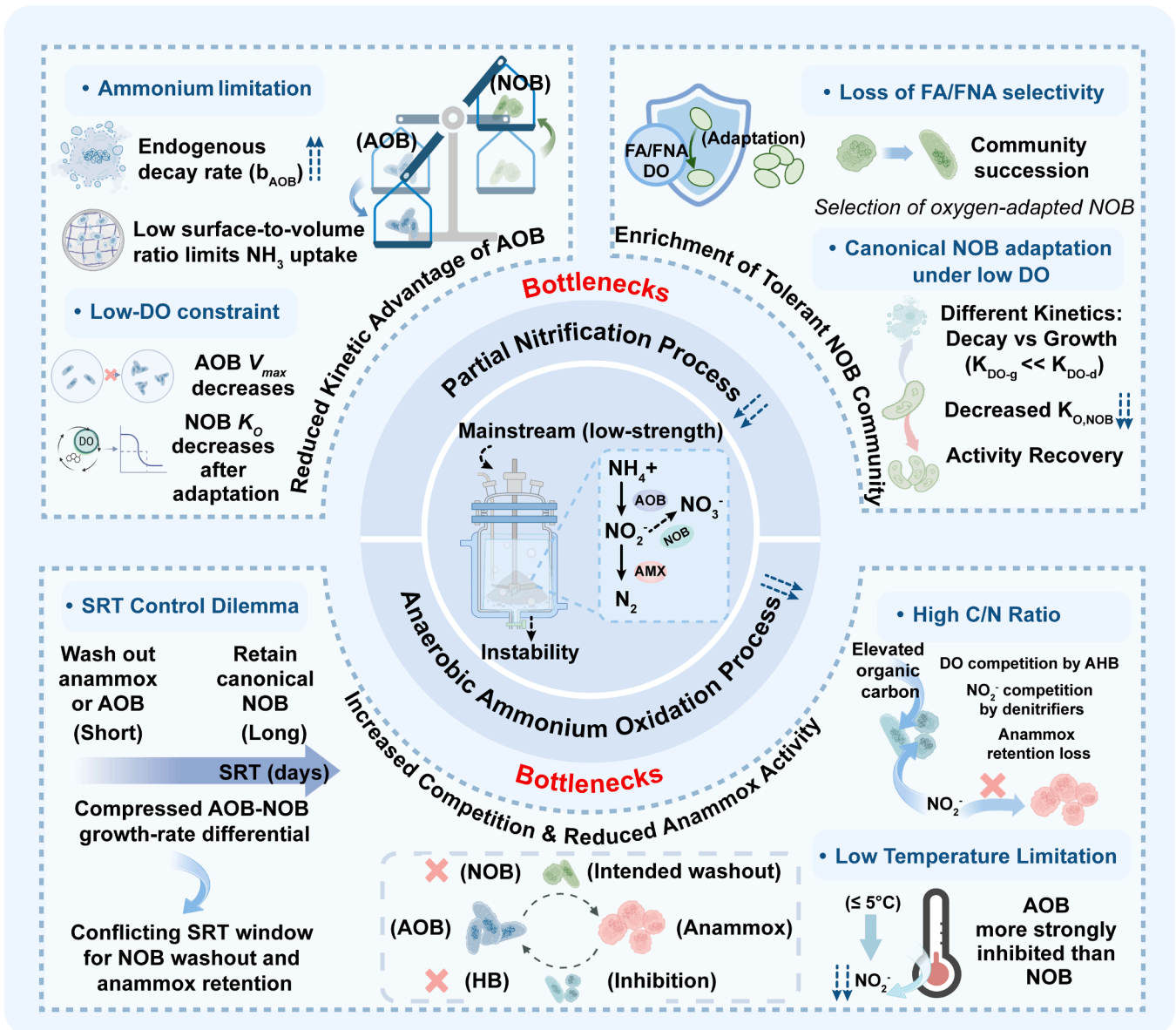


Fig. 1. System-level bottlenecks of AOB-dominated mainstream one-stage PN/A under low-strength and variable wastewater conditions, highlighting AOB decay, adaptive canonical NOB rebound, and constrained nitrite availability to anammox.

2016). The relatively low surface-area-to-volume ratio of typical AOB cells additionally restricts ammonia uptake under substrate-limited conditions. For example, representative AOB such as *N. oligotropha* have a cell volume approximately fiftyfold greater than that of other high-affinity ammonia oxidizers such as *Ca. Nitrosotenuis uzonensis*, whereas their surface area is only about tenfold greater. This size-related allometric mismatch reduces the membrane area available for ammonia transport relative to cellular volume and delays activity recovery even when ammonium concentrations transiently increase (French et al., 2021). At the reactor scale, these physiological constraints impair AOB retention. Low growth rates under substrate limitation restrict the formation of dense granules and stable biofilms, and AOB persist as loosely aggregated flocs that are vulnerable to hydraulic shear-induced washout (Chen et al., 2019). Active AOB biomass declines progressively, and mainstream nitrification becomes increasingly dependent on small and poorly retained AOB populations.

Ammonia limitation is further reinforced by the oxygen-restricted conditions required in mainstream PN/A. To maintain anammox activity, mainstream PN/A reactors are typically operated at DO concentrations below 1.0 mg/L, which imposes an additional physiological constraint on AOB. Under these conditions, the maximum specific oxygen uptake activity of AOB ($V_{max,AOB}$) declines sharply. Reported *Nitrosomonas* $V_{max,AOB}$ values decrease from 17.24 to 3.72 mg O₂/(gVSS-h) when DO is reduced from oxygen-replete conditions to 0.3-0.8 mg/L (Duan et al., 2024). The decline in maximum oxygen uptake activity is not accompanied by an adaptive increase in AOB oxygen affinity. The apparent oxygen half-saturation constant of *Nitrosomonas* AOB ($K_{O,AOB}$) remained at 0.21 mg/L under prolonged oxygen limitation (Wang et al., 2023). Therefore, low-DO aeration exacerbates ammonium limitation by imposing a separate respiratory bottleneck, accelerating the loss of active AOB biomass and further destabilising AOB-mediated nitrification in mainstream PN/A.

2.2. Mainstream conditions compromise the operational selectivity of conventional NOB suppression

After substrate limitation weakens AOB-dependent nitrification, the selectivity of low-DO aeration becomes a critical constraint on mainstream PN/A stability. Low-DO operation, including maintaining DO at 0.2-1.0 mg/L or intermittent aeration, is widely used as a NOB-control strategy in PN/A systems. However, long-term reactor evidence from mainstream low-DO operation indicates that oxygen restriction progressively undermines the selective advantage of AOB over canonical NOB. The shift in selective pressure reflects differences in the oxygen dependencies of growth and decay between NOB and AOB. In the kinetic model reported by Liu and Wang (2015), the half-velocity constant for NOB decay ($K_{DO,d} = 0.69$ mg/L) was much higher than that for NOB growth ($K_{DO,g} = 0.08$ mg/L). As DO declines, NOB decay is reduced more strongly than NOB growth. At 0.3 mg/L DO, the NOB growth inhibition factor remained as high as 0.79, indicating only moderate growth suppression. By contrast, the corresponding AOB constants showed less separation between growth and decay responses ($K_{DO,g} = 0.29$ mg/L, $K_{DO,d} = 0.48$ mg/L). Declining DO conditions constrain AOB growth without providing a comparable retention advantage, limiting the selective retention of active AOB biomass (Fig. 1).

