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Research Paper

Micro-aeration assisted with electrogenic respiration enhanced the microbial catabolism and ammonification of aromatic amines in industrial wastewater

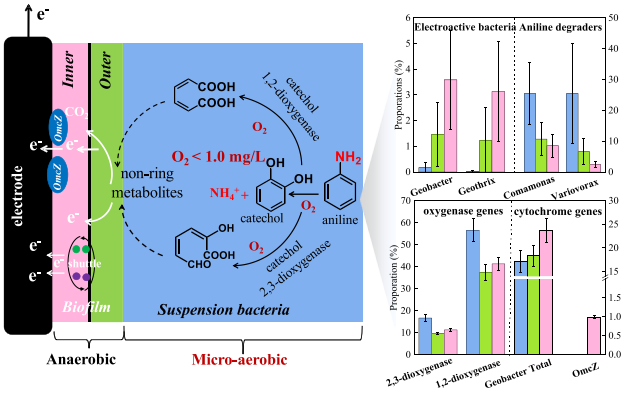
Ke Shi [a], Haoyi Cheng [a], Carolyn R. Cornell [b], Haiwei Wu [a], Shuhong Gao [a], Jiandong Jiang [c], Tiejun Liu [a], Aijie Wang [a], Jizhong Zhou [d,e,f,g], Bin Liang [a,*]

[a] State Key Laboratory of Urban Water Resource and Environment, Shenzhen Key Laboratory of Organic Pollution Prevention and Control, School of Civil & Environmental Engineering, Harbin Institute of Technology Shenzhen, Shenzhen 518055, China
[b] Department of Civil and Environmental Engineering, Rice University, Houston, TX 77005, USA
[c] Key Lab of Agricultural Environmental Microbiology, Ministry of Agriculture, College of Life Sciences, Nanjing Agricultural University, 210095 Nanjing, China
[d] Institute for Environmental Genomics and Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK 73019, USA
[e] School of Civil Engineering and Environmental Sciences, University of Oklahoma, Norman, OK 73019, USA
[f] School of Computer Science, University of Oklahoma, Norman, OK 73019, USA
[g] Earth and Environmental Sciences, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

HIGHLIGHTS

- Aniline (AN) could be degraded under micro-aerobic bioanode system.
- Microbial functional differentiation occurred in suspension and electrode.
- Functional genes were more sensitive than 16S rRNA genes to DO stimulation.
- AN degraders in suspension positively correlated with electroactive bacteria.
- AN degraders were potential hosts of dioxygenase catalyzing ammonification.

GRAPHICAL ABSTRACT



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ABSTRACT

Improvement of refractory nitrogen-containing organics biodegradation is crucial to meet discharged nitrogen standards and guarantee aquatic ecology safety. Although electrostimulation accelerates organic nitrogen pollutants amination, it remains uncertain how to strengthen ammonification of the amination products. This study demonstrated that ammonification was remarkably facilitated under micro-aerobic conditions through the degradation of aniline, an amination product of nitrobenzene, using an electrogenic respiration system. The microbial catabolism and ammonification were significantly enhanced by exposing the bioanode to air. Based on 16S rRNA gene sequencing and GeoChip analysis, our results indicated that aerobic aniline degraders and

* Corresponding author.
E-mail addresses: liangbin1214@163.com, liangbin1214@hit.edu.cn (B. Liang).

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electroactive bacteria were enriched in suspension and inner electrode biofilm, respectively. The suspension community had a significantly higher relative abundance of catechol dioxygenase genes contributing to aerobic aniline biodegradation and reactive oxygen species (ROS) scavenger genes to protect from oxygen toxicity. The inner biofilm community contained obviously higher cytochrome *c* genes responsible for extracellular electron transfer. Additionally, network analysis indicated the aniline degraders were positively associated with electroactive bacteria and could be the potential hosts for genes encoding for dioxygenase and cytochrome, respectively. This study provides a feasible strategy to enhance nitrogen-containing organics ammonification and offers new insights into the microbial interaction mechanisms of micro-aeration assisted with electrogenic respiration.

1. Introduction

Refractory nitrogen-containing organics (e.g., nitrobenzene, azo dye, and amide compounds) derived from industrial production and discharge are the limiting factor for wastewater denitrification [14,39,43,49,60]. Moreover, if untreated, organic nitrogen in wastewater cannot meet government's effluent standards and guarantee ecology security of aquatic ecosystems. The aromatic compounds with electron-withdrawing group (e.g., nitro group, azo bond) need to be firstly reduced under anaerobic conditions. Traditional biological treatment struggles to degrade these compounds due to the low electron cloud density and large steric hindrance formed by the benzene ring and nitrogen coupling [8]. Recently, electrostimulation has proved a feasible wastewater treatment approach to enhance the C-N bond cleavage/nitro group reduction and facilitate the amination (refractory organic nitrogen to aromatic amine, Fig. 1) [19,23,27,40,46,47]. However, the daughter products (e.g., aromatic amines) still require ammonification (aromatic amine to NH_4^+) to ultimately eliminate the environmental risks. Considering the initial stage of aromatic aniline oxidation is not thermodynamically favorable under anaerobic conditions based on Gibbs energy [6], oxygen is essential to accelerate benzene ring structure cleavage [15].

In electrogenic respiration systems, oxygen is generally considered to be unfavorable because it has the potential to act as a competitive electron acceptor, which could adversely impact current production and pollutant reductive degradation [3,4,54]. The presence of oxygen would also be toxic to marginally aerotolerant or strictly anaerobic bacteria such as methanogens [35]. However, several recent studies reported that the addition of very small amounts of air to anaerobic biological systems has positive impacts on hydrolysis, volatile fatty acid (VFA) production, and controlling process stability [52,62]. Micro-aeration based anaerobic processes have the potential to create unique niches to balance the metabolic activity of anaerobes and aerobes, where aerobes could utilize oxygen while anaerobes were protected from oxygen toxicity [36,55]. Integrating anaerobic reduction with aerobic oxidation reactions could also facilitate energy conversation and pollutant mineralization [41]. Furthermore, it is feasible that the electrostimulated system integrated with micro-aeration might enhance pollutants degradation efficiency and power output [6,9–11,56]. For instance, an anode biofilm under microaerobic condition significantly promoted pyridine bio-mineralization. The species related to pyridine biodegradation such as *Desulfovibrio* and *Dokadonella* were enriched, which was believed to contribute to better reactor performance [20]. Generally, microorganisms are an essential component and play significant roles in biological treatment process [50]. Linking community composition, functional potential, and interaction networks with biological processes is believed to be critical to understanding ecology mechanisms and controlling system performance [58]. However, the patterns in the specific niches (e.g., electrode or suspension) with respect to the taxonomic and functional profiles of bacterial community as well as their relationship remain poorly understood.

