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Biochar

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Biochar as an electron bridge: mechanistic insights into enhanced anammox performance in wastewater treatment

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Abstract

Anaerobic ammonium oxidation (anammox) process constitutes an energy-efficient nitrogen removal pathway critical for sustainable wastewater treatment. Anammox bacteria possess extracellular electron transfer capabilities relevant to their metabolism and potential interactions within microbial consortia. Enhanced electron transfer has been associated with anammox bacteria enrichment and improved nitrogen removal efficiency. Biochar addition has been reported to enhance electron transfer in anammox systems. This review summarizes the current understanding of biochar-induced electron transfer mechanisms during anammox processes. The proposed mechanisms include: (1) extracellular-polymeric-substance-mediated enrichment of redox-active compounds that facilitate biofilm-mediated electron transfer, (2) direct interspecies electron transfer through conductive biochar networks, and (3) mediated interspecies electron transfer via redox-active functional groups on biochar surfaces. Future research should integrate machine learning with mechanistic investigations to improve the prediction of biochar performance and guide the rational design of biochar materials. Such research efforts may provide a framework for optimizing electron transfer pathways and for future validation in scalable anammox systems.

Highlights

- Biochar supports extracellular electron transfer in anammox systems.
- Biochar stimulates EPS secretion, enriching redox-active compounds in the biofilm.
- Biochar provides conductive surfaces and redox-active groups for DIET and MIET.

Keywords Anammox, Electron transfer, Biochar, DIET, EET, MIET

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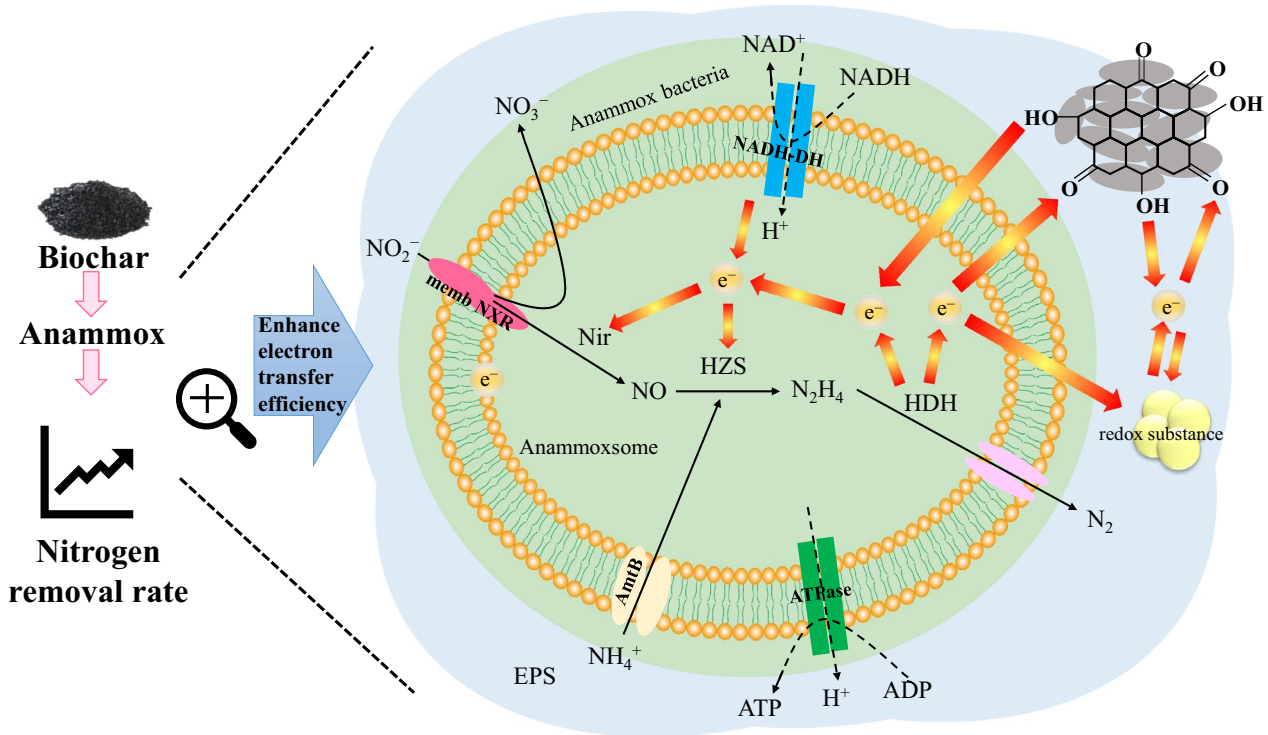
Graphical Abstract



1 Introduction

Anaerobic ammonium oxidation (anammox) is a microorganism-mediated process wherein ammonium (NH_4^+) is oxidized to dinitrogen gas (N_2) under anoxic conditions, utilizing nitrite (NO_2^-) as the terminal electron acceptor (Mulder et al. 1995; Vandegraaf et al. 1995; Xie et al. 2022). This autotrophic pathway involves enzymatic intermediates, such as nitric oxide (NO) and hydrazine (N_2H_4) and is catalyzed by specialized planctomycete bacteria (Strous et al. 1999; Xie et al. 2022). In contrast to the conventional nitrification/denitrification process, the anammox process reduces aeration energy requirement by 50–60%, eliminates the need for exogenous organic carbon, and lowers operational cost by up to 90% (Chen et al. 2016). However, anammox bacteria exhibit a significantly slower growth rate than conventional denitrifying bacteria, with a maximum specific growth rate of 0.0027 h^{-1} and a doubling time of 10–12 days (Chen et al. 2021; Wang et al. 2022a). The anammox process is mediated by extracellular electron transfer (EET) (Zhen et al. 2024). The prolonged doubling time of anammox bacteria may be due to their environmental sensitivity (Chen et al. 2023b; Li et al. 2021; Ren et al. 2022) and limited availability of the electron acceptor NO_2^- during the anammox process (Dou et al. 2025). These intrinsic constraints impede robust process scaling and industrial implementation of the anammox process. To advance the practical application of the anammox process in municipal wastewater treatment and optimize microbial electron transport kinetics, researchers have explored exogenous additives, such as divalent metal ions (e.g., Mn^{2+} and Zn^{2+}) (Li et al. 2018), N_2H_4 (Ganesan and Vadivelu 2019), and biochar (Huang et al. 2022). Biochar significantly enhances electron transfer efficiency and anammox performance through multiple mechanisms, including facilitating EET, providing sites for microbial attachment, and promoting enzymatic activity. In contrast, other exogenous additives are associated with limitations, such as high biotoxicity, dosage control challenges, inhibition risks, and secondary pollution potential, which hinder their practical application in engineering contexts.

Among the various exogenous additives investigated, biochar has emerged as a particularly promising material for enhancing EET in anammox systems. Biochar, a carbon-rich porous material produced through the thermochemical conversion of biomass under oxygen-limited conditions (typically 300–700 ℃), exhibits hierarchical pore architecture and high specific surface area (Xie et al.

2022; Zhu et al. 2025). It contains redox-active moieties, including poly-condensed aromatic structures and quinone functional groups (Chen et al. 2025b). Moreover, depending on the feedstock source and pyrolysis temperature, biochar can serve as a conduit for electron transfer, and as an acceptor and donor (Cai et al. 2022; Osman et al. 2022). However, biochar may not function as an efficient electron shuttle because of its limited solubility, particulate nature, and pseudocapacitive behavior, which involves irreversible redox reactions of certain redox-active species (Prévoteau et al. 2016; Yuan et al. 2017). This contrasts with canonical electron shuttles (e.g., lipophilic ubiquinones and hydrophilic flavins), which undergo reversible redox cycling (Fan et al. 2024). Biochar enhances microbial electron transfer efficiency by directly donating electrons and facilitating electron exchange through conductive pathways or redox-active surface functional groups (Chen et al. 2018; Fu et al. 2024). Biochar-derived redox-active surface functional groups, including quinone groups and phenolic O–H moieties, may mediate electron transfer by reversibly accepting and donating electrons, thereby facilitating EET within microbial consortia related to the nitrogen cycle (Xie et al. 2022). A systematic search was conducted in the Web of Science database using combinations of the keywords "anammox", "biochar", "extracellular electron transfer", and "extracellular polymeric substances", yielding 4232 articles published between 2020 and 2025. Analysis of these articles using VOSviewer software generated a relationship map (Fig. 1) illustrating the connections among anammox, biochar, EET, and extracellular polymeric substances (EPS). This figure represents the principal keyword co-occurrence network within the research theme, depicting the multifaceted mechanisms underlying biochar-enhanced anammox performance. Moreover, biochar concurrently exerts significant influence across multiple dimensions, especially on microbial ecology and EET pathways. It plays a pivotal role in facilitating microbial interactions, modulating electron shuttling processes, and supporting syntrophic relationships among the anammox bacteria. Biochar-amended anammox reactors have exhibited significantly higher abundances of core functional genes involved in anammox metabolism. For instance, in a previous study, the average copy numbers of *hzsA* (hydrazine synthase), *hdh* (hydrazine dehydrogenase), *nirS* (cd_1-type nitrite reductase), and *napA* (periplasmic nitrate reductase) genes in biochar-amended anammox reactors were 5.6-, 8.7-, 9.4-, and 4.2-fold higher than those in the non-biochar control, respectively (Xu et al. 2021b). Integration of biochar and anammox sludge within a fixed-bed column reactor resulted in a maximum total nitrogen removal efficiency of 90.5% (Mojiri et al. 2020).