The growth-decay imbalance provides a kinetic basis for canonical NOB persistence under low-DO operation. During prolonged exposure, low-DO conditions promote the retention and selection of oxygen-adapted NOB populations with increased oxygen affinity. When DO was reduced to 0.2 mg/L, the apparent half-saturation constant of NOB ($K_{O,NOB}$) decreased by 62%, from 1.01 ± 0.17 mg/L to 0.38 ± 0.05 mg/L (Duan et al., 2024). This shift improves oxygen acquisition and supports continued nitrite oxidation under oxygen-limited conditions. Consistent with this oxygen affinity shift, in situ NOB volumetric activity recovered from 43% to 77% of the initial level during sustained low-DO exposure. The functional recovery was accompanied by the

community-level enrichment of adaptive *Nitrospira* NOB. As oxygen limitation persists, NOB biomass increases threefold from 0.02 to 0.06 mg-cell/mg-N, whereas AOB biomass increases only twofold, from 0.05 to 0.10 mg-cell/mg-N (Wang et al., 2021). At the community level, *Nitrospira* relative abundance increases nearly 20-fold, whereas AOB abundance rises by only approximately 20% (Wang et al., 2021). These observations indicate that low-DO aeration mainly imposes transient activity suppression rather than durable NOB exclusion. Under sustained oxygen restriction, canonical NOB are retained and partially recover their nitrite-oxidation activity despite reduced total nitrification.

FA/FNA inhibition provides only limited compensation selectivity for NOB control during low-DO aeration under mainstream conditions (Fig. 1). Reported thresholds for selective NOB inhibition are 5-15 mg NH₃-N/L for FA and 1.6-4.2 mg HNO₂-N/L for FNA (Duan et al., 2019), whereas FA concentrations in mainstream sewage typically remain below 2 mg-N/L because of low ammonium concentrations (Van Tendeloo et al., 2021). FNA accumulation is likewise constrained because attenuated AOB-driven nitrification limits nitrite availability, keeping FNA below 1 mg-N/L (Feng et al., 2024). Below selective inhibition thresholds, FA/FNA exposure coincides with declining nitrite accumulation and NOB retention rather than sustained NOB exclusion. In a membrane-aerated biofilm reactor, operation below the FA/FNA threshold failed to maintain nitrite accumulation, as reflected by a decline in nitrite accumulation rate (NAR) from 92.9% to 81.7% (Wang et al., 2023). Consistently, a low FA concentration of 0.37 mg-N/L shortened the NOB doubling time from 5.4 to 1.7 days, placing NOB growth within typical mainstream SRT ranges and thereby favouring retention over washout (Wang et al., 2021). Together, low-DO adaptation and insufficient FA/FNA accumulation indicate that conventional NOB suppression is unlikely to provide durable nitrifier selectivity under mainstream PN/A conditions.

2.3. Partial nitrification instability disrupts nitrite partitioning toward anammox under mainstream selection pressures

2.3.1. SRT imposes a selection conflict between nitrifier washout control and anammox biomass retention

Selective NOB washout through short-SRT control (e.g., 4.3-9.7 days) has been demonstrated in sidestream PN/A systems, where the slightly higher maximum growth rate μ_{max} of AOB ($\mu_{max,AOB} = 1.05-1.4$ d⁻¹) relative to NOB ($\mu_{max,NOB} = 0.91-1.31$ d⁻¹) (Munz et al., 2011b) provides a kinetic basis for selective washout. However, under mainstream conditions, reduced substrate availability lowers the maximum substrate utilization capacity V_{max} in both groups, with AOB undergoing a larger reduction than NOB (Duan et al., 2024). Mainstream conditions narrow the growth-rate differential between AOB and NOB, restricting the SRT operating range for selective NOB washout (Zhi et al., 2023). Short-SRT control requires coordinated regulation of substrate availability and biomass retention to preserve AOB-mediated nitrification while limiting NOB accumulation (Fig. 1).

Anammox biomass retention imposes a competing SRT requirement. Because anammox bacteria have a low maximum specific growth rate ($\mu_{max,AMX} = 0.0027$ h⁻¹), their stable retention requires SRT values longer than 14.3 days, and reported stable-operation studies have commonly maintained SRTs of 20-40 days or more (Ma et al., 2025). However, such extended SRTs also favour the retention of K-strategist canonical *Nitrospira* NOB, which are well adapted to low-substrate mainstream environments (Zaman et al., 2021). Enrichment of *Nitrospira* diverts nitrite flux toward nitrate production, constraining nitrite availability for anammox. Stabilizing mainstream PN/A requires a selection strategy that sustains ammonia oxidation, constrains canonical NOB activity, and preserves nitrite availability for anammox under long-SRT operation.

2.3.2. Low temperatures and elevated C/N ratios constrain anammox nitrite availability through impaired nitrification and heterotrophic competition

Low temperature constrains AOB more strongly than canonical NOB and further limits the nitrite supply to anammox. Within the conventional nitrification temperature range of 7.2–28.3°C, AOB and NOB exhibit comparable temperature response coefficients of 1.099 and 1.098, respectively (Zhang et al., 2014). However, when temperature drops below 5 °C, their temperature sensitivities diverge. Young et al. (2017) reported that single-cell ammonia-oxidizing activity (VAOBRR) retained only 6% of the mesophilic-temperature activity in AOB, compared with 13% single-cell nitrite-oxidizing activity (VNOBRR) in NOB. The observed specific activity at low temperatures reaches only 30.4%–37.9% of the values predicted by the Arrhenius van't Hoff equation (Wu et al., 2016), indicating cold-induced impairment beyond thermodynamic rate limitation. The molecular response also fails to compensate for the loss of ammonia-oxidation activity under low-temperature exposure. The decoupling was reflected by a 1.2-fold increase in *amoA* transcript abundance despite a threefold decline in ammonia oxidation activity (Young et al., 2017). During longer-term operation, AOB *amoA* gene abundance has been reported to decrease from 1.8×10^8 to 0.2×10^8 copies/g (Jiang et al., 2023). This decline indicates weakened AOB dominance and reduced nitrification capacity, limiting the effectiveness of recovery through aeration or substrate adjustment alone.

Elevated influent carbon-to-nitrogen (C/N) ratios constitute a system-level constraint in mainstream PN/A. The C/N range generally considered favourable for PN/A stability is 0.6–1.2 (Regmi et al., 2014), whereas municipal sewage commonly fluctuates between 2.3 and 12.0 (Zhang et al., 2015). Under these carbon-enriched conditions, heterotrophic proliferation constrains PN/A by reducing oxygen availability for AOB and redirecting nitrite away from anammox. Aerobic heterotrophic bacteria (AHB) compete with AOB for dissolved oxygen, which weakens AOB-driven nitrite production. As the C/N ratio rises to 1.5, the AHB community becomes enriched in high oxygen-affinity taxa such as *Rubrivivax* and *Haliscomenobacter*, with $K_{O,AHB}$ decreasing from 0.39 to 0.27 mg/L, lower than the reported $K_{O,AOB}$ values of 1.4 to 0.54 mg/L (Cho et al., 2022). The resulting oxygen-uptake advantage has been associated with a 20% decrease in the ammonia oxidation rate (AOR) (Zhang et al., 2024). Heterotrophic denitrifiers compete with anammox for nitrite when organic carbon is available, because their reported μ_{max} of 1.2 d⁻¹ exceeds the 0.065–0.33 d⁻¹ range for anammox (Bi et al., 2015; Li et al., 2020), while excessive heterotrophic growth disrupts granule or biofilm structure and reduces anammox biomass retention. When the C/N ratio exceeds 2.5, denitrifiers proliferation converts dense granules into loose flocs and increased sludge volume index (SVI) (Miao et al., 2018). The deterioration in settleability reduces effective biomass retention and restricts the niche space available for anammox growth. Therefore, elevated C/N destabilizes mainstream PN/A by simultaneously suppressing AOB-driven nitrite production, diverting nitrite toward denitrification, and weakening the biomass-retention conditions required for anammox activity.