The advances in high-throughput sequencing of culture-independent technologies (e.g., 16S rRNA gene sequencing and GeoChip) and bioinformatics approaches (e.g., molecular ecology network), have greatly allowed for a shift from descriptive studies to mechanistic and predictive frameworks for harnessing beneficial microbial communities and desired outcomes. These high-resolution nucleic acid-based molecular methods have also revolutionized our knowledge and manipulation of wastewater treatment biotechnology [33,42,57,65,67–70]. Research priorities for sludge/biofilm microbiomes include illustrating the microbial functional mechanisms that mediate the improved operational performance. For example, metagenomic data in combination with network analysis showed electrostimulation strengthened microbial associations and helped generate a feasible approach to regulate a sludge microbiome for more efficient pollutant degradation [47,48]. Thus, we are attempting to explore the microbial community composition, function, and associations in a micro-aeration assisted with electrogenic respiration, determine the nitrogen-containing organics

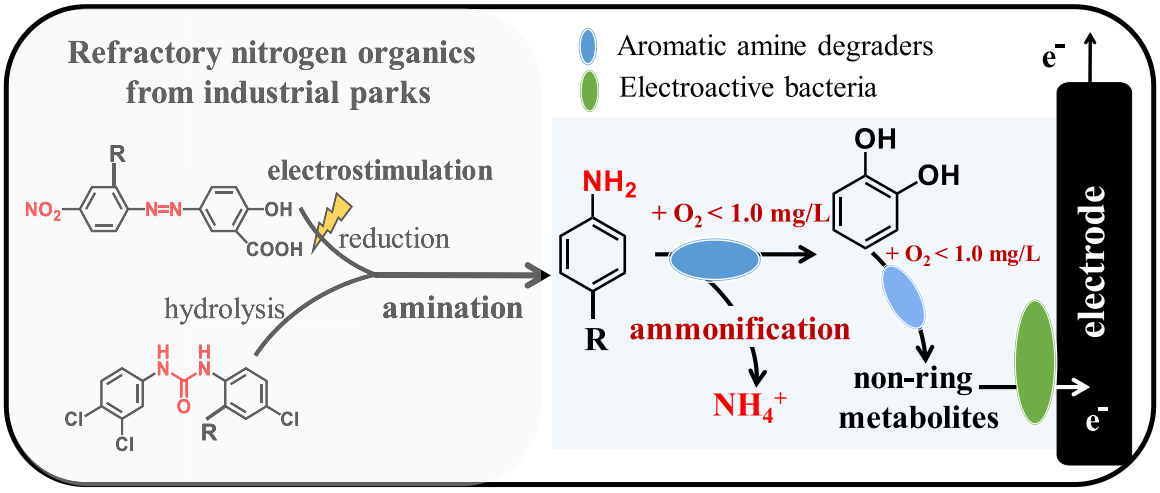

Fig. 1. Hypothesis for nitrogen-containing organics from amination to ammonification by micro-aerobic conditions assisted with electrostimulation.

ammonification efficiency, and bridge the gap between microbial community and system performance.

In this study, we constructed a micro-aeration assisted with electrogenic respiration system and investigated the aniline (typical amination product from nitroaromatics, amide, and azo compounds) degradation efficiency as well as current production by discerning bioanode microbial communities. The anode biofilm, classified as an inner and outer layer, and suspension were characterized using a microarray GeoChip (v4.6) [45] and 16S rRNA gene sequencing. Key functional bacteria and genes involved in electron transfer and benzene-ring cleavage were also analyzed. We hypothesize that (i) aniline could be substantially catabolized under micro-aerobic conditions and the small molecule metabolites utilized for current generation, (ii) micro-aeration would significantly change the taxonomic and functional microbiome structure and selectively enrich different functional members in each niche (i.e. suspension, outer biofilm, and inner biofilm), and (iii) metabolic cascade collaboration of aerobic and anaerobic functional bacteria would promote aniline biodegradation and current generation simultaneously (Fig. 1). This study demonstrates a promising technique for refractory nitrogen-containing organics deconstruction and ammonification and provides new insights into microbial associations in response to electrostimulation coupled with micro-aeration.

2. Materials and methods

2.1. Micro-aerobic reactors setup and operation

A dual-chamber bioelectrochemical system (BES) reactor (175 mL of each) was configured with identical glass chambers separated by a cation exchange membrane (Ultrex CMI-7000, Membranes International, U.S.). Graphite fiber bush was used as both anode and cathode with the saturated calomel electrode (SCE, + 247 mV vs standard hydrogen electrode, SHE) used as the reference electrode to measure anode potential. The anode and cathode were connected through a 1000 Ω resistor. One tube connected with a valve was inserted into the anode chamber to meet the micro-aerobic (dissolved oxygen, DO = 0.1–1.0 mg/L) [36,62] or anaerobic conditions. At the acclimation stage, the valve was opened and nutrient medium (50 mM phosphate buffered saline (PBS), 3.74 mM NH_4Cl, 1.74 mM KCl, 10 mL/L Wolf's vitamins, 10 mL/L Wolf's trace elements, pH = 7) containing 2.8 ± 0.1 mM aniline filled the anode chamber. The anode chamber was inoculated with activated sludge (a domestic wastewater treatment, Harbin, China) and effluent from a VFA feeding microbial electrolysis cell. The cathode chamber was filled with 50 mM PBS amended with 100 mM potassium ferricyanide. The generated current was determined by a data acquisition system (Model 2700, Keithley Instru. Inc., U.S.) which measured the voltage of the resister and then converted it to current based on Ohm's law. The anode solution was replaced when the current decreased. After stable current generation, half of the bioreactors were operated under anaerobic conditions by turning off the gas valve to cut off the air into the anodic headspace as the control group. The anaerobic anode control group is essential for examining the importance of micro-aerobic anode enhancing the oxidation of aniline.