Biochar serves as an effective biofilm carrier, enhancing biomass retention and resistance to oxidative stress (Gao et al. 2022). Ma et al. (2022) summarized the effects of various exogenous additives on anammox performance, whereas Zhang et al. (2022) focused on operational strategies and material-assisted enhancement of anammox processes (Ma et al. 2022; Zhang et al. 2022). More recently, Zhen et al. (2024) reviewed the roles of conductive materials and electron mediators in EET-dependent anammox. However, the specific mechanisms by which biochar mediates EET have not been systematically evaluated. Unlike previous reviews focused on additives or operational strategies for anammox enhancement, this review specifically focuses on biochar-mediated EET. It integrates EPS-associated redox activity, direct interspecies electron transfer (DIET), and mediated interspecies electron transfer (MIET) into an electron-transfer-oriented framework and evaluates how biochar properties may be rationally optimized for anammox systems. In addition, this review extends beyond mechanistic discussions by exploring machine-learning-assisted biochar design, as well as life-cycle assessment (LCA) and techno-economic assessment (TEA) perspectives for future applications. Here, "electron bridge" refers to the role of biochar either as a conductive interface that physically links microbial electron donors and acceptors or as a redox-active mediator that transiently accepts and donates electrons. This broader term includes, but is not limited to, classical electron-shuttle behavior. Biochar has attracted increasing research attention due to its low cost, tunable surface chemistry, and potential conductivity. However, its performance can vary with feedstock, aging, contaminant release, and reactor conditions. This review examines biochar-mediated electron transfer processes in anammox systems, particularly focusing on the roles of redox-active functional groups, intrinsic conductivity, EPS-associated electron transfer, and biochar–microbe interfacial interactions. By establishing a unified framework linking material properties, microbial responses, and interspecies electron-transfer pathways, this review aims to provide mechanistic insights into the rational optimization of biochar and to support the development of more efficient and stable anammox-based wastewater treatment systems.

2 Fundamental electron transfer mechanisms in anammox bacteria

Anammox bacteria possess a unique cellular architecture characterized by three compartments: the paryphoplasm, the riboplasm, and the anammoxosome (Moss et al. 2018). These compartments are separated by the intracytoplasmic membrane and the anammoxosome membrane. The anammoxosome is the exclusive intracellular

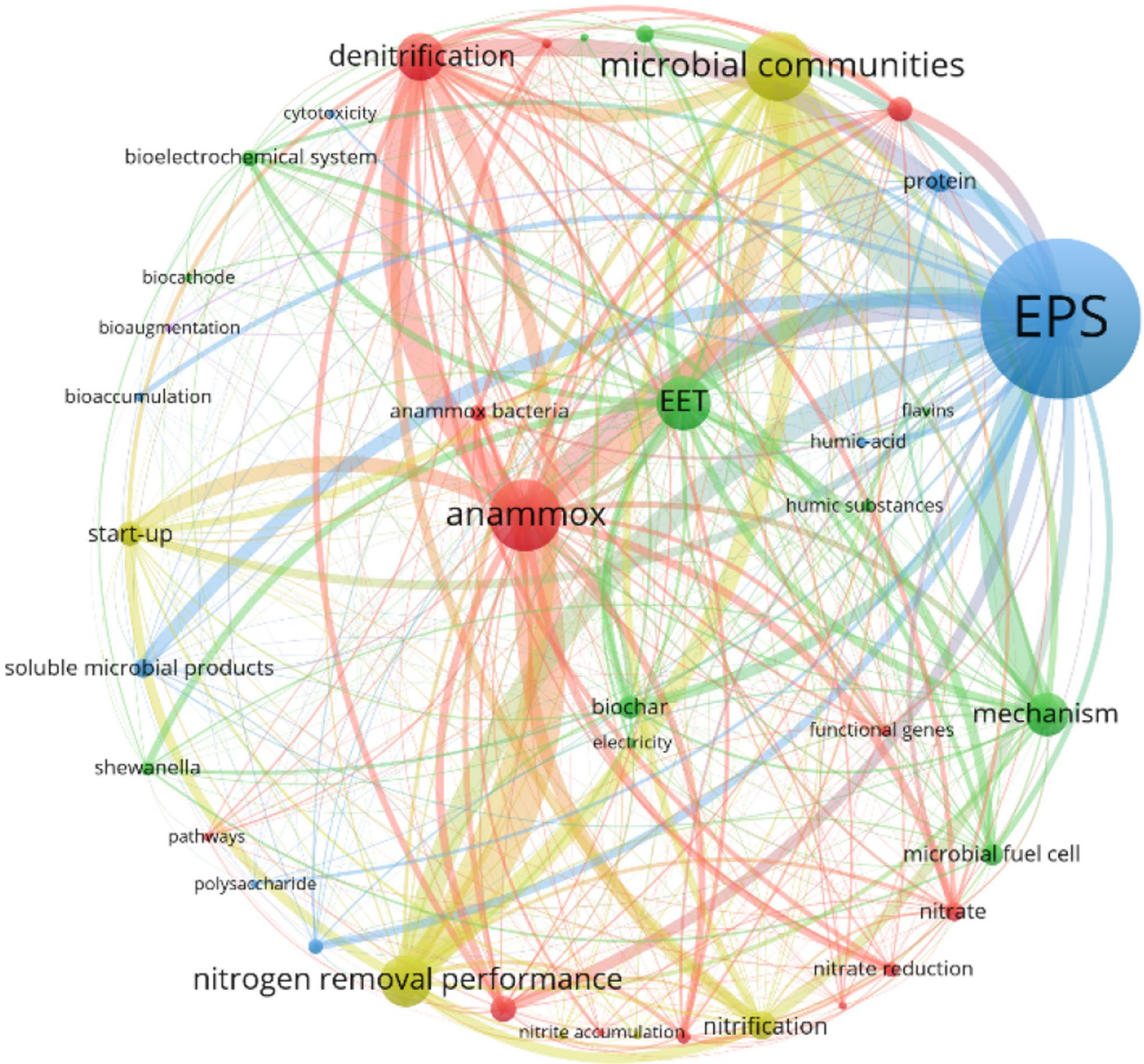


Fig. 1 A bibliometric analysis map visualizing the relationships among anammox, biochar, EET, and EPS

site for anammox and harbors the key metabolic enzymes required for this process (De Almeida et al. 2015; Versantvoort et al. 2022). This structure has been identified in all known anammox bacteria discovered to date (Cuecas et al. 2024; Lau et al. 2016; Liao et al. 2022). Because biochar primarily enhances anammox through EET, only the structural and metabolic features directly related to electron generation and transport are discussed below.

NO_2^- serves as the primary electron acceptor, while N_2H_4 is mainly produced within the anammoxosome (Okabe et al. 2021). Based on these findings, researchers have proposed a possible metabolic pathway for anammox bacteria (Jetten et al. 1998; Ponce-Jahen et al. 2024). Several metabolic models have been proposed for anammox bacteria. Here, only the key reactions relevant to intracellular electron generation are briefly summarized, as they provide the basis for understanding subsequent EET processes and the role of biochar. First, nitrite reductase (Nir) reduces cytoplasmic NO_2^- to NH_2OH, which is then transported into the anammoxosome by hydrazine hydrolase (HH). Within the anammoxosome, NH_2OH reacts with NH_4^+ to form N_2H_4, which is subsequently oxidized by hydrazine oxidoreductase (HZO) to produce N_2. The metabolic and electron-transfer

pathways related to these reactions are shown in Fig. 2a. EET enables microorganisms to exchange electrons with extracellular solid-phase electron acceptors or donors and is the primary mechanism through which conductive materials, including biochar, influence microbial metabolism (Tanaka et al. 2018). However, owing to the intrinsic non-conductivity of microbial cell membranes, intracellularly generated electrons must traverse this insulating barrier to reduce extracellular electron acceptors—a process mediated by specialized electron transport pathways (Ponce-Jahen et al. 2024). This mechanistic challenge in EET represents a key research focus. Investigations have revealed that electrons generated from intracellular organic matter oxidation are relayed to the outer membrane via the redox activity of multiple C-type cytochromes distributed across the cell membrane, periplasm, and outer membrane (Akram et al. 2021). These cytochrome assemblies form transmembrane conduits that bridge electrically insulating cellular barriers (Akram et al. 2019; Ferousi et al. 2019; Xiao and Yu 2020). In addition to transmembrane electron conduits formed by C-type cytochromes, EET also occurs via flavin-mediated electron shuttling and conductive microbial nanowires (Gao et al. 2015). Under bio-electrochemical conditions, anammox bacteria can couple ammonium oxidation to EET pathways, thereby