3. Comammox enrichment as niche-based regulation in mainstream PN/A

3.1. Low ammonium availability favours comammox participation in ammonia oxidation

In mainstream wastewater with low ammonia concentrations, constrained AOB activity undermines the stability of ammonia oxidation during the partial nitrification step of PN/A process (Duan et al., 2023; Wang et al., 2020). Comammox bacteria have been proposed as an alternative ammonia-oxidizing guild because their kinetic and physiological traits differ from those of canonical AOB under ammonia-limited conditions (Pinto et al., 2016; Vilardi et al., 2024). This role is primarily

supported by their high apparent ammonia affinity. Candidatus *Nitrospira inopinata* and Candidatus *Nitrospira kreftii* exhibit apparent half-saturation constants for ammonia with $K_{m(app),NH_3}$ values of 0.040 to 0.063 $\mu\text{mol-N/L}$ (Kits et al., 2017). These values are approximately 480 to 3,800 times lower than those reported for cultured *Nitrosomonas* AOB, typically 30–150 $\mu\text{mol N/L}$ (Costa et al., 2006) (Fig. 2). This higher ammonia affinity supports comammox-mediated ammonia oxidation at substrate concentrations that constrain canonical AOB activity. Persistence under substrate limitation is also consistent with the lower decay coefficient (*b*) reported for *N. inopinata* (0.05 d⁻¹) compared with canonical *Nitrosomonas* AOB (0.15 d⁻¹) (Mehrani et al., 2021). The lower maximum specific growth rate μ_{max} of comammox bacteria relative to AOB (0.15 d⁻¹ compared with 0.57 d⁻¹ for AOB) further reflects a K-strategist growth pattern that favours persistence under low ammonium availability.

In addition to these kinetic traits, comammox bacteria encode Rh-type ammonia transporters rather than the *AmtB*-type transporters commonly associated with canonical AOB (Palomo et al., 2018). Molecular simulations suggest that hydrogen-bonding interactions within ammonium-binding sites in comammox strengthen substrate binding under low ammonium availability (Guo et al., 2024). These ammonia acquisition systems help explain the high apparent ammonia affinity observed in cultured comammox. However, their relevance under fluctuating mainstream loads depends on their contributions to ammonia oxidation in reactors.

Reactor observations support comammox participation in ammonia oxidation under low-ammonium mainstream conditions. In an integrated fixed-film activated sludge (IFAS) system treating municipal wastewater with 20–40 mg-N/L ammonia, comammox bacteria accounted for more than 75% of overall ammonia oxidation activity (Vilardi et al., 2023). Ammonia oxidation remained stable after AOB inhibition by 1-octyne, while nitrite accumulation ratio reached 44.8–66.7%. The persistence of ammonia oxidation after 1-octyne inhibition supports comammox contribution to ammonia oxidation, whereas the observed nitrite accumulation ratio indicates transient extracellular nitrite release in the comammox-enriched system. Importantly, this nitrite accumulation suggests that complete ammonia oxidation at the cellular level does not necessarily translate into immediate stoichiometric nitrate formation at the reactor scale. Instead, when ammonia oxidation exceeds subsequent nitrite oxidation, a transient nitrite fraction become available for partitioning toward anammox.

3.2. Comammox niche occupation as a durable ecological constraint on canonical NOB in mainstream PN/A

During mainstream PN/A operation, conventional NOB suppression is difficult to sustain because low-DO aeration and FA/FNA inhibition provide only conditional and often transient control. Comammox enrichment offers an alternative route based on ecological niche occupation. Under low ammonium availability, oxygen limitation, and attached-growth biomass retention, comammox populations can be selectively retained while the ecological niche space available to canonical NOB is correspondingly reduced.

Mainstream PN/A imposes selection pressures that preferentially retain nitrifiers with high substrate affinity and strong persistence traits. Comammox *Nitrospira* can occupy low-substrate nitrifying niches that would otherwise support canonical NOB, while maintaining ammonia oxidation. This mechanism reframes NOB control from transient activity inhibition toward niche-based competition within the *Nitrospira* lineage. Evidence from full-scale systems supports this interpretation (Fig. 3). Wastewater comammox MAGs are frequently associated with the *Ca. Nitrospira nitrosa* cluster and exhibit lower strain-level diversity than strict AOB and most canonical *Nitrospira* NOB. This pattern is consistent with selective adaptation of specific comammox lineages to mainstream nitrogen-removal systems (Cotto et al., 2020). Their persistence is further supported by higher growth yield, metabolic versatility, and

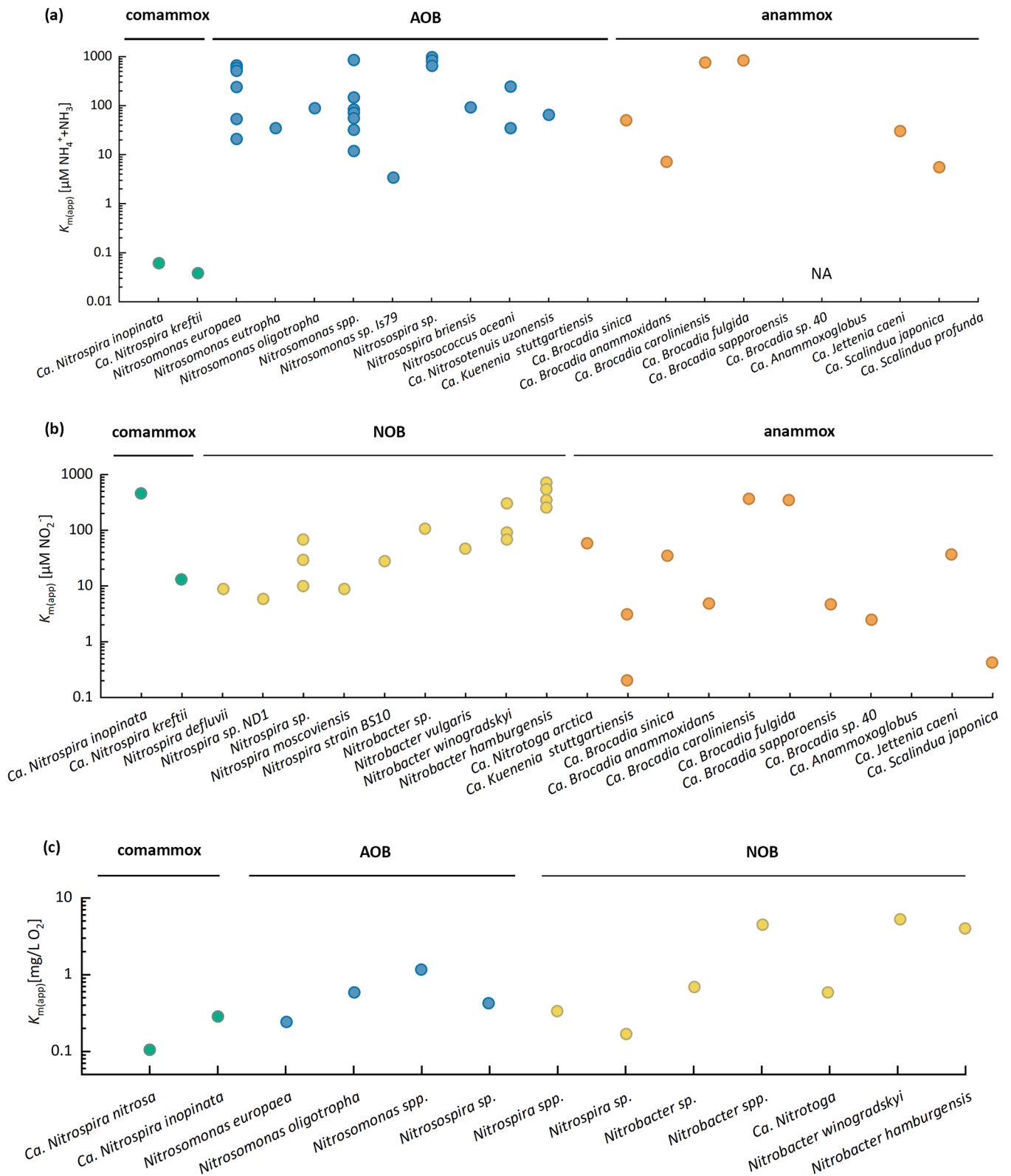


Fig. 2. Reported apparent half-saturation constants ($K_{m(app)}$) for ammonium (a), nitrite (b), and oxygen (c) among comammox bacteria, ammonia-oxidizing bacteria (AOB), anammox bacteria, and canonical nitrite-oxidizing bacteria (NOB). These kinetic values indicate potential substrate-affinity differences under defined experimental conditions and should be interpreted together with reactor-scale factors such as substrate concentration, mass transfer, biomass structure, and residual NOB activity.