2.2. Chemical analysis

Aniline concentration (0.1–3.0 mM) for the anode chamber was determined by high-performance liquid chromatography (HPLC, 2695, Waters, U.S.). The aniline degradation efficiency and power density were calculated as previously described [6]. DO (0–20 mg/L) in anolyte was measured using a DO sensor (WTW GmbH, Germany). Total organic carbon (TOC) concentration (0.1–20.0 mM) in anolyte was measured with a TOC analyzer (Fusion TOC, Tekmar-Dohrmann, U.S.). NH_4^+ concentration (0.1–3.0 mM) was determined according to the standard methods [18].

2.3. GeoChip hybridization, 16S rRNA gene sequencing, and data analysis

At the end of the chemical test, three biological samples were collected from the suspension (S), inner electrode biofilm (In), and outer (Ou) biofilm respectively for micro-aerobic group. The total genomic DNA was extracted according to established methods [66]. DNA purity and quantity were determined by a Nano-Drop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, U.S.) and by a PicoGreen using FLUOstar Optima (BMG Labtech, Jena, Germany), respectively. The primer pair consisting of forward the primer 515 F (5′-GTGCCAGCMGCCGCGG-3′) and the reverse primer 806 R (5′-GGACTACHVGGGTWTCTAAT-3′) targeting the V4 hypervariable regions of bacterial 16S rRNA gene was selected for high throughput sequencing [24].

Additionally, extracted DNA from each sample was used for GeoChip v4.6 functional gene analysis as described elsewhere [17,25,26,45]. In short, DNA was labeled with random priming and Cy-3 dye using the Klenow fragment of DNA polymerase I in a MAUI hybridization station (BioMicro, Salt Lake City, UT, U.S.). Once hybridization was complete, slides were washed and imaged using a NimbleGen MS200 scanner (Roche, Madison, WI, U.S.) The images were processed using Imagene software (6.1 premium version, Biodiscovery, El Segundo, CA, U.S.). Signal intensities were measured based on the scanned images, and spots with signal-to-noise ratios (SNR) lower than 3 were removed before statistical analysis.

Sequencing data of 16S rRNA gene amplicons were analyzed by removing PhiX sequences, joining paired-end reads using Flash [32], trimming ambiguous reads (N), removing short sequences (< 240 bp), and screening for chimeras using UCHIME [12]. Next, the 16 S rRNA gene sequences were classified into operational taxonomic units (OTUs) at a 97% sequence similarity threshold. The taxonomy of the 16S rRNA gene sequences was assigned by the RDP classifier with 50% confidence. Detrended correspondence analysis (DCA) and three nonparametric multivariate tests (multiple-response permutation procedure (MRPP), permutational multivariate analysis of variance (Adonis), and analysis of similarity (ANOSIM)) were calculated using R v4.2.1 to compare microbial and functional structure in the suspension and electrode biofilm. Hierarchical clustering analysis was conducted using CLUSTER v3.0 and visualized using TREEVIEW to assess separation of functional genes in suspension and electrode biofilm. Co-occurrence associations among microbial community and functional genes were explored by computing all pairwise Spearman's coefficients and the network analysis was visualized by Gephi (0.9.2) to show the microbial associations and potential genes host [63]. The *P* value of the difference between treatments was calculated using a two-tailed unpaired Student's *t*-test.

3. Results and discussion

3.1. Micro-aerobic bioreactors performance

To establish the micro-aerobic condition in the bioreactors, the anode headspace was opened to the atmosphere resulting in an initial DO concentration of 0.61 ± 0.17 mg/L. Once the micro-aerobic anode successfully acclimated as detected by stable current generation, the anodes were operated under anaerobic (control) and micro-aerobic conditions, respectively. Micro-aerobic conditions obviously increased aniline degradation efficiency and power density compared to the control (Fig. 2a). Additionally, the current generation lagged behind the aniline degradation under micro-aerobic conditions. The measured TOC was always higher than the calculated value of aniline (Fig. 2b), indicating some organic metabolite production along with aniline degradation. In contrast, the acclimation of the anaerobic bioanode was not successful with much lower current when aniline was the sole substrate, suggesting it could hardly be directly used as a substrate for electroactive bacteria. The concentration of NH_4^+ under micro-aeration

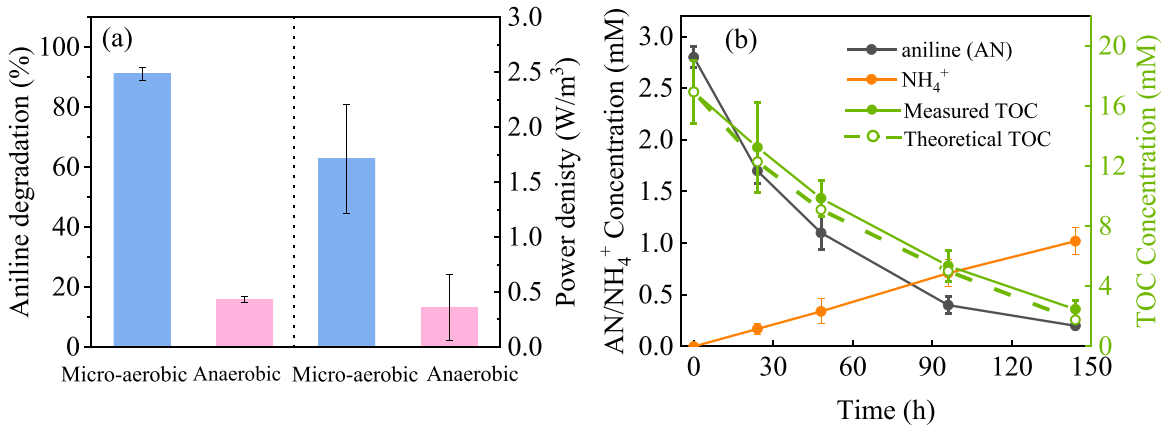

Fig. 2. Bioreactor performance. Aniline degradation (left) and electrical power output (right) (a), and the concentration of NH_4^+ and TOC under micro-aerobic condition (b).

