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Discussion

The roads taken and not taken: Trends of anammox-based wastewater treatment in China

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Anaerobic ammonium oxidation (anammox) is a nitrogen-transforming process mediated by a group of chemolithoautotrophic bacteria that use nitrite (NO_2^-) as electron acceptors to oxidize ammonium (NH_4^+) in an anoxic environment. Since its discovery in the 1990s, anammox has attracted worldwide research interest regarding anammox microbiology, its role in the biogeochemical nitrogen cycle, and its practical application for wastewater treatment (Table S1). Anammox research has proliferated in China over the past two decades (Fig. S1), and much progress has been made, including (1) developing high-rate and integrated anammox systems to treat high-strength wastewater and/or mainstream sewage to achieve efficient nitrogen removal while allowing process stability and transferability [1–5], (2) elucidating anammox inhibitory mechanisms [6,7], and (3) understanding the behavior of anammox consortia [8–10]. The National Natural Science Foundation of China (NSFC) is the most highly acknowledged funding agency that promotes this research field. Here, by glancing at the NSFC-funded grants, the author summarizes the advances in anammox research in China and envisions new directions in anammox research.

1. Current status of grants supporting anammox for wastewater treatment in China

As of May 2022, NSFC has granted over 100 awards related to the anammox process in the scope of wastewater treatment. These grants were mainly awarded to the Department of Engineering & Materials Sciences (number of grants = 90), followed by the Department of Chemical Sciences (22), the Department of Life Sciences (8), and the Department of Earth Sciences (3). Among the 183 keywords identified (Table S2), the top ten keywords were "coupled process" (frequency = 42), "PN/A, partial nitritation-anammox" (18), "process control" (15), "microbial ecology" (13), "tertiary BNR" (11), "inhibition" (10), "optimization" (7), "physiology of anammox bacteria", "SADN, sulfur-driven autotrophic denitrification" (7), and "sulfur and nitrogen removal" (7). An examination of the co-occurrence of the keywords (Fig. 1) showed that "coupled process" was investigated concurrently with the highest frequency, pairing with "PN/A" (co-occurrence = 21), "process control" (20), "tertiary BNR" (14), "PdN/A, partial denitrification-anammox" (12), "denitrification" (12), and "sulfur and nitrogen removal" (10). The area of coupled processes is widely investigated because NO_2^- must first be supplied by ammonia-oxidizing bacteria (PN/A) or denitrifiers (PdN/A) and is subsequently converted to dinitrogen (N_2) by anammox bacteria. Other co-occurring keywords were related to coupled processes (e.g., denitrifying anaerobic methane oxidation (DAMO), dissimilatory nitrate reduction to ammonium (DNRA), anammox coupled to Fe(III) reduction (Feammox)), microbial ecology (e.g., anammox hotspot, synergism), nitrogen species (e.g., NO, N_2O, nitrite, nitrate, hydroxylamine), inhibition (e.g., antibiotics, oxygen, nano metals), physiology of anammox bacteria (e.g., anammoxosome, cytochrome *c*, HZO enzyme), carbon metabolism (e.g., mixotrophy, endogenous carbon), electron acceptors and donors (e.g., iron, manganese, sulfur, sulfate), electro-anammox (e.g., bioelectrochemical system (BES), electrode), process intensification (e.g., biofilm carrier, granulation), bacterial quorum sensing (e.g., signaling molecules), analytical methods (e.g., stable isotopic probing, Raman spectroscopy, 10x genomics), greenhouse gas emission control (e.g., N_2O emission), bioreactor design (e.g., EGSB, MBR), and wastewater type (e.g., mainstream sewage, saline wastewater, leachate, coking wastewater).

2. The majority and the hidden gems

The vast majority of the NSFC grants were funded to provide a comprehensive understanding of the metabolic interactions in anammox consortia in response to environmental stimuli so that efficient nitrogen removal can be achieved. These research projects relied almost exclusively on the top-down approaches to obtain the desired functions by manipulating the ecosystem processes [11]. The top-down framework follows the steps of (1) ecosystem design (e.g., PN/A, PdN/A, SADN-anammox, DAMO-anammox), (2) process evaluation (e.g., environmental parameters modulation), (3) microbiome characterization (e.g., 16S rRNA gene sequencing of the best-performing consortia), and (4) community-level investigation of microbial interactions. This framework has become a paradigm in the era of meta-omics.

Alternatively, the bottom-up approaches target the molecular-

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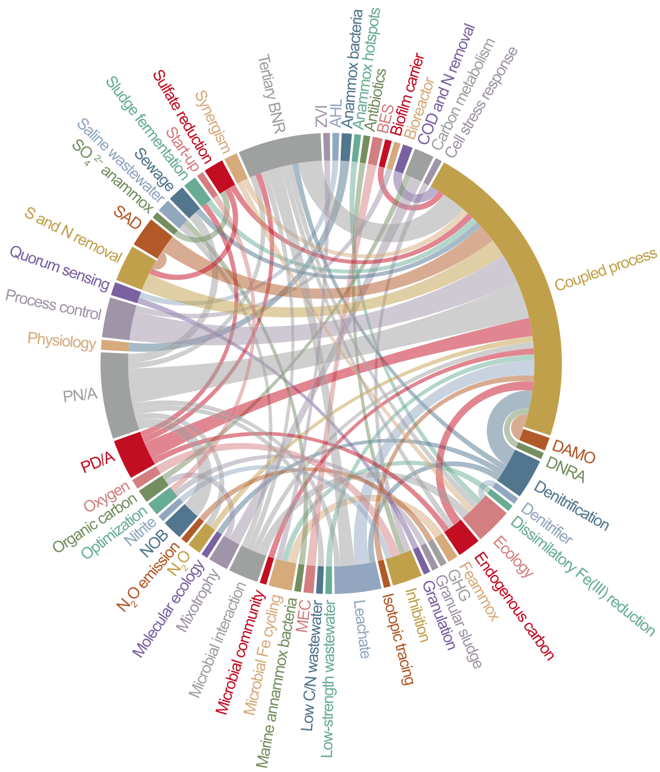