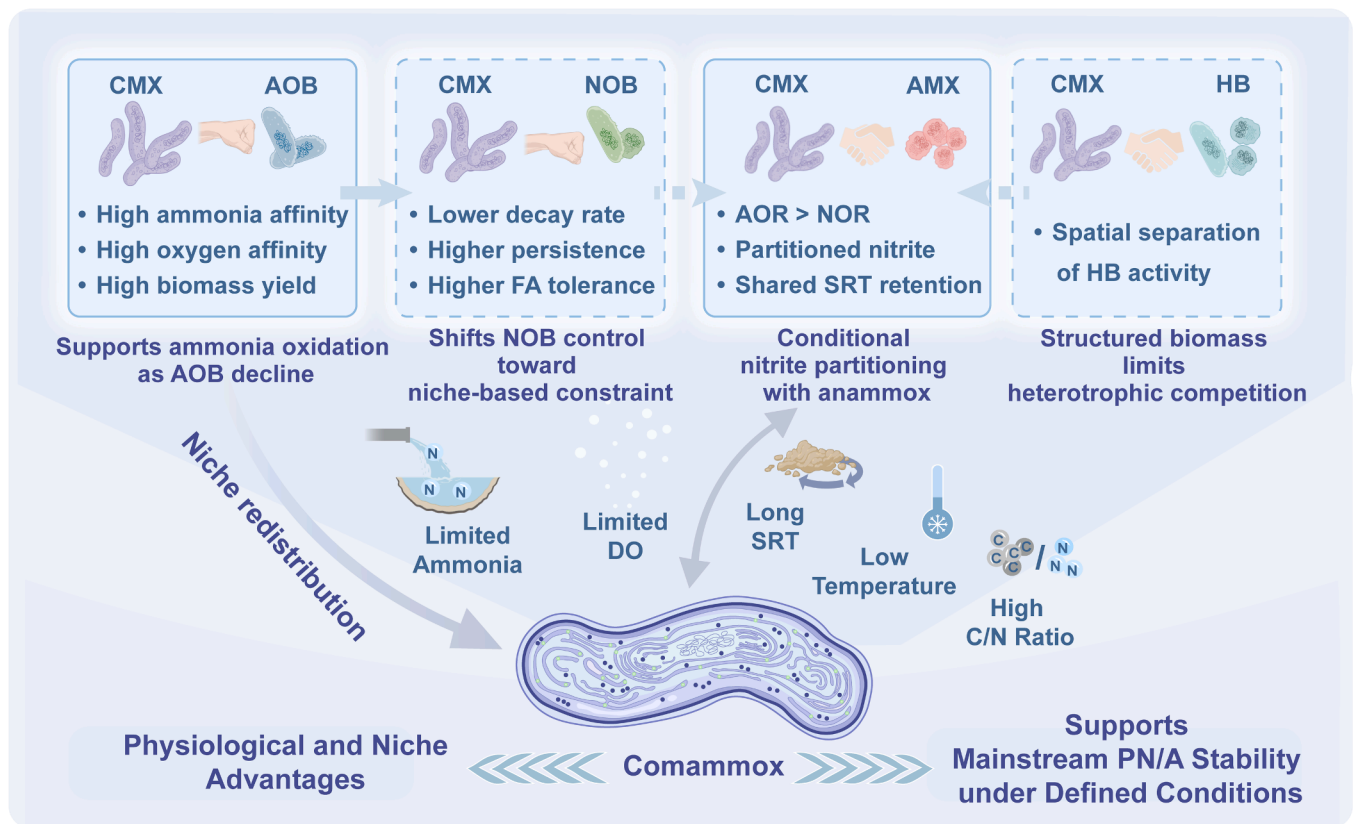


Fig. 3. Conceptual framework for comammox-mediated niche redistribution in anammox-dominated one-stage mainstream PN/A. The framework links comammox-mediated ammonia oxidation, niche-based constraint of canonical NOB, nitrite partitioning with anammox, and biomass-structured mitigation of heterotrophic competition.

differences in ammonia and nitrite affinity relative to canonical nitrifiers. Together, these traits provide an ecological basis for sustained comammox occupation of low-substrate niches that restricting canonical NOB establishment.

3.2.1. Kinetic and physiological traits favour comammox over canonical NOB under low-DO selection

The kinetic basis for comammox persistence relative to canonical NOB under low-DO mainstream operation has been characterised through comparisons of nitrite-oxidizing communities (Brotto et al., 2025). In a comammox-enriched bioreactor, the mixed nitrite-oxidizing community exhibited an apparent oxygen half-saturation constant K_O of 0.11 mg O₂/L, compared with 0.19 mg O₂/L in a canonical NOB-dominated guild (Mei et al., 2023). The lower K_O is consistent with higher apparent oxygen affinity under sustained oxygen limitation. A similar comparison reported a lower decay coefficient for the comammox-enriched nitrite-oxidizing community (0.09 d⁻¹) than for the canonical NOB-dominated community (0.14 d⁻¹). This difference indicates greater biomass persistence under prolonged low-DO operation. In addition, the maximum specific growth rate μ_{max} of cultivated comammox bacteria was 0.20 ± 0.01 d⁻¹, comparable to that of canonical NOB (Mehrani et al., 2022). Under low-DO mainstream conditions, lower endogenous decay and higher apparent oxygen affinity support comammox retention even when growth-rate advantages are limited.

The physiological basis for the kinetic pattern involves respiratory adaptation and energy-conserving metabolism. Comammox bacteria encode cytochrome *bd* oxidase, whose heme *d* cofactor confers a high oxygen-binding affinity and enables aerobic respiration under low-DO conditions (Lawson and Lückner, 2018; Palomo et al., 2018). In contrast, canonical *Nitrospira* NOB typically encode aa₃-type

heme-copper oxidases, which exhibit a lower apparent affinity for oxygen. This respiratory adaptation is coupled with the larger Gibbs energy yield of complete ammonia oxidation (-349 kJ/mol) relative to nitrite oxidation alone by canonical NOB (-74 kJ/mol) (Lawson and Lückner, 2018). The genomic and bioenergetic features provide a mechanistic basis for the preferential retention of comammox over many canonical NOB in oxygen-limited bioreactors.

3.2.2. Comammox-mediated substrate competition strengthens NOB control under low FA conditions

Under low FA/FNA conditions, direct chemical inhibition of canonical NOB is weak, and substrate competition becomes a more important selection mechanism. Mainstream FA concentrations rarely reach the thresholds required for direct NOB inhibition. Under these conditions, comammox enrichment reflects a substrate-competitive advantage over canonical NOB.

Comammox bacteria retain ammonia-oxidizing activity across the FA range typical of mainstream sewage (Fig. 3). Comammox *amoA* expression remained near 90% at 2.9 mg-N/L FA, while canonical NOB *nrxAB* transcript abundance decreased to 56% (Shao and Wu, 2021). Comammox activity declined sharply only when FA exceeded approximately 8.9 mg N/L, compared with pronounced NOB repression above 5.8 mg N/L. This broader FA tolerance range, together with high ammonia affinity, allows comammox to remain active under conditions that weaken most canonical NOB (Ran et al., 2025; Tang et al., 2023). Thus, under defined mainstream selection pressures, comammox bacteria reduce the dominance of adaptive canonical NOB more persistently than FA/FNA-based suppression.

3.3. Long SRT drives competing retention pressures among comammox, anammox, and canonical NOB

The coexistence of comammox and anammox bacteria in mainstream PN/A requires extended SRT because anammox bacteria have low maximum specific growth rates and require long biomass retention for stable maintenance. Comammox bacteria exhibit K-strategist growth kinetics that align with this retention requirement. This shared dependence on extended biomass retention allows comammox-mediated ammonia oxidation and anammox-mediated nitrogen removal to be maintained within the same one-stage reactor.