conditions was increased with aniline degradation, further indicating the occurrence of ammonification (Fig. 2b). However, the NH_4^+ concentration was lower than the theoretical concentration. Since aniline was the sole carbon and nitrogen source, the NH_4^+ was likely oxidized in the presence of oxygen and utilized by microbial metabolism. Together, the results suggest that aniline could be more efficiently catabolized under micro-aerobic conditions and the small-molecule metabolites were used by electroactive bacteria to extracellularly transfer electrons instead of aniline.

3.2. Response of taxonomic and functional microbiome to micro-aerobic stimulation

To prove how was aniline degraded and metabolites acted as the substrate for current generation, DCA was carried out to elucidate how micro-aerobic conditions affect the taxonomic (16 S rRNA gene) and functional gene structure as well as the composition of suspension and bioanode communities. The suspended microbial communities were noticeably separated from the anode biofilm microbial communities, especially the inner biofilm communities (Fig. 3). Although the inner biofilm communities have some overlap with the outer biofilm communities, the functional gene structure of the inner biofilm clearly differed from the outer biofilm. Overall, there was a greater observable difference in the suspended and anode biofilm functional gene structure compared to the taxonomic structure. This reinforced recent works that the shift of functional gene structure was more significant among each niche than16S rRNA gene structure [31,53]. This is likely due to the high heterogeneity of sampling environments, low taxonomic resolution, or high noise associated with amplicon sequencing [69]. Also, the microbial responses to environmental stress could be more sensitive at the functional gene level than the taxonomic level. Furthermore, three nonparametric multivariate statistical methods (ANOSIM, PERMANOVA, and MRPP) strongly indicated the taxonomic community structure and functional gene structure significantly differed between the inner biofilm and suspension compared with the outer biofilm based on statistical analysis and *P* value (Table 1). The genes related to electron transfer and stress responses also showed a significantly higher dissimilarity between inner biofilm and suspended communities (Table 1).

3.3. Potential functions of dominant genera

A total of 465 genera were classified among the suspension, inner and outer electrode biofilm communities. As shown in Fig. 4, *Comamonas* and *Variovorax* were noticeably enriched in the suspension with their relative abundance totaling more than 50% of the community. Previously reported *Comamonas* harboring gene *tad* (aniline-degrading gene) was enriched with DO supply, which might be the main reason for efficient aniline removal [59]. For a much more stable and high-performance system of aniline removal, *Variovorax* also showed higher relative abundance. In addition, Breugelmans et al. found a

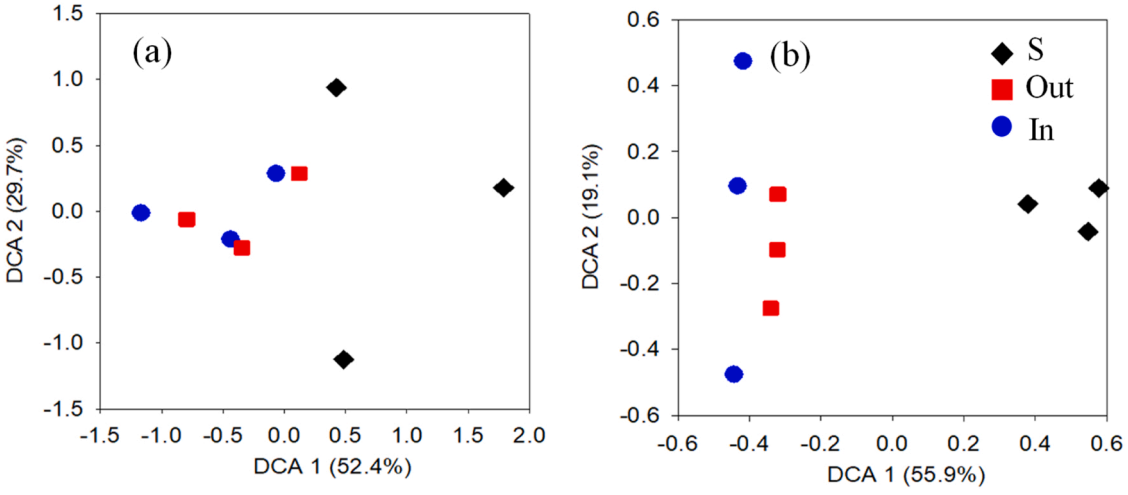

Fig. 3. Taxonomic and functional gene community structure. Detrended correspondence analysis (DCA) based on Bray-Curtis distance of identified OTUs from 16S rRNA genes sequencing (a) and all functional genes (b) in inner biofilm (In), outer biofilm (Out), and suspension (S).

Table 1
Significance test of the effect of micro-aerobic condition on the bioanode microbial community and functional structure with three different statistical approaches (Bold values indicate $P < 0.1$).