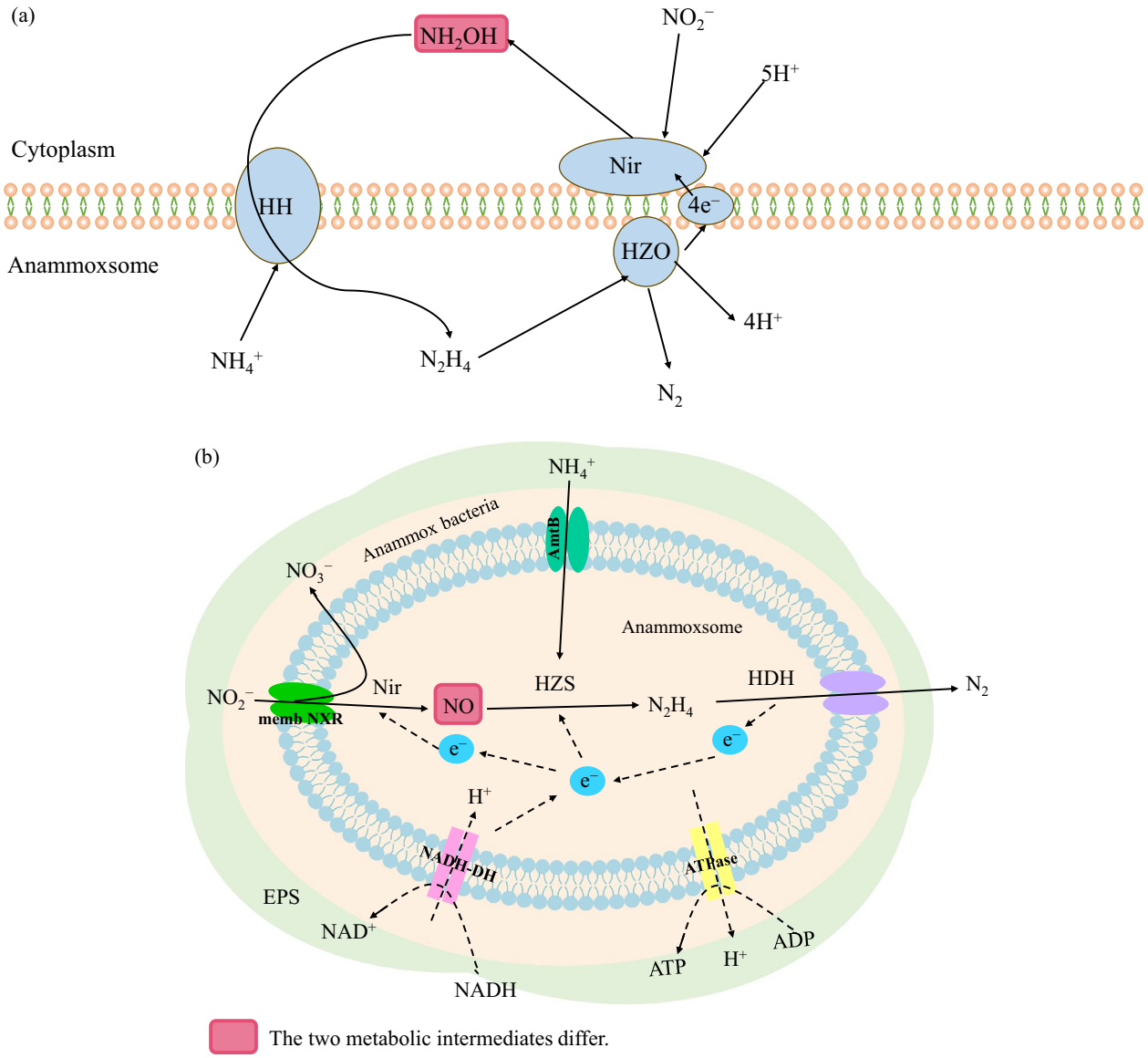


Fig. 2 **a** Anammox metabolism model; **b** Metabolism model of Anammox based on *Candidatus Kuenenia stuttgartiensis*

transferring intracellular electrons to electroactive interfaces (e.g., electrodes). Previous studies have experimentally demonstrated the electroactivity of anammox bacteria. Within microbial electrolysis cells (MEC), anammox bacteria simultaneously mediate ammonium (NH_4^+) oxidation and EET to insoluble acceptors, such as graphene oxide electrodes. Experimental evidence confirms that these electroactive biofilms generate anodic currents by transferring electrons to graphene oxide while oxidizing ammonium (Shaw et al. 2020). In addition, ^{15}N isotope-labeling experiments and transcriptome analysis have shown that anammox bacteria are electrically active and can interact with solid carbon to completely oxidize NH_4^+ to N_2 (Shaw et al. 2020; Speth et al. 2016). These findings collectively demonstrate that anammox bacteria possess intrinsic electroactivity, providing the mechanistic basis for biochar-mediated EET enhancement. The electron transfer mechanism discussed here is markedly distinct from the pathway based on classical conductive pili of *Geobacter* (Kartal et al. 2011; Lovley 2017; Shaw et al. 2020). Given the intrinsic electroactivity of anammox bacteria, biochar can further facilitate electron transport by providing conductive surfaces and reversible redox-active functional groups. Functional groups on the biochar surface, especially quinone/hydroquinone groups, can give and receive electrons through reversible redox reactions (Sun et al. 2017). The biochar-induced enhancement of anammox activity may be attributed to two distinct electron transfer pathways: (1) electrons derived from intracellular NH_4^+ oxidation are transferred to extracellular electron acceptors through direct c-type cytochromes (Emilia Rios-Del Toro et al. 2018; Shaw et al. 2020); and (2) biochar particles act as insoluble electron shuttles, facilitating interspecies electron transfer between anammox bacteria and anaerobic respirers involved in denitrification or Fe(III) reduction (Zhang et al. 2023c; Zhou et al. 2016). These processes correspond to DIET and MIET, respectively.

However, based on the genomic analysis of *Kuenenia stuttgartiensis*, Kartal et al. (Kartal and Keltjens 2016) proposed an alternative metabolic pathway for anammox bacteria, as shown in Fig. 2b, suggesting that NO rather than NH_2OH serves as the key intermediate. In this model, the key enzymes of anammox bacteria are localized in the anammoxosome, where it generates proton motive force across its membrane, driving ATP synthesis through membrane-bound ATPase. Nir catalyzes the reduction of NO_2^- to nitric oxide (NO), while hydrazine synthase (HZS) facilitates the enzymatic conversion of NH_4^+ to hydrazine (N_2H_4). Subsequently, hydrazine dehydrogenase (HDH) oxidizes N_2H_4 to N_2, concurrently producing 4 electrons that enter the electron transport chain. These reactions can be presented as follows:

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$$

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^-$$

$$NO_2^- \rightarrow NO_3^- + 2H^+ + 2e^-$$

Although the two metabolic models differ in their proposed intermediates, both support intracellular electron generation and ultimately converge on EET processes, which can be facilitated by conductive biochar materials. The characteristic red color of anammox biomass has been attributed to the abundance of C-type cytochromes embedded within the EPS matrix. EPS is a key structural and electrochemical component governing biochar-mediated EET in anammox biofilms. It consists of proteins (PN), polysaccharides (PS), glycoproteins, humic substances, and nucleic acids (Xu et al. 2022a). The EPS matrix has a hydrogel-like architecture, which is structurally and functionally analogous to the mucin-like extracellular matrices of biofilms and multicellular organisms. This matrix forms a selectively permeable barrier around anammox bacteria, safeguarding cellular integrity while facilitating nutrient diffusion and enhancing substrate accessibility to cells within microbial aggregates (Boleij et al. 2018). Since EPS can strongly bind toxic pollutants and has certain water-holding capacity and antioxidant activity, it can buffer the adverse environmental effects (Gao et al. 2015). C-type cytochrome has been identified as a key component of electron transfer reactions necessary to facilitate key catabolic reactions, such as ammonium oxidation and nitrite reduction during anammox (Akram et al. 2021). N_2H_4 oxidation is catalyzed by HDH, a C-type cytochrome complex containing an extensive electron transfer network. This molecular framework facilitates rapid long-distance electron movement across the complex (Akram et al. 2019). The PN/PS ratio serves as a critical indicator for evaluating the composition and functional properties of EPS. PN (primarily comprising microbial-secreted enzymes and structural proteins) contributes to adsorption, catalysis, and intermolecular cross-linking, while PS (such as glucans and cellulose) confers structural integrity, water retention capacity, and adhesion property. The PN/PS ratio fundamentally dictates the architecture and matrix stability of EPS. Both PS and PN cooperatively regulate microbial aggregation and biofilm development. PS acts as a "scaffold" for biofilm formation, facilitating microbial adhesion and stabilizing three-dimensional structures

through hydrogen bonding and hydrophobic interactions (Decho and Gutierrez 2017). However, it has been reported that exopolysaccharides in the EPS layer can significantly suppress EET by increasing charge transfer resistance (Gao et al. 2019). Conversely, PN mediates cell–cell signaling and surface recognition, orchestrating microbial aggregation (Muhammad et al. 2020). In particular, C-type cytochromes, specific PN components, directly participate in the redox reactions associated with anammox catabolism (e.g., hydrazine oxidation), enabling long-range electron transfer through conjugated heme structures (Bird et al. 2019). Low PN/PS ratios are associated with EPS matrices dominated by a gel-like polysaccharide network, which enhances mechanical strength and hydration capacity while increasing diffusion resistance, potentially suppressing electron transfer efficiency (Gao et al. 2019; Vu et al. 2009). In contrast, high PN/PS ratios favor conductive EPS architectures, with protein-rich matrices enhancing electron transfer through redox-active components (e.g., C-type cytochromes) (Zhang et al. 2023c).