Reactor studies support the SRT-dependent retention and enrichment of comammox bacteria under mainstream conditions (Fig. 3). Comammox *Nitrospira* were poorly retained at SRTs below 10 days under low-substrate conditions (Mehrani et al., 2023), whereas extending the SRT to 20 days was associated with an increase in their relative abundance to 69% of the *Nitrospira* community (How et al., 2020). In an oxidation ditch reactor, increasing SRT from 23.2 days to 26–60 days was accompanied by a 33-fold increase in comammox *amoA* gene copy number (Park et al., 2021). In a long-term study, comammox bacteria became progressively enriched within the ammonia oxidizers over 215 days of operation. Comammox *amoA* gene copy number reached 50 times that of canonical AOB *amoA*, and the relative abundance of comammox *Nitrospira* was 13 times higher than that of AOB *Nitrosomonas* (Hou et al., 2024; Roots et al., 2019). These reactor-scale

patterns are consistent with the slow-growing strategy of comammox, including their relatively low μ_{max} (0.15–0.20 d⁻¹) and higher biomass yields relative to canonical AOB (Kits et al., 2017; Mehrani et al., 2022). The reported patterns indicate that the SRT range required for anammox retention is compatible with comammox enrichment, but long-SRT operation can also retain canonical *Nitrospira* NOB when additional niche-control mechanisms are absent.

3.4. Rate imbalance between comammox ammonia oxidation and nitrite oxidation enables nitrite partitioning toward anammox

Despite the nitrite-oxidizing capacity of comammox encoded by *nxrAB* genes, comammox-derived nitrite can become available extracellularly when intracellular ammonia oxidation exceeds subsequent nitrite oxidation. This rate imbalance allows comammox activity to coexist with sustained anammox function in comammox-enriched mainstream PN/A systems (Fig. 4). Therefore, Their interaction depends on whether released nitrite is taken up by anammox before being reoxidized to nitrate by comammox.

The mechanistic basis for this partitioning is the rate imbalance between ammonia oxidation and nitrite oxidation within comammox cells. For *Nitrospira inopinata*, the maximum specific ammonia oxidation rate reached 2.57 mg N/(gVSS-h), exceeding the corresponding nitrite oxidation rate of 1.56 mg N/(gVSS-h) (Wang et al., 2020). This rate differential means that nitrite production transiently exceeds

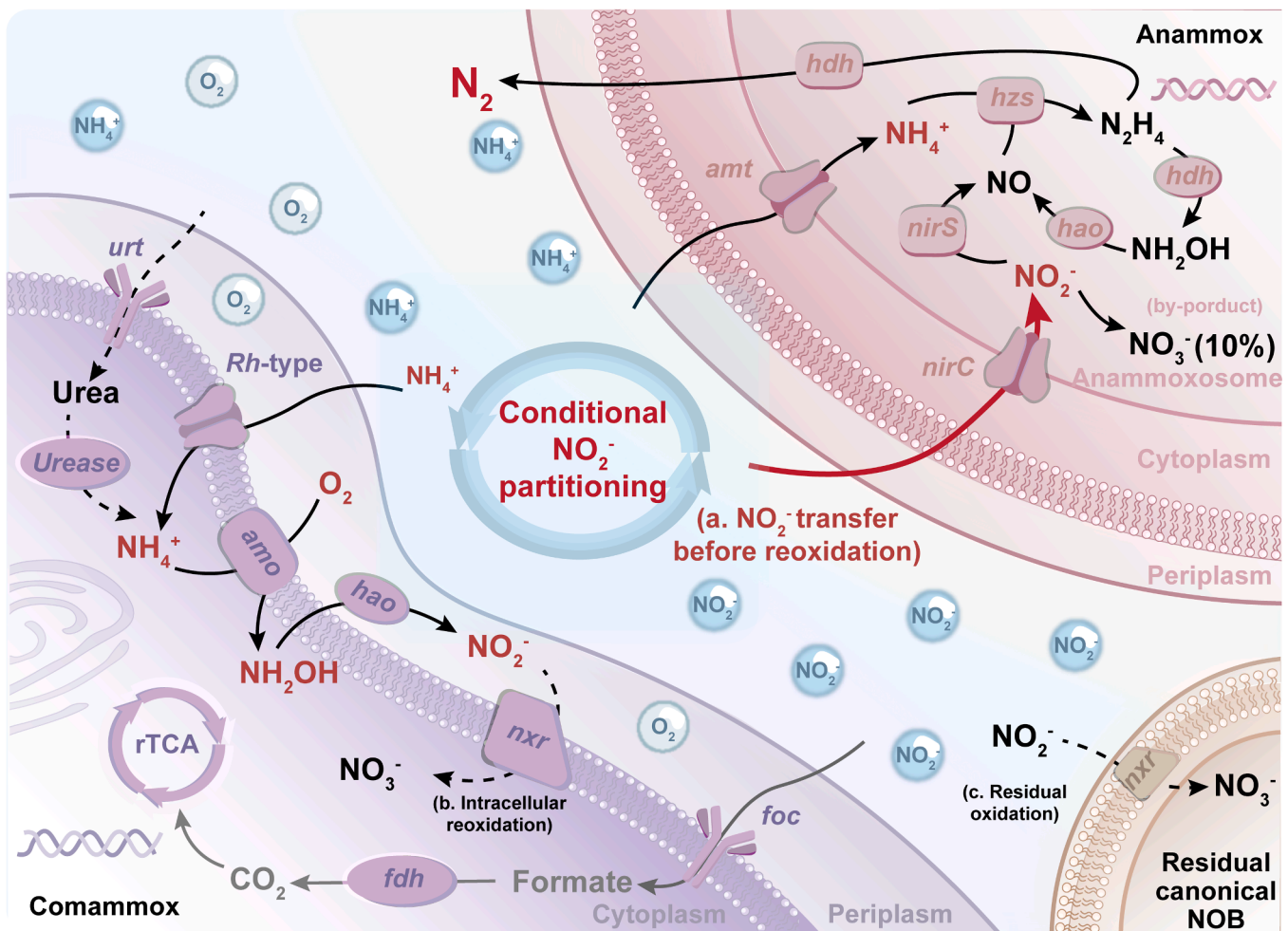


Fig. 4. Boundary-defined nitrite partitioning between comammox and anammox bacteria in one-stage mainstream PN/A systems. Comammox-derived nitrite is transferred to anammox, reoxidised by comammox, or further oxidised by residual canonical NOB depending on spatial proximity, diffusion, oxygen distribution, and community composition.

intracellular nitrite oxidation, generating a transient intracellular nitrite pool. A portion of this pool is then released to the extracellular space before further intracellular oxidation occurs, providing the cellular and kinetic prerequisite for anammox access to comammox-derived nitrite.

Strain-level kinetic variation further limits generalization based solely on apparent nitrite affinity. The apparent nitrite affinity differs markedly among cultured comammox species. *Nitrospira inopinata* has a high apparent nitrite half-saturation constant, with a $K_{m(app),NO_2}$ value of 449.2 $\mu\text{mol/L}$, whereas *Nitrospira kreffii* has a much lower $K_{m(app),NO_2}$ value of 12.5 $\mu\text{mol/L}$ (as summarized in Fig. 2) (Kits et al., 2017; Sakoula et al., 2021). Reported $K_{m(app),NO_2}$ values for anammox *Candidatus Brocadia* range from 0.761 to 2.99 $\mu\text{mol/L}$ (Mehrani et al., 2023; Zhu et al., 2024). These values indicate that anammox has a pronounced advantage in nitrite uptake over low-affinity comammox strains such as *N. inopinata*. However, this advantage becomes less pronounced in the presence of comammox strains with higher nitrite affinity, such as *N. kreffii*. Therefore, comammox strain composition becomes a key determinant of nitrite partitioning, and limited strain-level resolution in mainstream reactor communities restricts kinetic interpretation of nitrite partitioning based on apparent nitrite affinity alone.

Reactor observations are consistent with this nitrite-partitioning interpretation (Fig. 4). Early comammox studies identified *Nitrospira nitrosa* and *Nitrospira nitrificans* as complete ammonia oxidizers in engineered enrichment systems, establishing their capacity for complete nitrification rather than demonstrating direct nitrite transfer to anammox bacteria (Daims et al., 2015; van Kessel et al., 2015). Reactor studies detected comammox colonisation in full-scale moving bed biofilms under low nitrogen loading (Suarez et al., 2024). Comammox abundance also increased along rotating biological contactors as ammonia availability declined (Zheng et al., 2022). In one-stage PN/A reactors, substantial ammonia oxidation by comammox was observed when anammox-mediated nitrogen removal reached $70.1 \pm 2.7\%$ (Shao and Wu, 2021). These observations indicate that comammox activity is compatible with sustained anammox function.