	Jaccard Dissimilarity						Bray Curtis Dissimilarity					
	Adonis		Anosim		MRPP		Adonis		Anosim		MRPP	
	F	*P*	*R*	*P*	*δ*	*P*	*F*	*P*	*R*	*P*	*δ*	*P*
All functional Genes (In vs S)	5.45	**0.055**	0.96	**0.096**	0.28	0.109	6.28	**0.001**	0.96	0.104	0.18	0.155
All functional Genes (Out vs S)	6.34	**0.067**	1.00	**0.098**	0.27	0.102	7.43	**0.001**	1.00	**0.092**	0.17	**0.095**
16 S rRNA 97% cutoff (In vs S)	1.35	**0.001**	0.41	0.108	0.65	**0.091**	1.54	**0.001**	0.30	**0.099**	0.63	0.103
16 S rRNA 97% cutoff (Out vs S)	1.29	**0.001**	0.37	**0.093**	0.65	0.108	1.32	0.155	0.15	0.316	0.62	0.317
e^- transfer related genes (In vs S)	4.67	**0.028**	0.96	0.102	0.30	**0.096**	5.44	**0.016**	1.00	0.103	0.19	**0.088**
e^- transfer related genes (Out vs S)	5.82	**0.049**	1.00	0.098	0.28	**0.081**	6.56	**0.033**	1.00	**0.087**	0.17	**0.100**
Stress genes (In vs S)	5.80	**0.001**	1.00	0.101	0.37	0.109	7.67	**0.016**	1.00	**0.090**	0.24	**0.084**
Stress genes (Out vs S)	6.32	**0.023**	1.00	0.102	0.36	0.106	8.49	**0.012**	1.00	**0.100**	0.23	0.111

Abbreviations: Suspension (S), Outer biofilm (Out), Inner biofilm (In).

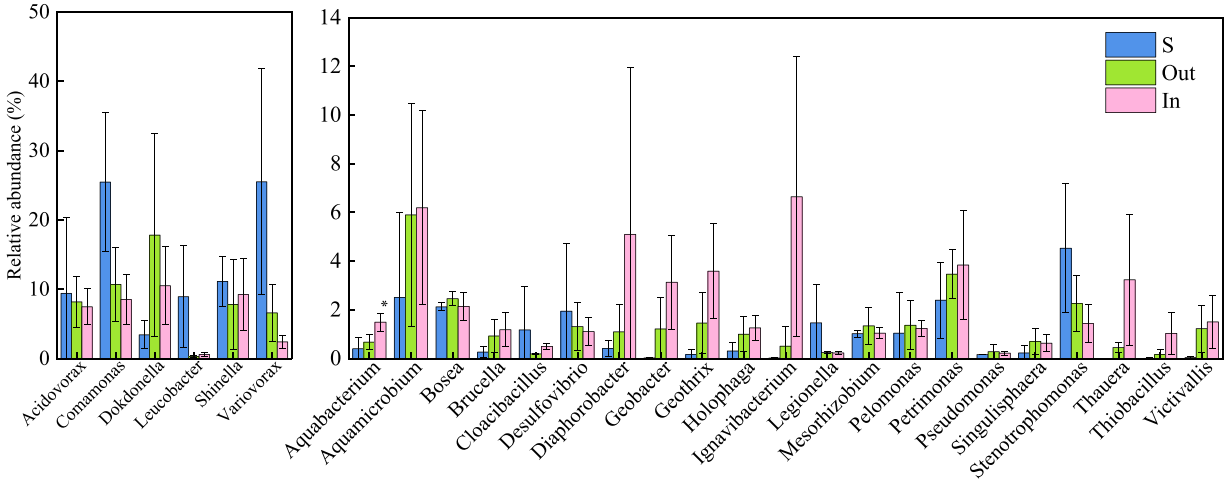

Fig. 4. The dominant genera in the suspension and anode communities under micro-aerobic condition. Bars represent the standard deviation from three biological replicates.

multicomponent aniline dioxygenase enzyme in 3,4-dichloroaniline (DCA) degradation in *Variovorax* sp. strain WDL1 [2,37,5]. Other bacteria such as *Stenotrophomonas* and *Leucobacter* which have the ability to aerobically degrade aniline were also enriched in the suspension [61, 71]. The increased presence of known aniline degraders in suspension explains the enhanced aniline ammonification by benzene ring cleavage. Although anaerobic aniline degradation is slow and thermodynamically unfavorable, a previous study also reported it was degraded by *Ignavibacterium* and *Acidovorax* under anaerobic conditions [44]. Consistently, *Ignavibacterium* was distinctly enriched in the inner electrode biofilm with low oxygen concentration. Key electroactive bacteria were also dominant in the electrode biofilm including *Geobacter*, *Geothrix,* and *Aquamicrobium* [30,51]. The relative abundance of *Geobacter* and *Geothrix* were higher in the inner biofilm than in the outer biofilm likely attributed to the fact that they can directly transfer electrons to the electrode in the absence of oxygen. *Geobacter* spp. are typically the most abundant known exoelectrogens, which transfer electrons through conductive pili or outer-membrane cytochromes to the anode. Also, *Geothrix fermmentans* can utilize the electrode as an electron acceptor to transfer electrons by secreting two different redox electron shuttles as electron mediators [34].

Based on the taxonomically identified aniline degraders and electroactive bacteria, the potential function of these dominant genera discussed above was proposed. Firstly, most of the dominant genera (e.g., *Comamonas* and *Variovorax*) capable of aerobically degrading aniline were enriched in the suspension due to the existence of limited DO. Considering the DO dilution and utilization from suspension to the electrode, DO concentration decreased to the lowest level in the inner electrode biofilm. Subsequently, the relative abundance of the aerobic aniline degraders in outer electrode biofilm were lower than in the suspension, but higher than those of the inner biofilm. The outer biofilm might be the transition area between the suspension and inner niches. Finally, most of the genera identified with electrochemical activity (e.g., *Geobacter*) were dominant in the inner biofilm utilizing the electrode as an electron acceptor under an anaerobic environment. Although dominant functional species were selectively enriched in different niches based on oxygen stress, the functional genes outline need to be further investigated considering the different functional gene structure in each niche was more significant than the taxonomic microbiome structure.