Anammox bacteria achieve nitrogen removal through their unique cellular components, particularly the anammoxosome, and the associated intracellular and electron transfer processes. Two primary metabolic models have been proposed: a classical pathway involving hydroxylamine intermediates and a NO-based pathway supported by genomic studies of *Kuenenia stuttgartiensis*. These processes occur synergistically to enhance metabolic efficiency and adaptability under varying environmental conditions. C-type cytochromes associated with EPS play a critical role in electron transfer, though efficiency is influenced by EPS composition (e.g., PN/PS ratio). Therefore, alterations in EPS composition may substantially affect EET. EPS is proposed to facilitate electron flux, particularly when enriched in redox-active proteins, flavins, or conductive extracellular structures. These intrinsic EET routes provide the mechanistic basis for understanding how biochar may intervene in anammox systems, either by modifying EPS-associated redox chemistry or by providing conductive and redox-active interfaces for interspecies electron transfer. In summary, the EET mechanisms in anammox systems are highly complex and environmentally adaptive, necessitating further multi-omics investigations to elucidate the relevant molecular regulatory networks. However, this discussion focuses only on the structural components and metabolic pathways directly involved in electron generation, transport, and extracellular transfer, as these processes constitute the mechanistic basis for understanding how biochar enhances anammox performance through EET.

3 Enhancement mechanisms of biochar-mediated electron bridging in anammox

3.1 Role of biochar properties in anammox electron transport

Biochar has been shown to enhance electron transport efficiency in anammox systems (Yue et al. 2026). However, the efficacy of biochar is governed by its inherent physicochemical properties, including surface functionalities (e.g., quinone/phenolic groups) and electrical conductivity. Feedstock type and pyrolysis temperature dictate the composition of functional groups on the surface of biochar and, consequently, its electron transport efficiency. These temperature-dependent transformations in surface functional groups modulate the electronic exchange capacity (EEC), representing the combined electron-donating capability (EDC) and electron-accepting capability (EAC) of biochar (Zhang et al. 2023b). For poplar-derived biochar, carbonyl functionalities predominate at 300–500 °C, whereas pyrolysis at 550–650 °C promotes substantial carbonaceous restructuring into aromatic networks (Zhang et al. 2020). At pyrolysis temperatures of 300–400 °C, biochar exhibits a stronger electron-donating ability than electron-accepting ability, indicating its electron donation tendency. Above 550 °C, progressive graphitization dominates, yielding biochar with increased aromatic condensation and reduced electron transfer capacities (Yuan et al. 2022; Zhang et al. 2023b). In another study, a biochar was prepared using three distinct feedstocks—wheat straw, barley grass, and pine wood—at varying pyrolysis temperatures (Zhang et al. 2018). For each given feedstock, increasing pyrolysis temperature from 200 °C to 350/400 °C enhanced the EDC of biochar (Zhang et al. 2018). Within the temperature range of 200–400 °C, EDC exhibited strong positive linear correlations with the phenolic hydroxyl group content of biochar. Conversely, the biochar prepared at pyrolysis temperatures above 650 °C exhibited fewer oxygen-containing functional groups but formed condensed aromatic structures. This structural shift resulted in minimal variations in EDC regardless of the initial feedstock, as the conjugated π-electron system became the dominant contributor to EDC. Concomitantly, the electrical conductivity of biochar increased (Zhang et al. 2018). Owing to its demonstrable EEC, biochar facilitates electron transfer in redox-driven processes (Lu et al. 2022; Wang et al. 2022c). Essentially, biochar produced at 300–400 °C exhibited abundant electron-donating moieties (phenolic/hydroquinone groups), conferring high EEC and EDC but limited EAC (Wang et al. 2022b). This redox profile can enhance anammox metabolism by augmenting the electron-accepting function of anammox biomass and EPS (Wang et al. 2022b; Xu et al. 2020). Conversely, biochar prepared through pyrolysis above

500 °C exhibited stronger electron-accepting characteristics owing to its enhanced EAC, potentially impeding anammox metabolism through electron competition (Xu et al. 2020). Nevertheless, the tunable redox properties of biochar make it an adaptable participant in anammox-associated electron transfer processes.

3.2 Biochar in the anammox electron transfer pathway

Current evidence suggests that the redox-active functional groups and electrical conductivity of biochar enable three non-exclusive proposed mechanisms for biochar-enhanced electron transfer in anammox systems: (1) EPS-mediated redox enrichment through the accumulation of redox-active proteins and electron-transfer compounds within the biofilm matrix; (2) DIET via conductive biochar networks; and (3) MIET through redox-active surface functionalities capable of reversible electron exchange. EPS-mediated electron transfer refers to electron movement through redox-active extracellular polymers, while DIET refers to direct electron exchange through conductive biological or mineral/material interfaces. MIET refers to electron transfer mediated by diffusible or surface-bound redox-active compounds. These three mechanisms are not mutually exclusive and may coexist within the same anammox system.

3.2.1 *EPS-mediated electron shuttling: cytochrome enrichment and biofilm optimization*

Biochar may enhance anammox efficiency by promoting EPS secretion and potentially modulating electron transfer pathways (Fig. 3). Bacterial EPS proteins, including C-type cytochromes, exhibit electrochemical redox functionality and intrinsic electron transfer capabilities (Li et al. 2016). Notably, in electroactive microorganisms such as *Shewanella oneidensis* MR-1, EPS components directly facilitate EET by serving as transient interfacial matrices (Li et al. 2016; Xiao et al. 2017). Therefore, biochar has been proposed to contribute to extracellular electron flux by forming conductive interfaces that complement redox-active biomolecules (e.g., membrane cytochromes) (Bu et al. 2022).

In anammox systems, hydrazine oxidation is mediated by HDH. This soluble cytochrome complex is proposed to organize multi-heme clusters into an electron-conducting pathway that facilitates charge transfer (Akram et al. 2019). Consequently, these redox-active proteins are thought to be localized within the anammoxosome membrane and participate in electron transfer processes. EPS of anammox bacteria contain a C-type cytochrome like that present in HDH. Previous studies have indicated that biochar amendment is associated with increased abundance of redox-active exoproteins, a higher PN/PS ratio

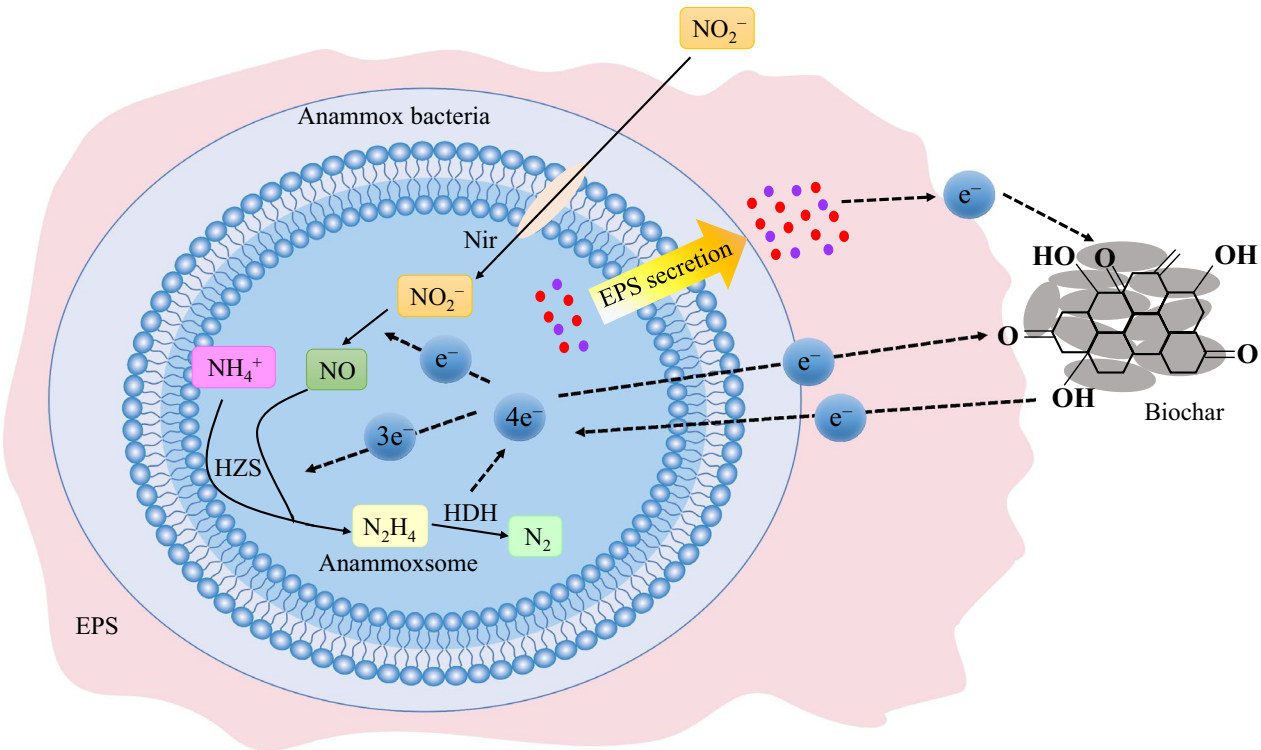


Fig. 3 Mechanisms of EPS secretion stimulated by biochar addition for anammox process