3.5. Comammox integration constrains nitrifier-associated N_2O formation through pathway-specific mechanisms

Mainstream PN/A systems generate N_2O through multiple nitrogen-conversion pathways, with the dominant pathway varying across reactor configurations and operating conditions. In PN/A reactors, N_2O production is commonly associated with the partial nitrification stage. In one PN/A bioreactor, 97.5% of N_2O originated from the partial nitrification reactor (Okabe et al., 2011b). In a single-stage PN/A system, isotopic analysis assigned approximately 73% of N_2O production to nitrifier denitrification and 27% to hydroxylamine oxidation (Chen et al., 2020). These observations identify AOB nitrifier denitrification and hydroxylamine (NH_2OH) oxidation as the main nitrifier-associated N_2O formation pathways in PN/A systems (Terada et al., 2017). In structured PN/A biomass, anaerobic microzones with residual organic carbon penetration also contribute to additional N_2O formation by supporting heterotrophic denitrification within anammox granules or biofilms, whereas abiotic conversion of extracellular NH_2OH provides an additional N_2O source (Duan et al., 2024). Comammox integration can reduce N_2O formation by decreasing the contribution of canonical AOB nitrifier denitrification and hydroxylamine oxidation.

The reduced N_2O potential of comammox bacteria is supported by genomic and physiological evidence. Comparative genomic analyses indicate that *Nitrospira inopinata* and other characterized comammox *Nitrospira* lack the canonical NO reductase systems required for enzymatic NO reduction to N_2O , constraining the denitrification route typically associated with canonical AOB (Kits et al., 2019). In addition, cultured and enriched *N. inopinata* lack cytochrome P460 (*cytL*), an enzyme implicated in N_2O formation from hydroxylamine in canonical AOB. Accordingly, the N_2O detected from *N. inopinata* is attributed mainly to abiotic conversion of extracellular NH_2OH , rather than to

canonical nitrifier denitrification. Han et al. (2021) reported similar trends, with lower N_2O and NO_y production by *N. inopinata* than by canonical AOB. The reduced emission has been attributed to differences in hydroxylamine turnover associated with hydroxylamine oxidoreductase.

The mechanistic distinction is reflected in N_2O yields at the culture level. In pure culture, *N. inopinata* produced low N_2O during ammonia oxidation, with yields of $0.07 \pm 0.01\%$ (Kits et al., 2019) and about 0.1–0.2% under different substrate conditions reported by Han et al. (2021). NH_2OH addition assays further showed that the $N_2O/\text{NH}_2\text{OH}$ yield of *N. inopinata* was $0.63 \pm 0.05\%$, lower than the yields measured for canonical AOB such as *Nitrosomonas nitrosa* ($2.17 \pm 0.22\%$) and *Nitrosomonas europaea* ($7.14 \pm 0.25\%$) (Han et al., 2021). Reactor and modelling evidence extend this low- N_2O pattern to engineered nitrification systems. In a comammox-enriched nitrifying reactor, the N_2O emission factor was 0.35%, compared with 2.2% in a canonical AOB-enriched reactor, and N_2O emissions decreased as the relative abundance of comammox increased within the ammonia-oxidizing community (Ren et al., 2024). In a weakly acidic low-DO nitrifying reactor, a 20% reduction in N_2O yield coincided with an increase in the comammox/AOB *amoA* ratio from 6.6 to 17.1 (Li et al., 2021). Model analysis of granular PN/A similarly predicted minimal N_2O production of 0.1% in a comammox-mediated PN/A configuration, compared with 1.7–2.8% in a canonical AOB-mediated PN/A configuration, although the predicted reduction depended strongly on DO and influent ammonium control (Zhu et al., 2024). Together, these findings support comammox enrichment as a potential strategy for reducing nitrifier-derived N_2O formation when comammox dominate over canonical AOB within the ammonia-oxidizing community. However, effective N_2O mitigation requires identifying the dominant N_2O production pathways under specific operating conditions. These pathways include nitrifier-driven hydroxylamine oxidation, abiotic conversion of extracellular NH_2OH , and heterotrophic denitrification, with their relative importance governed by DO, C/N, and biomass structure.

4. Translating comammox–anammox coupling from conceptual integration to mainstream PN/A implementation

For engineering implementation, comammox enrichment should be treated as an operationally conditional strategy for shaping the nitrifying-community structure, rather than as an independent treatment solution for mainstream PN/A. Comammox enrichment reshapes ammonia oxidation, competition with canonical NOB, and nitrite partitioning to anammox, but these effects remain constrained by kinetic traits, spatial organization, and community composition. Further development should integrate reactor-configuration design, residual nitrate control, pathway-resolved molecular detection, nitrogen flux validation, and process modelling under fluctuating mainstream loading conditions.

4.1. Operational constraints for mainstream comammox–anammox PN/A

4.1.1. Limited selectivity of long SRT for enriching comammox over canonical *Nitrospira* NOB

The key limitation is that extended SRT also supports the retention of canonical NOB *Nitrospira*. This constraint represents an operational limitation for concurrent comammox–anammox retention, rather than a defining feature of long-SRT operation. Selective enrichment of comammox is more consistently associated with extended SRT when combined with sustained low DO and ammonium loading (Gottshall et al., 2021; Vilardi et al., 2023; Park et al., 2021). These patterns show that extended SRT alone does not determine selection within the nitrite-oxidizing community. Prolonged oxygen and FA/FNA limitation also selects for NOB populations with enhanced oxygen affinity and stress tolerance. Under these conditions, comammox and canonical NOB

co-occur and exhibit temporal variation in dominance (Suarez et al., 2024). Thus, long SRT establishes the potential retention of comammox and anammox, whereas DO and substrate availability govern the persistence of canonical NOB as active competitors within the same functional guild.

4.1.2. Low-temperature operation requires direct verification of comammox activity

Low temperature reinforces the ecological advantage of comammox in mainstream PN/A by suppressing AOB-mediated nitrification. This creates a niche for comammox as an alternative ammonia-oxidizing guild under cold mainstream conditions, although direct evidence from reactor-scale PN/A systems remains limited.

Environmental evidence supports this temperature-associated niche differentiation. In groundwater, temperature explained 31.17% of the variation in comammox community structure, and comammox *Nitrospira* relative abundance increased as temperature declined to 5–10 °C (Zhang et al., 2020). In oligotrophic upstream environments, comammox clade A and clade B accounted for 34–87% of total ammonia oxidizers and exceeded the AOB contribution at low temperature (Liu et al., 2020). Across environmental samples, comammox abundance was negatively correlated with temperature, with Spearman's r values of -0.63 to -0.75 (Tang et al., 2023). Tang et al. (2023) also detected active ammonia oxidation by comammox clade B in freeze-thawed wetland soil, alongside greater comammox co-occurrence network complexity below 15 °C. These observations establish environmental relevance, but not reactor-scale functional stability in mainstream PN/A systems.

The primary unresolved issue is stable performance of comammox ammonia oxidation and anammox activity at reactor scale. Hydrogel immobilisation of comammox–anammox biomass achieved $89.4 \pm 4.3\%$ nitrogen removal at 5 °C in laboratory-scale operation (Li et al., 2023), indicating that engineered spatial organisation partially mitigates low-temperature kinetic constraints. However, performance under seasonal temperature variation, variable loading, and long-term biomass retention remains unverified. Therefore, implementation in cold climates should be regarded as promising but insufficiently validated for full-scale mainstream PN/A application.