3.4. Functional genes

GeoChip hybridization analysis was further used to investigate the response of microbial functional genes to oxygen stimulation. The first step in biological mineralization of aniline was transformed to catechol and further cleaved by various dioxygenases, both meta and ortho cleavages could be occurred [29]. Importantly, the enhanced expression of gene encoding catechol 2,3-dioxygenase and meta-pathway promoter operated under oxygen-limited conditions [21]. Catechol 1,2-dioxygenase could catalyze the intradiol cleavage of aromatic ring at ortho position of catechol, which could produce cis-caroxylic acid with incorporation of two atoms of molecular oxygen into the substrate [16]. The two pathways may be co-occurred because both of genes encoding for catechol 1,2- and 2,3-dioxygenases were detected in the microbial communities. Oxygenase activated by the presence of oxygen is essential to enhance benzene ring structure cleavage [7]. Therefore, the total abundance of the genes encoding for catechol dioxygenase was significantly higher in the suspension community (Fig. 5a), which is consistent

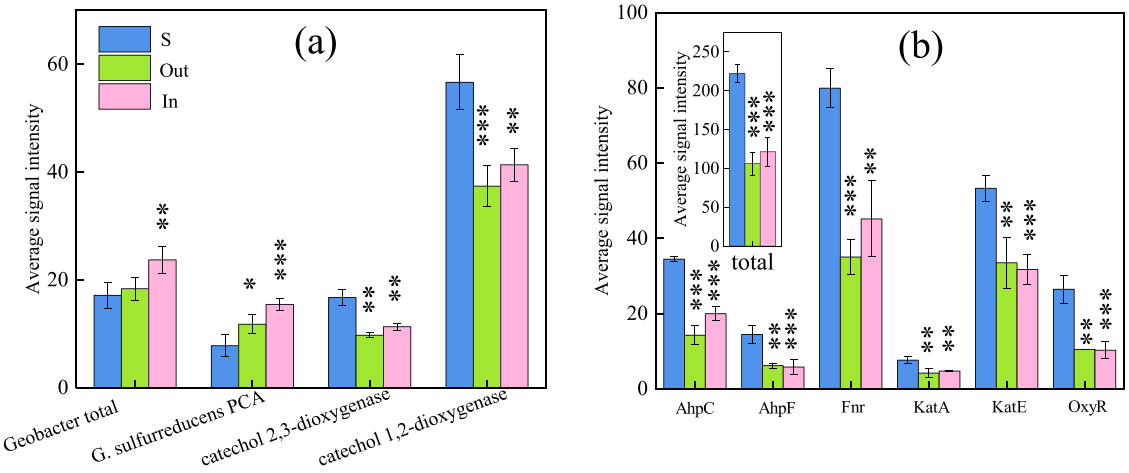

Fig. 5. Comparison of the signal intensity of genes encoding for cytochrome *c* and catechol dioxygenase (a), anti-ROS enzymes (b) (Bars represent the standard deviation from three biological replicates. Student's t-test between suspension and outer biofilm as well as inner biofilm, * $P < 0.1$, ** $P < 0.05$, *** $P < 0.01$).

with the suspension distinctly harboring higher aerobic aniline degraders (e.g., *Comamonas* and *Variovorax*). Hierarchical clustering analysis of catechol-dioxygenase genes showed that the suspension microbial communities were well separated from electrode biofilm communities, and there was also a clear difference between the inner and outer electrode biofilms (Fig. S1). Specifically, two dioxygenase genes from *Aspergillus niger* (145230746, *ortho*) and *Roseobacter* sp. (126717171, *ortho*), were unique in the electrode biofilm communities (Fig. S1). Additionally, several of the dioxygenase genes (i.e, gene ID 167034310, 154162742, and 56799003) were only detected in the inner biofilm communities. Lastly, dioxygenase gene-carrying bacteria such as *Comamonas testosteroni* (117998411, *ortho*) and *Chloroflexus aggregans* (118046180, *meta*) were exclusively present in the suspension communities, which agreed with the taxonomic composition. We also focused on cytochrome *c* proteins since they are a key component for direct electron transfer in dissimilatory anaerobic respiration of extracellular electron acceptors [22]. In this study, a total of 90 cytochrome *c* genes from several electroactive genera (*Geobacter*, *Shewanella*, *Pseudomonas*, *Desulfovibrio*, and *Rhodobacter*) were detected among all the communities. Although the total collective signal intensity for cytochrome *c* genes did not significantly differ between the suspension and electrode communities, the total signal intensities from *Geobacter* and *G. sulfurreducens* PCA were significantly higher in the inner biofilm communities (Fig. 4a). This was consistent with the higher relative abundance of *Geobacter* based on 16S rRNA gene sequencing results. Additionally, the cytochrome *c* genes from *Geobacter* were well separated between suspension and electrode biofilm communities (Fig. S2). Particularly, cytochrome *c* belonging to OmcZ genes from *Geobacter sulfurreducens* PCA (39997174) was uniquely detected in the inner biofilm. Significant investigations of extracellular electron transfer (EET) by anode biofilm mainly composed of *G. sulfurreducens* have implicated diverse redox active outer membrane c-type cytochrome. Whole genome analysis of gene transcript abundance of OmcB and OmcE showed higher expression in cells grown on electrode with oxidizing potentials. After simultaneous deletion of these genes, adaption by this strain is accompanied by up-regulation of OmcZ with current generation [38].

In the micro-aerobic environment, facultative and aerobic microorganisms consume and partially reduce oxygen molecules by generating reactive oxygen species (ROS), which could damage the microbial cell membrane, protein, and DNA [13]. Regulatory genes encoding for hydroperoxide reductase and catalase produced by aerobic or facultative bacteria are involved in oxygen shock response to allow them to thrive in an aerobic condition. The total abundance of anti-ROS genes was significantly higher ($P < 0.05$) in the suspension than those in the electrode biofilm (Fig. 5b). Specifically, this included the genes coding for AhpC, AhpF, Fnr, KatA, KatE, and OxyR. The distribution of oxygen response genes was consistent with the DO dilution profile in the biological system, which implies the suspended microbial communities have higher oxygen tolerance.

Collectively, oxygen stimulation enhanced the relative abundance of genes related to aerobic aniline biodegradation in the suspended microbial communities, and it did not inhibit the electrode biofilm respiring activity. The abundance of functional genes associated with EET such as cytochrome *c* were significantly higher in the inner electrode biofilm communities to satisfy terminal current output. In comparison, the abundance of anti-ROS genes was significantly higher in suspension than in electrode communities. Other than the adaptive response to oxygen, co-existence and synergistic interaction between facultative and anaerobic microbes could be another aerotolerant strategy.