(Wang et al. 2022b), and enhanced microbial EET (Wang et al. 2022a). Biochar addition has also been reported to be associated with increased EPS secretion by anammox bacteria, resulting in a significant increase of 30–40% in EPS secretion compared to the control group (Wang et al. 2022b). However, some studies have revealed that biochar amendment did not consistently enhance EPS production. In reactors operated under long-term conditions and subjected to nitrogen loading shocks, biochar produced at different pyrolysis temperatures failed to induce a sustained stimulatory effect on total EPS yield (Xu et al. 2020). Biochar may also modulate the electrochemical properties of anammox sludge. Specifically, the electron transfer capability (ETC) of EPS increased by 73.8%, accompanied by reduced charge transfer resistance (Xu et al. 2021b). These changes may facilitate electron flux through conductive networks formed between redox-active biochar surfaces and C-type cytochromes, potentially improving nitrogen conversion kinetics through enhanced hydrazine dehydrogenation and nitrite reduction (Xu et al. 2021b). At a dosage of 10 g L^{-1}, biochar amendment caused a 51% increase in anammox activity compared with the control, which was hypothesized to be associated with the 30–40% increase in EPS secretion by anammox bacteria (Wang et al. 2022b). Quantification further revealed a significant 44.1% increase in extracellular EDC compared to the control group. PN and PS within EPS are considered important factors governing the surface charge, hydrophobicity, and three-dimensional architecture of bacterial aggregates. These physicochemical modifications may increase cell density, thereby facilitating intercellular communication and coordinated metabolic cooperation under high-density conditions, which subsequently improve microbial metabolic rates and functional activity (Hou et al. 2015).

Biochar can enhance anammox performance by promoting EPS secretion. The underlying effect may arise not only from increased EPS production, which facilitates microbial aggregation and higher cell density, thereby strengthening intercellular interactions, but also from the ability of biochar to stimulate the secretion of redox-active extracellular PN and increase the PN/PS ratio. This shift may promote the transformation of EPS from a PS-rich structural matrix into a more electrochemically active, PN-dominated matrix. Such a transition has been associated with enhanced electron transfer capability and reduced charge transfer resistance, suggesting that EPS may function not only as a passive protective hydrogel but also as a conductive extension of the cellular electron transfer network. The presence of C-type cytochromes homologous to HDH in EPS suggests that these extracellular PN can synergistically interact with the redox-active surface of biochar to form a continuous electron transfer pathway between cells and external conductive materials. This interaction may facilitate the release and transport of electrons generated from intracellular metabolism, thereby potentially alleviating electron transfer limitations in key reactions, such as hydrazine oxidation.

3.2.2 DIET: conductive network formation

DIET enables electron exchange between microorganisms through direct physical contact or conductive materials (e.g., biochar) without the involvement of soluble intermediates (Summers et al. 2010). This process generally relies on membrane-bound cytochromes or conductive pili, while biochar may serve as a conductive bridge that physically connects microorganisms, thereby facilitating electron transfer. In addition, surface redox mediation involves surface functional groups (such as oxygen-containing functional groups) of biochar that participate in redox reactions as electron donors or acceptors. Research suggests that the electron transfer efficiency depends, at least in part, on the redox properties of these surface functional groups (Saquing et al. 2016).

Biochar may indirectly support DIET by increasing microbial proximity through attachment sites, whereas direct DIET requires electron transfer through conductive biochar networks. The electrical conductivity of biochar may facilitate DIET among electroactive microorganisms by establishing conductive networks that support redox interactions through C-type cytochromes or microbial nanowires (Yu et al. 2021). Owing to its distinct structural configurations, biochar may facilitate DIET through π-conjugated domains and redox-active surface moieties (Chen et al. 2014). The redox-active surface moieties of biochar can serve dual roles as electron donors and acceptors, thereby potentially functioning as important mediators for electron transfer. Moreover, with reported EDC and EAC values of 2.64 mmol g^{-1} and 0.79 mmol g^{-1}, respectively, biochar appears to possess balanced redox capacities suitable for its role in electron transfer processes. Notably, biochar has been reported to function as an electron donor in redox reactions and as a mediator for nitrate reduction during anammox (Fu et al. 2024). Biochar may also serve as an engineered microbial habitat that improves anammox performance through accelerated granulation, selective enrichment of anammox bacteria, and enhanced expression of functional genes (Xie et al. 2022; Yu et al. 2021). Recent studies have reported that biochar amendment enhanced the ETC of anammox bacteria and improved their nitrogen removal efficiency by 4.0–11.6%. Furthermore, biochar may function as a bidirectional redox mediator by donating electrons for nitrite reduction while accepting electrons during hydrazine oxidation, thereby potentially

facilitating nitrite conversion via EET (Pang et al. 2023). The carbon/oxygen-containing functional groups (such as –OH, C=O, C–O) have shown linear correlations with the EDC of biochar and may participate in EET through electron donation and acceptance (Xu et al. 2022b; Zhang et al. 2019). Interfacial redox interactions between surface moieties of biochar and electron-transfer proteins of anammox bacteria may enhance enzymatic nitrogen conversion kinetics, potentially improving nitrogen removal performance (Harris et al. 2010). Figure 4 illustrates that biochar may function as an electron shuttle or donor in EET processes. Its redox-active surface functional groups (e.g., quinone/hydroquinone moieties) can participate in reversible redox reactions, facilitating electron exchange between anammox bacteria and extracellular acceptors. This mechanism likely enhances interspecies electron transfer and supports metabolic coupling within microbial consortia. Overall, current evidence suggests that biochar may serve as an electron shuttle or terminal acceptor associated with HDH-mediated hydrazine oxidation, thereby contributing to EET processes and potentially enhancing nitrogen removal performance of anammox systems (Zhen et al. 2024).

3.2.3 MIET: quinone-facilitated redox cycling

MIET involves electron exchange between microorganisms through soluble redox mediators, such as quinones and flavins. These mediators shuttle between oxidized and reduced states, facilitating electron transfer from donors to acceptors. Biochar may promote MIET by adsorbing or releasing these mediators and functioning as an electron shuttle between microorganisms and Fe(III) minerals, similar to humic substances, sulfur-containing compounds, and certain minerals. Biochar has also been reported to stimulate iron reduction through electron transfer among cells, biochar, and mineral phases (Kappler et al. 2014). Anammox bacteria contain core enzymes within their anammoxosomes. The proton motive force likely drives proton translocation across the anammox membrane, coupling with ATPase activity to support ATP synthesis. Certain redox-active molecules may cross cell walls and membranes, thereby potentially accessing intracellular enzymes involved in electron transfer processes. The electron shuttling capacity of low-temperature biochar (300–400 °C) and humic acid may facilitate ammonium oxidation in anammox bacteria, potentially improving respiratory efficiency by coupling intracellular hydrazine synthesis (from NH_4^+) with nitrite reduction, where nitrite serves as the terminal electron acceptor (Aishvarya et al. 2019). Biochar can also enhance anammox electron flux via shuttle-mediated EET. In a previous study, biochar addition led to enhanced anammox electron flux, accompanied by a 29.7–36.4% reduction in UASB startup duration and a