4.1.3. High C/N operation requires spatial separation of heterotrophs and anammox niches

Elevated influent C/N represents a critical operational constraint on mainstream PN/A, because municipal sewage often has C/N ratios of 2.3 to 12.0, exceeding the 0.6 to 1.2 range generally considered favourable for stable PN/A operation (Xu et al., 2020). Under elevated organic carbon conditions, heterotrophic bacteria (HBs) compete with anammox bacteria for nitrite (Koch et al., 2019). Comammox enrichment alone cannot resolve this competition because it does not prevent heterotrophic denitrification-driven nitrite consumption before nitrite reaches anammox-active zones. Maintaining comammox-mediated ammonia oxidation and anammox nitrite access under elevated organic carbon conditions requires reactor designs that spatially separate heterotrophic activity from comammox and anammox activity.

Coexistence among comammox bacteria, HBs, and anammox bacteria has mainly been demonstrated in biofilms structured by redox and substrate gradients. Such organisation separates aerobic organic carbon oxidation from microaerobic ammonia oxidation and anaerobic anammox activity, thereby limiting the exposure of anammox to organic carbon while preserving comammox activity (Miao et al., 2016; Koch et al., 2019). In MABR systems operated at a C/N ratio of 6, this configuration supported approximately 81.3% total nitrogen removal through coupled nitrification and denitrification pathways (Cui et al., 2025). In sequencing batch biofilm reactors operated at C/N ratios of 2 to 4, the maximum specific activity of anammox increased 4.8-fold, accompanied by improved biomass retention and extracellular polymeric substance production (Li et al., 2022).

Current reactor evidence supports this coexistence mainly at C/N

ratios of approximately 2 to 6, but this range is configuration-dependent. At higher organic loading, anammox retention and nitrite availability are compromised, particularly in systems without stable biofilm or aggregate structure. Therefore, reactor-specific validation is needed to define organic-loading limits, oxygen-transfer regimes, and nitrite-partitioning stability under elevated C/N conditions.

4.2. Reactor configurations for biomass retention and spatial organization of comammox and anammox

4.2.1. Structured carriers and hydrogel matrices for spatial biomass retention

The implementation of comammox–anammox systems requires reactor media that retain slow-growing biomass while maintaining oxygen and substrate gradients required for stable nitrite partitioning. Low dissolved oxygen and extended SRT favour comammox enrichment, but competition with canonical *Nitrospira* NOB and functional coupling with anammox depend on controlled retention and spatial organization of attached biomass. Current biofilm models have not yet fully resolved this design space for mainstream PN/A (Suarez et al., 2024; Wang et al., 2021; Zhao et al., 2022), so carrier selection remains a central engineering determinant.

Hybrid biofilm systems provide the most established platform for this purpose. IFAS systems combine suspended sludge activity with attached growth niches. In IFAS reactor, comammox accounted for approximately 71% of ammonia oxidation in the attached phase, compared with 29% in the suspended phase (Vilardi et al., 2024). Likewise, sequencing batch biofilm reactors (SBBRs) with foam cube carriers have achieved near-complete COD and nitrogen removal with shorter start-up periods than conventional MBBR-based PN/A systems (Zhao et al., 2022). These findings indicate that attached growth can stabilize biomass retention and improve mass-transfer conditions for comammox–anammox operation.

Gel immobilisation provides stronger spatial control by entrapping target biomass within defined structure. Comammox and anammox gel beads have sustained ammonia removal after start-up periods of 60 to 86 days (Gottshall et al., 2021; Li et al., 2023), while biofilm-supported gel systems have achieved simultaneous partial nitrification, partial denitrification, and anammox under mainstream conditions within approximately 40 hours (Zeng et al., 2020). These approaches are especially relevant under low temperature or elevated C/N, where biomass washout and loss of functional segregation are more likely.

Additive manufacturing offers a further route to control carrier geometry. Three-dimensionally printed carriers are designed with defined surface area, pore connectivity, and diffusion length, allowing oxygen penetration and nitrite transfer to be tailored to process requirements. Gyroid and other triply periodic minimal surface structures are attractive because they combine high interfacial area with continuous internal channels. Pilot-scale domestic wastewater treatment with printed polypropylene gyroid carriers has demonstrated stratified biofilm formation and nutrient removal (Nishi et al., 2026), and printable hydrogels provide additional control over porosity and surface functionality (Farsheed et al., 2023). Thus, retention media should be evaluated as spatial-control interfaces rather than passive biomass carriers. Hybrid carriers, gel matrices, and printed media provide increasing control over diffusion length, oxygen exposure, and community localization, which are central to maintaining comammox and anammox activity under variable mainstream loading.

4.2.2. Inoculum selection, substrate regime, and feeding strategy for targeted co-enrichment

Co-enrichment strategies define the initial community composition for nitrite partitioning between comammox and anammox by maintaining both functional groups under low-oxygen and low-substrate conditions (Mehrani et al., 2023). However, because nitrite derived from comammox can be rapidly consumed by anammox or reoxidised by

comammox and canonical NOB, effluent nitrite concentrations cannot resolve nitrite flux from comammox to anammox. Co-enrichment should be interpreted as a prerequisite for functional coupling, rather than direct evidence of established coupling.

The inoculum source determines the initial availability of comammox populations and accelerates their establishment during enrichment. Activated sludge from wastewater treatment plants remains the most common source and has yielded comammox relative abundances of 22.79–42.9% within microbial communities (Hou et al., 2024; Shao, 2024). Acid soil provides an alternative inoculum source with high *amoA* transcriptional activity (Hu et al., 2021). After inoculation into ammonia-fed bioreactors, comammox abundance reached 55.8% (Takahashi et al., 2020), facilitating comammox colonisation.

Substrate composition and feeding mode impose the main selective pressure during initial operation. Urea-containing wastewater is a targeted substrate for comammox enrichment (Vilardi et al., 2024). At 6 mg N/L urea, the relative abundance of comammox *amoA* increased from 50% to 85% (Zhao et al., 2021). Similarly, at 1.5 to 3.5 mg N/L urea, comammox abundance reached approximately twice that of AOB (Vilardi et al., 2022). Urea also offers a substrate advantage for anammox taxa that encode urease genes (*ureABC*) and urea transporters (*urtCDE*), including *Candidatus Scalindua* (Ganesh et al., 2018). Overall, substrate selection can accelerate early-stage co-enrichment, but sustained enrichment still depends on oxygen control, ammonium availability, and biomass retention.

4.3. Partial denitrification-mediated control of residual nitrate accumulation

Strictly autotrophic comammox–anammox PN/A systems generate residual nitrate because nitrate is generated through nitrite oxidation by comammox or residual canonical NOB, as well as through the stoichiometric nitrate by-product (10%) of anammox metabolism (Cui et al., 2021). Under stringent effluent nitrogen requirements, residual nitrate becomes a performance constraint even when ammonia removal and nitrite partitioning remain stable. Endogenous partial denitrification can serve as a supplementary nitrate-recycling pathway for residual nitrate control within PN/A-based systems.

In configurations integrating comammox, denitrifying HBs, and anammox, denitrifying HBs reduce nitrate to nitrite using influent organic carbon (Table 1). The nitrate-derived nitrite is then available for anammox consumption together with nitrite released from comammox (Zhang et al., 2022). Structured simultaneous partial nitrification, partial denitrification, and anammox (SPNDA) systems provide reactor-level evidence for this nitrate-recycling route. For example, Cui et al. (2023) employed an upflow anaerobic sludge blanket (UASB) reactor to achieve nitrogen removal of $92.9 \pm 1.1\%$ with effluent nitrate concentrations below 2 mg/L (Cui et al., 2023). Transcriptional analysis indicated active ammonia and nitrite conversion, supported by elevated expression of *hdh*, *hzo*, *hzsAB*, together with a threefold increase in the *narG/nirK + nirS* ratio. A sequencing batch biofilm reactor achieved 98.0% COD removal and 96.4% nitrogen removal in a stratified biofilm where comammox occupied the outer microaerobic zone, denitrifying heterotrophic bacteria occupied the intermediate zone, and anammox occupied the inner anaerobic zone (Li et al., 2022).