3.5. Co-occurrence associations between dominant bacteria and functional genes

In order to better identify the microbial associations of electrode and suspension microorganisms with oxygen shocking, network analysis was used to investigate the co-occurrence between the dominant bacteria and functional genes. Only the functional genera and genes discussed above were included in the analysis to focus on aniline ammonification and electrogenic respiration. The network consisted of 30 nodes and 138 edges based on significant correlations (Spearman's $r > 0.8$, $P < 0.05$) (Fig. 6). Among them, more than half of the links were positive, which could represent a cooperative relationship among different species. For example, the aerobic aniline degraders (e.g., *Stenotrophomonas*) showed positive associations with electroactive bacteria (e.g., *Geobacter*). The anti-ROS genes also had positive links with genes encoding for dioxygenase and cytochrome *c* protein. As previously proposed, if the genes and the co-occurred microbial taxa exhibited significantly similar abundance tendencies, the microbial taxa could be speculated as the potential gene hosts [64]. Accordingly, *Geobacter* was considered to be the possible host of genes encoding cytochrome, and *Stenotrophomonas* was likely carrying the catechol 2,3-dioxygenase genes. Many studies have identified that outer-membrane cytochrome *c* protein directly contacted the electrode and served as a key component in the process of EET [28]. Furthermore, genes encoding for anti-ROS protein were positively associated with *Geobacter*, implying the genera could be evolved with the ability to be aerotolerant. Anaerobes have evolved strategies that either minimize the extent of oxygen toxicity or restore metabolic function shortly after the disappearance of oxygen stress [36]. Therefore, the idea that anaerobes (e.g., *Geobacter*) in anoxic conditions

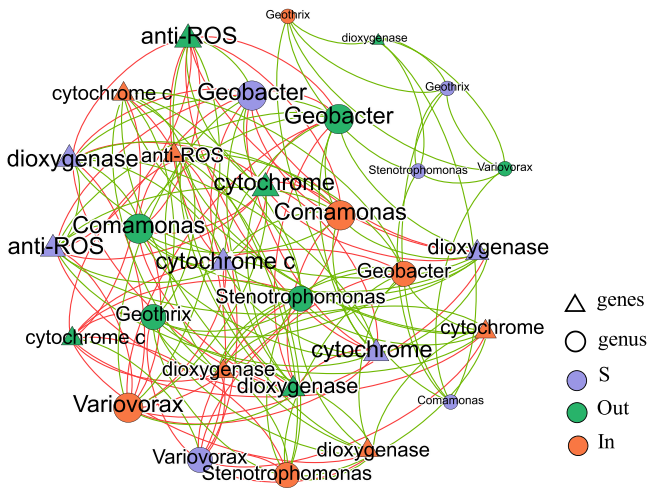

Fig. 6. The co-occurrence network patterns among the dominant taxa and function genes (green and red edges represent positive and negative associations).

develop ROS-scavenging systems to avoid oxygen toxicity is reasonable.

3.6. Outlook

The ammonification of refractory nitrogen-containing organics is essential to meet stricter effluent standards and achieve aquatic ecosystem health. Although electrostimulation could serve as a promising technology to accelerate amination (refractory organic nitrogen to aromatic aniline), ammonification (aromatic aniline to NH_4^+) remains difficult because oxygen is helpful for ammonification but not favorable for amination by electrostimulation. This study proposed a feasible scheme to assist ammonification processes by micro-aeration assisted with electrogenic respiration, which would not affect amination. The aromatic aniline could be more efficiently transformed to NH_4^+ in the presence of oxygen and the non-ring metabolites could serve as the substrate for electroactive bacteria to generate current. Although oxygen could act as a competitive electron acceptor inhibiting the current generation, it could be minimized by micro-aeration strategy. Also, DO was utilized and decreased to around 0.08 mg/L due to oxygen consumption in aniline ammonification. The micro-aeration assisted with electrogenic respiration system showed higher power density. The enhanced operational performance might be resulted from the shift of microbial communities.

Our data on microbial community composition, functional gene structure, and network associations further suggested that the ecological responses to micro-aeration could be coupled with electrogenic respiration as depicted below (Fig. 7). Firstly, the decrease of DO concentration from suspension to electrode biofilm markedly altered the taxonomic and functional gene structures of microbial communities, as shown by DCA-based ordination for both 16 S rRNA gene sequencing and GeoChip data. Three complimentary non-parametric multivariate statistical tests (Table 1) further supported the DCA results. Second, various functional members known to be involved in aniline degradation displayed different abundance at suspension and electrode biofilms. Specifically, most of the aerobic aniline degraders and degradation genes were significantly enriched in the suspension where there was a higher DO concentration. Due to the utilization and diffusion, the DO concentration was lower in the inner electrode biofilm. Consistently, the physiological activities of EET such as *Geobacter* and genes encoding for cytochrome *c* showed higher abundance in the inner biofilm. Finally, it seems that DO in the system not only changed community structure and composition, but also triggered microbial cooperation. The result of network analysis revealed that aerobic aniline degraders in the suspension displayed positive correlations with electroactive bacteria in the inner biofilm. Aniline degraders were considered to be potential hosts of dioxygenase genes contributing to benzene ring cleavage. The different impacts on the populations/genes for aromatic aniline ammonification and current generation could be important in maintaining the functional efficiency in micro-aeration assisted electrogenic respiration system. Considering aerobic or facultative bacteria could consume oxygen, the outer and inner electrode biofilms had lower DO concentration levels. Accordingly, the outer layer mainly consisted of facultative bacteria