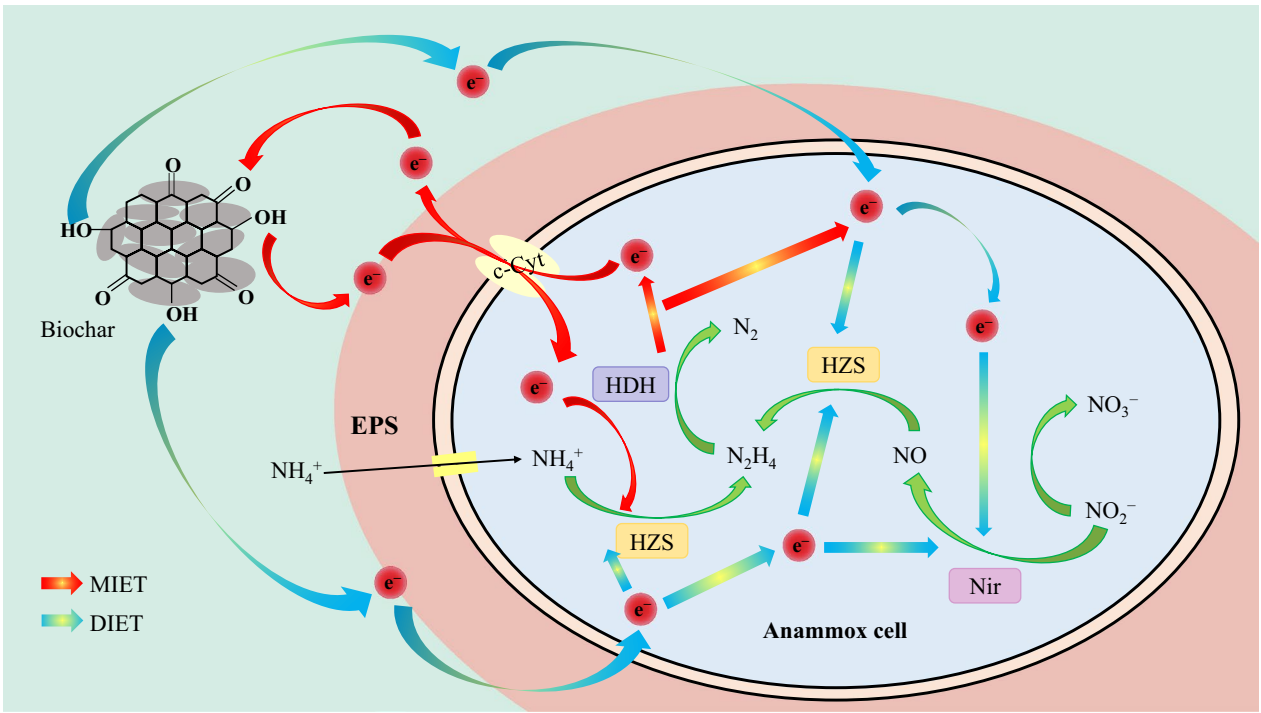


Fig. 4 Biochar promotes electron transport potential mechanism

9.3–14.7% increase in nitrogen removal compared to unamended controls (Chen et al. 2023a). The organic molecular architecture and particulate morphology of biochar may contribute to electron-shuttling behavior by facilitating interactions among microorganisms, biochar, and electrons (Fig. 4). As depicted in Fig. 4, biochar may enhance anammox performance by functioning as an insoluble electron shuttle or potential electron donor. It may adsorb and release redox mediators (e.g., flavins) or participate directly in electron transfer processes, thereby lowering energetic constraints associated with ammonium oxidation and nitrite reduction. These effects may enhance microbial electron flux and improve reactor stability under varying operational conditions. During anammox, biochar can function as an electron shuttle, facilitating electron transfer and potentially reducing the energetic demand associated with the oxidation of NH_4^+ and NO_2^- by anammox bacteria (Zhen et al. 2024).

Biochar has been widely reported to enhance the nitrogen removal performance of anammox systems, potentially through EPS secretion and EET. Corn stover-derived biochar (pyrolyzed at 300–550 °C) and bamboo-based biochar (400 °C) showed relatively favorable performance in previous studies, with the proposed mechanisms involving EET in *Candidatus Brocadia*-dominated consortia and enhanced expression of functional genes. When biochar was used as the sole electron acceptor, the expression levels of genes associated with anammox metabolism, including *amtB*, *hao*, and *hdh*, were upregulated. Metabolic pathway reconstruction of the dominant bacteria, *Candidatus Brocadia* and *Candidatus Kuenenia*, indicated that both bacteria possess key anammox-related genes, such as *amtB*, *hao*, and *hdh*. However, the genome of *Candidatus Brocadia* lacks the genes encoding nitrite reductase (NO-forming), *nirK*, and *nirS*, whereas *Candidatus Kuenenia* carries *nirK*. In the studied system, *Candidatus Brocadia* appeared to be more competitive than *Candidatus Kuenenia*, which may partly explain why the biochar-driven anammox process relies more strongly on the hydroxylamine pathway (Yue et al. 2026). Increasing the pyrolysis temperature may promote graphitization, thereby enhancing electrical conductivity of biochar while reducing its electron exchange capacity due to the loss of oxygen-containing redox-active functional groups. Consequently, biochar prepared at high temperature (>500 °C) generally exhibits superior electron transport properties. These temperature-dependent physicochemical characteristics, including electrical conductivity, surface functional groups, and porous structure, collectively influence the electron transfer efficiency of biochar in anammox systems. As discussed in Sect. 3.2.1, biochar-induced EPS enrichment may strengthen biofilm-associated redox transfer. Here, we focus on how the physicochemical properties of biochar influence DIET and MIET pathways. However, these pathways are unlikely to operate independently in biochar-enhanced anammox systems and are instead influenced collectively by biochar properties, reactor operating conditions, and microbial community. Firstly, the large surface area and porous structure of biochar provide favorable habitats for microorganisms, thereby promoting microbial enrichment. Following attachment to the biochar surface, anammox bacteria may secrete EPS to form early-stage biofilms. However, it remains unclear whether EPS enrichment precedes enhanced electron transfer or occurs as a consequence of improved electron transport (Kayoumu et al. 2025). As biofilm development progresses, microbial enrichment subsequently improves DIET efficiency. Alternatively, conductive biochar surfaces may initially promote electron transfer, which in turn stimulates EPS production and biofilm formation. The temporal relationship between these processes remains to be clarified. As DIET becomes progressively established and energy consumption decreases, the relative contribution of MIET may decline. As discussed in Sect. 3.1, the EEC of biochar is closely associated with pyrolysis temperature. Combined with the mechanisms described above, biochar produced via pyrolysis at 300–400 °C generally contains abundant electron-donating groups (e.g., phenolic/hydroquinone groups), exhibits relatively high redox activity, promotes EPS secretion, and facilitates MIET through electron-accepting behavior. In contrast, biochar produced at pyrolysis temperatures >700 °C typically possesses a higher degree of graphitization and electrical conductivity, which may favor DIET-related electron transfer processes. Current evidence supporting these mechanisms has been derived mainly from gene-expression analysis, EPS characterization, electrochemical measurements, and reactor-performance evaluations. Although these observations are consistent with enhanced EET, they are not pathway-specific. Direct confirmation of DIET or MIET requires electron-flux measurements, inhibitor-based experiments, isotopic tracing, or spatially resolved electrochemical analyses.

Previous studies have observed an association between biochar amendment and enhanced total nitrogen removal efficiency of the anammox system under all tested nitrogen loading conditions. However, the magnitude of improvement varied with the nitrogen load. For instance, in an anammox system with a nitrogen concentration of 100 mg L^{-1}, biochar addition (10 g L^{-1}) promoted nitrate reduction and improved nitrogen removal efficiency. Concurrently, the electron transfer capacity reached 0.89 meq e^{-1} g^{-1}, which was approximately three times higher than that of the control. Furthermore,

the abundance of *Candidatus Kueneia* increased significantly (Li et al. 2022). Another study observed a gradual decrease in anammox activity at total nitrogen concentrations above 250 mg L^{-1}, whereas biochar amendment increased the nitrogen concentration to 300 mg L^{-1}. This phenomenon was suggested to be associated with the enrichment of anammox bacteria, such as *Candidatus Brocadia* and *Candidatus Kueneia*, increased EPS production relative to the control, and a 2.6-fold increase in the copy number of *hzsB* gene (Chen et al. 2023a). Additionally, another study reported that, at a total nitrogen concentration of 440 mg L^{-1}, the anammox system exhibited higher EPS secretion and a larger population of anammox bacteria (Li et al. 2023). At relatively low nitrogen loads, oxygen-containing functional groups on the biochar surface (e.g., phenolic hydroxyl and quinone groups) may facilitate electron donation and potentially promote the reduction of nitrate to nitrite, which can subsequently be utilized by anammox bacteria, thereby contributing to nitrate recycling pathways. Biochar can also increase EPS secretion, providing additional attachment sites for microorganisms and potentially enhancing the abundance of anammox bacteria and denitrifiers. Although the enhancement effects of biochar are still observed under high nitrogen loading conditions, their magnitude appears to be weaker than that under low-load conditions. We hypothesize that high substrate load may cause localized imbalances in electron donors or nitrite, EPS restructuring, and reduced electron-flow efficiency. This should be tested using time-resolved nitrogen profiling, EPS characterization, and electrochemical monitoring. Therefore, for the treatment of high-strength wastewater, modified biochar or increased biochar dosage may be required to maintain enhancement effects. However, the benefits of increased biochar dosage for electron-transfer capacity should be balanced against potential drawbacks, including impaired mass transfer, altered sludge structure, and secondary contamination. The comparative effects of various types of biochar on anammox performance and their associated mechanisms are systematically summarized in Table 1.