Fig. 1. Keywords co-occurrence of the NSFC grants on anammox for wastewater treatment from 2000 to 2021 (data source: https://kd.nsfc.gov.cn/). Abbreviations: PN/A, partial nitritation-anammox; PD/A, partial denitritation-anammox; NOB, nitrite-oxidizing bacteria; MEC, microbial electrolysis cell; GHG, greenhouse gases; DNRA, dissimilatory nitrate reduction to ammonium; DAMO, denitrifying anaerobic methane oxidation; BES, bioelectrochemical system; AHL, acyl homoserine lactone; ZVI, zero valent iron; BNR, biological nitrogen removal; SAD, sulfur-driven autotrophic denitrification.

scale metabolism of cornerstone species. Here, the workflow starts with the physiological characterization of the anammox species, followed by genome-scale metabolic modeling. Unlike model organisms, anammox bacteria cannot be cultivated with conventional techniques such as colony picking. They must be enriched as granular biomass or planktonic cells in sequencing batch reactors or membrane bioreactors, respectively, and there are no standardized cultivation protocols. The cultivation and enrichment process is time-consuming, taking months to years to obtain a high-quality anammox isolate. In recent years, highly enriched (>95%) cultures, rapid growth (doubling time of 2–4 days), and extracellular electron transfer-dependent NH_4^+ oxidation have been reported for phylogenetically distant anammox species such as *Candidatus* Kuenenia stuttgartiensis (freshwater), Brocadia sinica (freshwater), and Scalindua sp. (marine/saline waters) [12,13]. These traits could inspire investigators to design and implement anammox-based technologies in an energy-harnessing manner. The discovery of these traits, however, was based on detailed knowledge about the physiology, genomics, and self-assembly behavior of anammox bacteria, and this information is crucial in a bottom-up approach workflow.

Other metabolic functions of anammox bacteria remain enigmatic, constituting a technical challenge but with scientific significance. For example, anammox bacteria are the only organisms known to naturally produce hydrazine (N_2H_4) as a metabolic intermediate, a powerful reducing agent ultimately used as rocket fuel. Future endeavors are needed to develop tools for the in vitro biosynthesis of hydrazine by converting wastewater NH_4^+ via cell-free hydrazine synthase. The formation of encapsulin nanocompartments is another trait that can potentially be used to convert anammox bacteria into a biomanufacturing platform for the facile synthesis of silver or gold nanoparticles [14]. The abovementioned aspects may constitute the next frontiers of anammox research. Some NSFC research grants have been funded to explore the versatile functionalities of anammox bacteria beyond wastewater treatment.

3. Future perspectives

Multiplexing strategies in amplicon sequencing approaches have enabled rapid and high-throughput microbial community analyses. This technology paves the way for studying the metabolic interactions of anammox in wastewater treatment. Furthermore, various coupled processes could be used to treat different wastewaters, thereby increasing the complexity. Research using top-down strategies has resulted in numerous publications (Fig. S1), while the bottom-up approach is the road less traveled for anammox research in China. More than 30 years ago, a Netherlands-based group started anammox research focusing on the enrichment of anammox bacteria. They have subsequently launched applicable PN/A processes (e.g., SHARON) for wastewater treatment and have extended their investigation to anammox physiology, cell biology, and biochemistry (Tables S1 and S3). Today, considering the active engagement of China, an integration of top-down and bottom-up approaches will be needed to further promote anammox research. First, an anammox-specific database should be developed to accumulate, archive, and update information about anammox bacteria and other coexisting microbes in the coupled processes. Such a database should include genomes, expression profiles, protein sequences, metabolites, and pathway reactions, similar to other comprehensive databases such as MiDAS [15]. Second, with advances in the sequencing of anammox bacteria, genome-scale metabolic networks should be reconstructed for selected organisms (e.g., Kuenenia stuttgartiensis). These networks could be converted into mathematical models to predict physiological properties. Furthermore, integrating the genome-scale models for dynamic flux analysis of anammox consortia (e.g., ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, heterotrophic denitrifiers, Comammox organisms) that cooperate or compete in wastewater could be used to predict microbial interactions under various environmental conditions for process optimization.

Regarding real-world applications, implementing a full-scale anammox process, particularly for mainstream sewage treatment, remains a large challenge in China. Global efforts have been dedicated to expanding full-scale sidestream anammox technology towards mainstream application [16]. The spontaneous growth of anammox bacteria with enhanced deammonification capability was recently reported in a full-scale wastewater treatment plant located in a temperate zone of China [17]. Anammox enrichment in the mainstream indicates its future prospects, yet long-term process stability and transferability require an effective bridging of thermodynamics, physiology, microbial ecology, and engineering principles. Integrating the top-down and bottom-up approaches will help produce functional anammox consortia for improved nitrogen removal. In this way, anammox biotechnologies are envisioned as a driver of innovation in water sector towards carbon neutral.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ese.2022.100221.

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