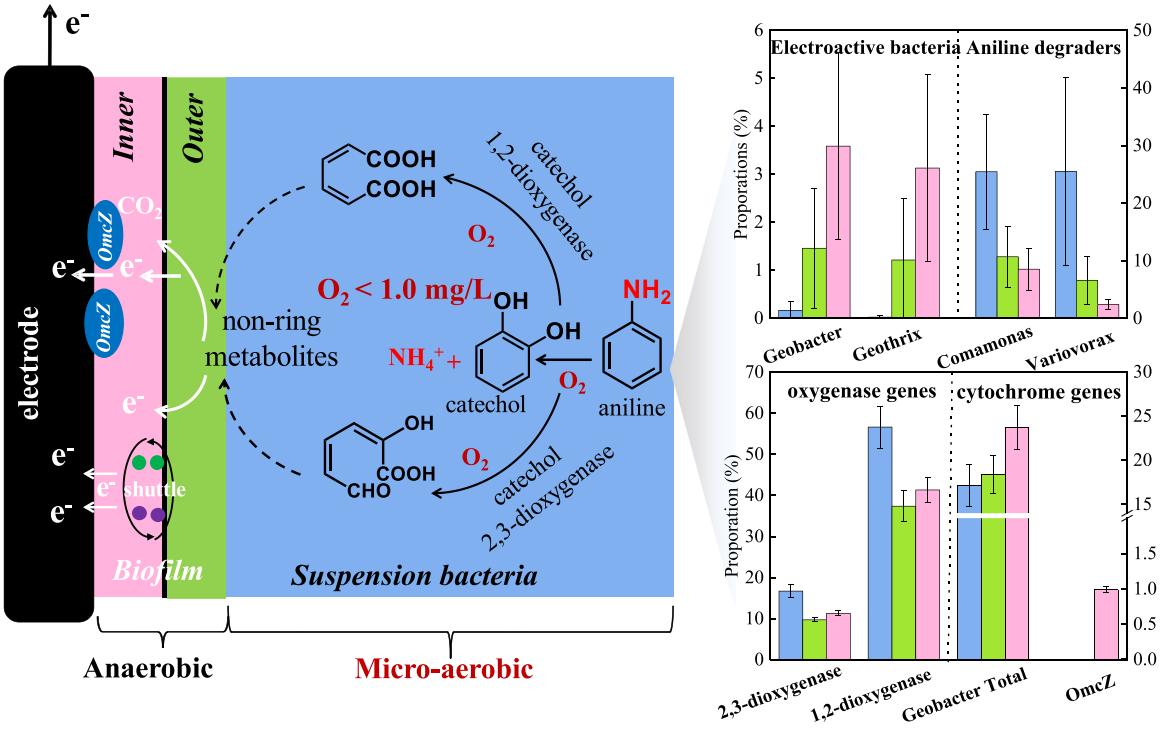

Fig. 7. Conceptual diagram for enhanced microbial ammonification by micro-aeration assisted with electrogenic respiration.

acting as both a physical and biological shield to protect the inner strict anaerobic bacteria from DO under micro-aerobic conditions. The co-existence of diverse bacteria and obvious enrichment of key genes shaped by DO were responsible for reliable aniline degradation.

Therefore, a conceptual model for refractory nitrogen-containing organics ammonification was proposed to overcome the limits of the low transformation in traditional wastewater treatment. In this mode, a whole hybrid hydrolysis acidification system directly introducing polarized electrodes and in-situ small amounts of air into the existing treatment construction is installed as a pretreatment in wastewater treatment plants. Several hybrid treatment technologies combined biological with chemical or physical treatment have been applied in wastewater treatment during the last few years [1]. However, there are universally acknowledged treatment issues including high economic cost, energy consumption, uncertainty and variability management for coupled processes. The integrated biological system, electrostimulated hydrolysis acidification assisted with micro-aeration regulation, would be promising and attractive because this approach could flexibly match existing wastewater treatment construction and significantly relieve the toxicity of refractory pollutants on the sequential secondary biochemical treatment unit designed to NH_4^+ removal through nitrification and subsequent denitrification processes. Simultaneously, it could reduce organic load and oxygen demands for aerobic biological process. More studies should emphasize the optimization of technique parameters (e.g., electrical power input, micro-aeration rate) and the investigation of metabolic mechanism using multi-omics (e.g., metagenomics, metatranscriptomics, metaproteomics) to manipulate the microbiomes for improving the treatment performance.

4. Conclusions

This study demonstrated that aniline could be catabolized to ammonia in the presence of minimal oxygen in an electrogenic respiration system. Microbial community and functional gene composition noticeably differentiated due to the oxygen gradient. Specifically, aerobic aniline degraders enriched in the suspension showed positive correlations with electroactive bacteria in the inner electrode biofilm. Also, the genes encoding for catechol dioxygenase which contributes to benzene ring cleavage were significantly greater in the suspension while cytochrome *c* genes that contribute to EET showed significantly higher abundance in the biofilm. Overall, this study provides a feasible approach to accomplish aromatic nitrogen ammonification and offers new insight into the response of microbial associations and functional evolution to micro-aeration assisted with electrogenic respiration.

Environmental implications

Refractory nitrogen-containing organics are the toxic factor for wastewater denitrification, which would suppress microbial metabolism and even induce aquatic eutrophication. This study proposed a feasible scheme to simultaneously accelerate amination and ammonification of organic nitrogen by micro-aeration assisted with electrogenic respiration. Network analysis indicated pollutant degraders were positively associated with electroactive bacteria and could be the potential hosts for genes encoding for dioxygenase and cytochrome, respectively. This approach could flexibly match existing wastewater treatment construction and significantly relieve the toxicity of organics on the sequential aerobic biological treatment.

CRediT authorship contribution statement

Ke Shi: Conceptualization, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Haoyi Cheng:** Conceptualization, Investigation, Writing – review & editing. **Carolyn R. Cornell:** Writing – review & editing. **Haiwei Wu**: Investigation, Writing – review & editing. **Shuhong Gao:** Writing – review & editing. **Jiandong Jiang:** Writing – review & editing, Funding acquisition. **Tiejun Liu:** Writing – review & editing. **Aijie Wang:** Supervision, Conceptualization, Writing – review & editing, Funding acquisition. **Jizhong Zhou:** Investigation, Writing – review & editing. **Bin Liang:** Conceptualization, Investigation, Writing – review & editing, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.130943.

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