Biochar has been reported to possess electron-donor and electron-acceptor capabilities, suggesting potential capacitive behavior and an ability to buffer electron transfer processes. Nevertheless, direct electrochemical verification within active biofilms is still required to distinguish capacitive effects from purely surface-mediated redox cycling. Although biochar can adsorb and release soluble redox mediators, such as flavins, excessive or irreversible adsorption may deplete these mediators and potentially inhibit MIET. Claims regarding the intrinsic electron shuttling capability of biochar, particularly

Table 1 Effects of different biochar on anammox process and its potential mechanism

Biochar feedstock	Anammox pyrolysis temperature (℃)	EAC/EDC (meq e^{-1} g^{-1})	Reactor type	Biochar dosage	Experimental duration	Key observed result	Dominant genus of anammox	Hypothesized mechanism	Reference
Bamboo	400	0.03/0.35	Serum bottles	10 g L^{-1}	40d	NRE:4.0–11.6%↑ NRR:5.6–18.0%↑	*Candidatus Brocadia*	EPS-mediated electron shuttling	(Wang et al. 2022b)
Corn stover	300	0.220/0.439	Serum bottles	10 g L^{-1}	60 h	Not reported	*Candidatus Kuenenia*	DIET	(Xu et al. 2021a)
Sawdust	300	0.79/2.64	Serum bottles	10 g L^{-1}	80 h	NRR:10.1%↑	*Candidatus Kuenenia*	DIET	(Li et al. 2022)
Corn stover	300	0.944/0.258	Serum bottles	10 g L^{-1}	60 h	NRE:17.5%↑ NRR:16.7%↑	*Candidatus Brocadia*	EPS-mediated electron shuttling	(Xu et al. 2022a)
		1.110/0.313	Serum bottles	10 g L^{-1}	60 h	NRE:34.6%↑ NRR:32.6%↑			
Coconut shell	400	0.458/3.370	Serum bottles	50 g L^{-1}	18 d	NRE:17.44%↑	*Candidatus Jettenia*	MIET	(Pang et al. 2023)
Coal biochar	550	0.211/3.060	Serum bottles	50 g L^{-1}	18 d	NRE:14.34%↑			
Corn stover	300	0.220/0.439	UASB	10 g L^{-1}	160 d	NRE:13.6%↑ NRR:12.99%↑	*Candidatus Kuenenia*	DIET	(Xu et al. 2020)
Corn stover	300	0.220/0.439	UASB	10 g L^{-1}	80 d	NRE:7.4–17.4%↑	*Candidatus Brocadia*	EPS-mediated electron shuttling	(Xu et al. 2021b)

↑: up

NRE nitrogen removal efficiency, *NRR* nitrogen removal rate, *B-EPS* the bound fractions of EPS, *S-EPS* the slime of EPS, *UASB* upflow anaerobic sludge blanket

those prepared at lower pyrolysis temperatures (300–400 °C), warrant further mechanistic investigation. Although this property is often compared with that of humic substances, the particulate and solid-phase characteristics of biochar suggest a distinct mode of action. The electron shuttle behavior of biochar differs fundamentally from that of dissolved electron shuttles, such as anthraquinone-2,6-disulfonate (AQDS) and flavins, although certain functional similarities exist. Firstly, both possess reversible redox-active functional groups that can serve as intermediate electron carriers by transferring electrons between microorganisms and terminal electron acceptors or donors (Zhang et al. 2023a). Secondly, the electron shuttling capacity of biochar likely originates primarily from surface quinone and phenolic functional groups (Xin et al. 2023). AQDS is a synthetic quinone compound, whereas flavins are naturally occurring nitrogen-containing heterocyclic compounds (e.g., riboflavin and flavin adenine dinucleotide) secreted by microorganisms. In contrast to these dissolved electron shuttles, biochar is a particulate solid material (Xin et al. 2023). During reactor operation, AQDS and flavins may be readily washed out with the effluent and, therefore, often require continuous replenishment. Compared to dissolved electron shuttles, biochar is less susceptible to washout and may provide a more persistent electron-transfer platform. Furthermore, biochar can also function as a microbial carrier and has been associated with increased production of EPS and flavins in certain microbial systems (Chen et al. 2024).

3.3 Current limitations in EET pathways

Current research demonstrates that biochar amendment in anammox reactors substantially enhances the EET capacity of anammox microbial communities, thereby optimizing the nitrogen removal process. Improper selection of biomass feedstocks or inappropriate conditions and methods for biochar preparation may lead to the generation of harmful constituents, such as heavy metals, polycyclic aromatic hydrocarbons (PAHs), environmentally persistent free radicals (EPFRs), dioxins, and perfluorinated chemicals (PFCs) (Xiang et al. 2021). Biochar surface contains both reversible and irreversible redox-active groups (Prévoteau et al. 2016). Over time, interaction with anammox bacteria can transform the irreversible fraction, contributing to biochar aging. This aging process may cause pore blockage and alter the specific surface area of biochar, which could subsequently affect microbial adhesion and biofilm formation, ultimately influencing its electron transfer efficiency (Wang et al. 2020b; Xiong et al. 2021). Furthermore, due to differences in feedstock heterogeneity and operating conditions, the elemental composition and surface functional groups of biochar can change after aging, with the number of oxygen-containing functional groups potentially increasing under specific conditions (Long et al. 2024). Therefore, there is currently no universal quantitative formula that links the extent of biochar aging to EET efficiency under varying conditions. However, the net effect of such changes on DIET within the anammox bacterial community remains unclear, requiring substantial evidence and further investigation. Simultaneously, aged biochar becomes more susceptible to biological degradation and physical breakdown; it may adsorb and subsequently release external contaminants, and could leach intrinsic pollutants (Mia et al. 2017; Xiang et al. 2021). These released toxins can directly inhibit the metabolic activity of anammox bacteria (Liu et al. 2024). Some researchers have reported that, after continuous addition of 5 mg L^{-1} of Cu^{2+} for 8 days in an anammox system, the nitrogen removal rate decreased to below 30% (Liu et al. 2025). Also, batch experiments using four aromatic compounds (o-cresol, p-nitrophenol, o-chlorophenol, and quinoline) revealed the irreversible toxic effects of o-cresol and o-chlorophenol, whereas p-nitrophenol and quinoline mainly caused reversible inhibition. Notably, o-chlorophenol (9 mg L^{-1}) reduced the specific anammox activity (SAA) by more than 60% (Ramos et al. 2015). Therefore, despite the ambiguous or even potentially positive role of altered surface chemistry in electron shuttling, the direct biocidal effect of released toxins likely predominates, leading to suppressed microbial activity and hindered metabolic electron flow, ultimately resulting in compromised nitrogen removal efficiency. Furthermore, biochar may promote electron transfer either by stimulating EPS secretion or by directly mediating electron transfer through surface functional groups. However, the temporal sequence of these processes remains unresolved. Therefore, further exploration of the temporal relationship between these two processes is necessary.

Although EPS-mediated electron shuttling, DIET, and MIET via quinone groups have all been proposed as potential pathways through which biochar enhances electron transfer in anammox systems, the current evidence remains largely indirect. Most mechanistic inferences have been drawn from electrochemical measurements, spectroscopic characterization, and microbial community analyses rather than from pathway-specific quantitative assays. Future research should integrate electron-flux measurements, isotope tracing, inhibitor experiments, and spatially resolved electrochemical analyses to determine the relative contributions of EPS-mediated transfer, DIET, and MIET. As summarized in Table 1, although

certain biochars exhibit higher EAC/EDC values or enhanced NH_4^+ removal, the relative contribution of each EET pathway varies considerably across studies, and direct comparisons are often complicated by inconsistent units, incomplete reporting, and differing experimental conditions. Consequently, the dominance of specific electron transfer pathways cannot be universally asserted. Nevertheless, substantial gaps remain in direct quantification of pathway contributions, particularly under varying degrees of biochar aging, surface chemistry, and reactor configurations.

4 Challenges and improvement strategies for biochar addition in anammox systems

Future research should proceed from mechanistic validation to material optimization and finally to sustainability assessment (Fig. 5). (1) For biochar preparation, strategic feedstock selection and optimization of pyrolysis temperature are essential to enhance intrinsic electrical conductivity and promote electron transfer. For instance, under identical pyrolysis conditions, cattle manure biochar demonstrated higher EEC and electrical conductivity than sawdust-derived biochar, which was primarily attributed to the abundance of oxygen-containing functional groups and redox-active metals (Wang et al. 2020a). Herbaceous biochar prepared at elevated temperatures also exhibits higher EEC and electrical conductivity compared to woody biochar, owing to enhanced graphitization and reduced charge transfer resistance (Kappler et al. 2014; Zhang et al. 2017). Therefore, key electrochemical properties of biochar, including EEC and electrochemical activity (ECA), can be assessed using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS), facilitating the screening and selection of biochars with higher EEC/EAC values, reducing charge-transfer resistance, and improving electrical conductivity. (2) The surface functional group characteristics of biochar are strongly influenced by its modification methods and treatment conditions, which in turn regulate its performance in anammox reactors. For example, the addition of KOH-modified biochar to an anammox reactor exposed to chloroquine phosphate (CQ) promoted the secretion of EPS with a higher proportion of α-helical structures, thereby alleviating the inhibitory effect of CQ on functional microbial communities (Hu et al. 2025). Metal-functionalized biochars generally exhibit more pronounced enhancement effects. In a previous study, iron-modified biochar significantly accelerated reactor start-up by stimulating the growth of functional microorganisms and outperformed conventional activated carbon in promoting anammox activity (Lu et al. 2021). In addition, iron- and zinc-modified