This pathway lowers effluent nitrate by recycling nitrate-derived nitrite to anammox, but its effectiveness depends on balancing carbon availability and biomass structure. Insufficient organic carbon limits nitrate-to-nitrite reduction, whereas excessive organic carbon intensifies oxygen and nitrite competition and impairs anammox retention. Therefore, stable operation requires structured biofilms or granules that maintain redox microenvironments and limit overgrowth of denitrifying heterotrophic bacteria.

4.4. Evidence standards for reactor-scale validation and predictive modelling

4.4.1. Reactor-scale validation requires functional evidence beyond molecular detection

Validation of comammox-enriched mainstream PN/A remains limited by the gap between molecular detection and in situ activity. Although *amoA*-targeted PCR assays distinguish comammox from canonical AOB and AOA, abundance estimates remain uncertain because primer coverage is incomplete and amplification bias is substantial. Reported primer coverage reaches approximately 95% of clade A and 92% of clade B sequences (Pjevac et al., 2017). The *Ntsp-amoA* 162F/359R primer set overestimates low-abundance clades by up to 32-fold relative to PCR-free metagenomic sequencing (Lin et al., 2020). In sludge and biofilm samples, 16S rRNA sequencing and *amoA* qPCR also yield inconsistent abundance profiles (McKnight et al., 2025). Therefore, gene abundance alone is insufficient to verify active comammox-associated ammonia oxidation, particularly during start-up, operational disturbance, and recovery.

Reactor-scale evidence remains limited because most comammox-enriched mainstream PN/A systems have been evaluated at laboratory or pilot scale. In full-scale reactors, ammonium loading, C/N ratio, dissolved oxygen, and temperature fluctuate simultaneously, creating conditions that are difficult to reproduce in short-term studies. Future studies should verify functional persistence rather than merely detect community presence. This requires evidence that comammox-mediated ammonia oxidation and anammox activity remain active during mainstream disturbances. It also requires confirmation that biomass structure maintains nitrite transfer to anammox despite residual NOB activity. Because process disturbances can alter N_2O formation pathways, validation should also quantify the relative contributions of nitrifier-associated, heterotrophic, and abiotic N_2O formation.

4.4.2. Predictive modelling of comammox–anammox nitrogen partitioning in mainstream PN/A

Process models are required to link pathway-resolved measurements to reactor-scale performance under variable mainstream loading (Liu et al., 2020; Wang et al., 2020). Existing biofilm models that incorporate comammox, canonical nitrifiers, anammox, and heterotrophic bacteria simulate substrate gradients, microbial succession, and effluent nitrogen speciation under defined operating conditions (Zhang et al., 2022). However, their predictive capacity remains limited under transient or unstable operating regimes. Therefore, multi omic and isotope-based datasets source should be used as model constraints for parameter estimation, calibration, and validation. Future models should integrate mechanistic biofilm modelling with data-driven prediction. For comammox and anammox mainstream PN/A, prediction should extend beyond effluent nitrogen to include community stability, nitrite partitioning, canonical NOB recovery, nitrate accumulation, and N_2O source attribution (Yu et al., 2024; Zhang et al., 2026; Guo et al., 2026). Therefore, integrating isotope flux and reactor operation data can support the transition from configuration specific demonstrations to transferable mainstream PN/A design.

5. Conclusion

Mainstream one-stage PN/A is constrained by the limited robustness of AOB-mediated nitrification, the reduced selectivity of canonical NOB suppression, and the narrowing of the anammox retention window under SRT, temperature, and C/N variability. These constraints are coupled through the nitrite budget, so they cannot be resolved by adjusting a single operating parameter within the conventional mainstream PN/A framework.

This review interprets comammox enrichment as niche regulation within constrained mainstream PN/A systems. Comammox bacteria contribute to ammonia oxidation under low-substrate conditions,

Table 1
Reported reactor configurations and operating conditions for comammox-associated PN/A and partial denitrification-anammox systems.

System		Reactor type	Biomass form	Influent ammonium (mg-N/L)	Influent COD (mg/L)	pH	Temperature (°C)	DO level (mg/L)	SRT (h)	HRT (h)	Nitrogen removal efficiency (%)	Reference
Comammox in PN/A system	<i>Ca. Nitrospira</i> and <i>Ca. Brocadia</i>	MBBR	Biofilm	0-200	/		NA	~30	0-24	~10-30	NA	(Suarez et al., 2024)
	<i>Nitrospira</i> and <i>Ca. Kueningenia</i>	RBC	Biofilm	NA	/	NA	NA	NA	NA	NA	NA	(Wang et al., 2022)
	<i>Ca. Nitrospira nitrificans</i> and <i>Ca. Jettenia/ Brocadia</i>	CSTR	Floccular	120-220	/	7.4-8.0	26.5-29.6	0.05-0.3	150-300	150-300	48.5-70.1	(Shao and Wu, 2021)
	<i>Ca. Nitrospira nitrificans</i> and <i>Ca. Kueningenia</i>	MBR	Floccular (40%)	60	/	7.63-8.15	24.9-31.9	0.14±0.09 - 0.36±0.31	NA	4-36	71.7 ± 5.6	(Shao, 2024)
	<i>Ca. Nitrospira nitrosa</i> and <i>Ca. Brocadia</i>	IFAS	Floccular and Biofilm	20–40	/	NA	NA	2-6	-	-	>71	(Vilardi et al., 2023)
	<i>Ca. Nitrospira inopinata</i> and <i>Ca. Brocadiaceae</i>	Batch	Hydrogel beads coated granules	~18	/	8.2	NA	4	-	-	~100	(Gottshall et al., 2021)
	<i>Nitrospira inopinata</i> and	Polycarbonate column reactor	Hydrogel beads	30	/	NA	10-25	0.2-0.3	-	3.9	98.9 ± 0.9 -99.6 ± 0.3 %	(Li et al., 2023)
Comammox in PDN/A system	<i>Nitrospira</i> , <i>Thauera</i> and <i>Ca. Brocadia</i>	SBR	Floccular	50	~185		30 ± 1	0.2-0.5	NA	1-4	92.78±7.79	(Chen et al., 2023)
	<i>Ca. Nitrospira</i> , <i>Thauera</i> and <i>Ca. Kueningenia/ Brocadia</i>	UASB	Granule	30	96.7 ± 8.1	NA	30 ± 1	0.24 ± 0.06-0.27 ± 0.18	-	4-6	92.9 ± 1.1	(Cui et al., 2023)
	<i>Ca. Nitrospira inopinata</i> , <i>Thauera</i> and <i>Ca. Kueningenia</i> (denitrifier)	SBBR	Biofilm	50	100-250	NA	30 ± 1	0.5	-	24	94.8	(Li et al., 2022)
	<i>Nitrospira nitrosa</i> , <i>Alphaproteobacteria</i> and <i>clone Anammox_26</i>	SBR	Floccular	5-40	NA	7.3–7.8	25 ± 3	0.05	12	24	71.7	(Xiang et al., 2025)

compete with canonical NOB within defined FA and DO ranges, persist under long-SRT regimes compatible with anammox retention, and participate in nitrite partitioning with anammox. These functions also define the boundaries of the strategy. Replacement of canonical NOB by comammox redistributes nitrite-oxidizing activity within the nitrifier community rather than removing the nitrite sink, and reported reactor outcomes include both anammox-supporting and nitrite-competing interactions.

Practical implementation requires biomass retention and spatial structuring, control of low temperature and elevated C/N, residual nitrate management when needed, and pathway-resolved validation under variable mainstream loading. Comammox–anammox mainstream PN/A should therefore be regarded as a promising strategy whose value depends on defined ecological, spatial, and operational boundaries.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used Grammarly, ChatGPT in order to improve language and readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Yanjin Luo: Writing – original draft. **Haoran Duan:** Conceptualization, Writing – review & editing. **Min Zheng:** Writing – review & editing. **Wei Wei:** Investigation. **Xueming Chen:** Writing – review & editing. **Bing-Jie Ni:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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.

Author on this paper Min Zheng serves as an editor of Water Research. The manuscript has been handled and reviewed independently by other editors of the journal, with Min Zheng having no part in, or visibility over evaluation and acceptance of this work.

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Data availability

No data was used for the research described in the article.

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