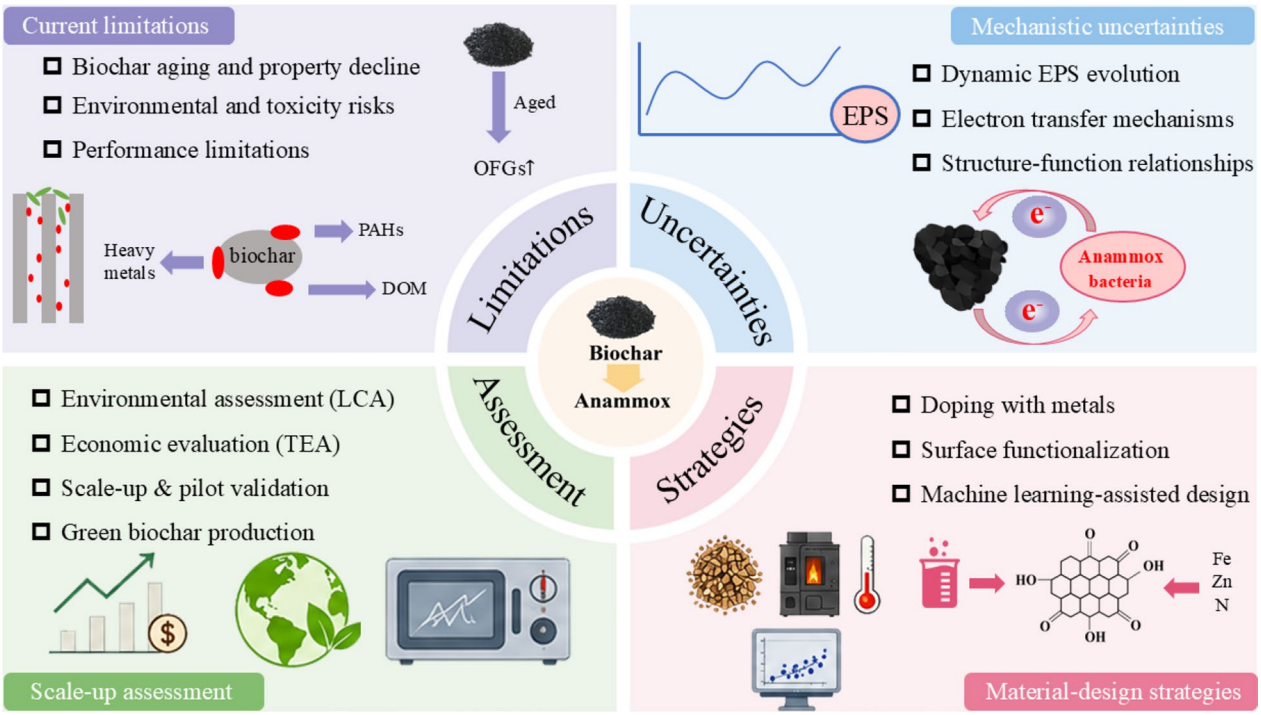


Fig. 5 Challenges and proposed improvement strategies for biochar addition in anammox systems

biochar composites further enhanced electron transfer within the reaction system due to their larger specific surface area and abundant redox-active functional groups (Liu et al. 2021). Therefore, targeted biochar modification strategies, such as metal/heteroatom doping and acid–base treatments, can effectively tailor the composition of surface functional groups, thereby optimizing electron transfer pathways and improving biochar applicability in anammox processes. (3) The optimization of biochar properties is often constrained by the diversity of feedstocks and the complex combinations of pyrolysis parameters and modification strategies, making conventional approaches time-consuming and inefficient. Therefore, efficient methods are required to identify optimal pyrolysis conditions. A representative example is the study conducted by Zhao et al. (2024), in which four representative machine learning models were developed to accurately predict key physicochemical properties of biochar, including specific surface area, average pore size, and total pore volume, under different acidic treatment conditions. In addition, some researchers have employed machine learning to develop composite index models for predicting the adsorption performance of biochar and identifying optimal pyrolysis conditions and feedstock selection (Chen et al. 2025a). These findings demonstrate that machine-learning-based prediction of biochar physicochemical properties can provide valuable guidance for optimizing pyrolysis conditions. (4) Biochar production is inevitably accompanied by energy consumption and carbon emissions. To advance its sustainable implementation, future studies should benchmark anammox performance within a LCA framework, with particular emphasis on global warming potential (GWP) and cumulative energy demand (CED). Specifically, the energy input required for biochar production (300–700 °C) should be carefully balanced against the energy savings achieved through reduced aeration and external carbon addition during the anammox process. Meanwhile, TEA should incorporate net present value (NPV) modeling by balancing the high initial costs of biochar synthesis with the long-term economic benefits associated with enhanced nitrogen removal efficiency. To further reduce the energy consumption associated with biochar production, emerging technologies such as microwave-assisted pyrolysis and hydrothermal carbonization (HTC) should be considered (Fernández et al. 2024; Huang et al. 2024; Wang and Wang 2019). (5) The dynamic evolution of EPS is a critical aspect for understanding biochar-enhanced electron transfer in anammox systems. However, in situ and real-time observation techniques for monitoring EPS dynamics within anammox systems are still under development. Therefore, future studies should focus on advanced characterization approaches, including confocal laser scanning microscopy (CLSM), fluorescence in situ hybridization coupled with CLSM (FISH–CLSM), and Cryo-SEM/Cryo-TEM, to investigate the dynamic evolution of EPS after biochar addition into anammox systems. Research efforts are required for elucidating the molecular basis underlying the interactions between EPS and c-type cytochromes associated with biochar, as well as clarifying the potential mechanisms of electron transfer between EPS and biochar. In addition, further investigations are needed to evaluate the changes in the surface functional groups of biochar before and after the involvement of biochar in anammox electron transfer processes, thereby improving the understanding of its aging dynamics. Based on the above challenges and corresponding improvement strategies, future research should systematically address current limitations and mechanistic uncertainties, advance rational biochar design, and evaluate sustainability for large-scale implementation, as summarized in Table 2.

Finally, further research is imperative to elucidate the dominant electron transfer pathways in biochar-enhanced anammox systems. Such mechanistic insights will guide targeted strategies to optimize electron flux and nitrogen removal efficiency in anammox systems.

5 Conclusions

As a redox-active material, biochar can enhance EET in anammox systems, thereby improving nitrogen removal performance. Current evidence supports three non-exclusive proposed mechanisms through which biochar may facilitate electron transfer: (1) EPS-mediated enrichment of redox-active compounds within the biofilm matrix; (2) DIET through conductive biochar interfaces; and (3) MIET through redox-active functional groups. These mechanisms may operate simultaneously, thereby collectively enhancing electron flux in anammox microbial communities. However, the relative contribution of each pathway remains uncertain and warrants further investigation using advanced electrochemical, molecular, and imaging techniques. Nevertheless, the relative contributions of EPS-mediated transfer, DIET, and MIET remain difficult to generalize, as they are influenced by biochar properties, aging state, microbial community structure, and reactor configuration. Future research should focus on leveraging machine learning to optimize the design and functionality of biochar and further elucidating its dominant electron transfer pathways during anammox, thereby achieving targeted improvements in nitrogen removal efficiency and operational stability of anammox systems.

Table 2 Future research framework for advancing biochar-enhanced EET in anammox systems

Research priority	Main challenge	Recommended future research	Expected outcome
Biochar optimization and stability	Biochar performance relies on feedstock, pyrolysis conditions and aging, while its long-term stability and environmental impacts remain poorly understood	Optimize feedstock selection and pyrolysis conditions to enhance EEC, electrical conductivity and graphitization of biochar, and evaluate its aging-induced changes in surface chemistry and electrochemical properties using CV, EIS, XPS, FTIR and Raman spectroscopy	Development of biochars with enhanced ETC, long-term stability and environmental safety
Surface functionalization and material engineering	Surface functional groups largely determine EET efficiency	Optimize metal/heteroatom doping and acid–base treatments to regulate redox-active functional groups and EET pathways	Improved EET efficiency and anammox performance
Biochar design assisted by machine learning	Empirical optimization is constrained by the large parameter space of feedstocks, pyrolysis and modification strategies	Develop machine learning models linking biochar preparation conditions with electrochemical properties and reactor performance to rapidly identify optimal biochar designs	Rational biochar design and reduced experimental effort
Mechanistic validation of EPS-mediated electron transfer	The dynamic evolution of EPS and dominant EET pathways remain poorly understood	Combine CLSM, FISH–CLSM, Cryo-SEM/Cryo-TEM, electrochemical analyses and multi-omics approaches to elucidate the interactions among EPS, biochar, and c-type cytochrome and distinguish DIET from MIET pathways	Better understanding of molecular mechanisms governing biochar-enhanced EET
Sustainability assessment and scale-up	Large-scale implementation requires balancing environmental impacts, economic feasibility and process performance	Integrate LCA, TEA, pilot-scale validation and low-energy biochar production technologies (e.g., microwave-assisted pyrolysis and hydrothermal carbonization)	Economically viable and environmentally sustainable large-scale application of biochar-enhanced EET

Author contributions
All authors contributed to the study conception and design. Writing—original draft, Investigation, Conceptualization by Wenya Zhao. Writing—review & editing by Wenqi Li, Yuheng Zhu, Yidi Li, Mabruk Adams, and Hanbo Chen. Writing—review & editing, Writing—original draft, Visualization, Funding acquisition, Conceptualization by Chongjun Chen. All authors read and approved the final manuscript.

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Data availability
The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interests
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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