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Responses of Aromatic-Degrading Microbial Communities to Elevated Nitrate in Sediments

Meiying Xu,[*,†,‡] Zhili He,[§] Qin Zhang,[†] Jin Liu,[†] Jun Guo,[†,‡] Guoping Sun,[†,‡] and Jizhong Zhou[§,∥,⊥]

[†]Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Institute of Microbiology, Guangzhou 510070, China

[‡]State Key Laboratory of Applied Microbiology Southern China, Guangzhou 510070, China

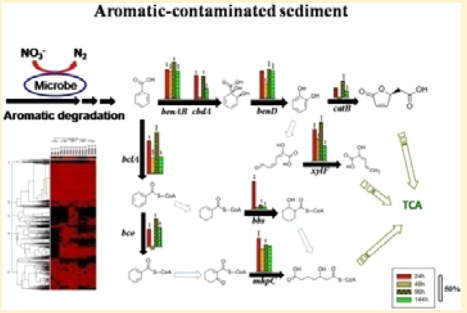
[§]Institute for Environmental Genomics and Department of Microbiology and Plant Biology, University of Oklahoma, Norman, Oklahoma 73019, United States

[∥]Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, United States

[⊥]State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing 100084, China

Supporting Information

ABSTRACT: A high number of aromatic compounds that have been released into aquatic ecosystems have accumulated in sediment because of their low solubility and high hydrophobicity, causing significant hazards to the environment and human health. Since nitrate is an essential nitrogen component and a more thermodynamically favorable electron acceptor for anaerobic respiration, nitrate-based bioremediation has been applied to aromatic-contaminated sediments. However, few studies have focused on the response of aromatic-degrading microbial communities to nitrate addition in anaerobic sediments. Here we hypothesized that high nitrate inputs would stimulate aromatic-degrading microbial communities and their associated degrading processes, thus increasing the bioremediation efficiency in aromatic compound-contaminated sediments. We analyzed the changes of key aromatic-degrading genes in the sediment samples from a field-scale site for *in situ* bioremediation of an aromatic-contaminated creek in the Pearl River Delta before and after nitrate injection using a functional gene array. Our results showed that the genes involved in the degradation of several kinds of aromatic compounds were significantly enriched after nitrate injection, especially those encoding enzymes for central catabolic pathways of aromatic compound degradation, and most of the enriched genes were derived from nitrate-reducing microorganisms, possibly accelerating bioremediation of aromatic-contaminated sediments. The sediment nitrate concentration was found to be the predominant factor shaping the aromatic-degrading microbial communities. This study provides new insights into our understanding of the influences of nitrate addition on aromatic-degrading microbial communities in sediments.



INTRODUCTION

Aromatic compounds comprise a wide variety of natural and synthetic compounds containing one or more aromatic rings. A significant number of persistent and toxic xenobiotics, such as benzene, toluene, ethylbenzene, xylene (BETX), chlorinated aromatics, heterocyclic aromatics, nitroaromatics, and polycyclic aromatic hydrocarbons (PAHs), belong to this family and pose a major environmental problem.[1] Because of their high hydrophobicity and persistence, aromatic compounds are apt to adsorb onto suspended particles and eventually settle to the sediment when these compounds are in an aquatic ecosystem. As a result, high concentrations of aromatic compounds are accumulated in the sediment and cause significant hazards to sedimentary organisms directly and to human health indirectly.[2,3] Therefore, degradation of aromatic compounds and remediation of contaminated sediments have drawn great attention recently.[4−7]

Owing to oxygen consumption in the covering water, major degradation processes for aromatic compounds in the sediment are anoxic. It was found that distinct redox compartments could be formed within anoxic hydrocarbon-contaminated aquifers, and indigenous microbial communities could perform biodegradation processes at different rates using locally available electron donors and acceptors according to the particular redox parameters.[3,8,9] The promiscuity of the catabolic enzymes allows bacteria to degrade, at least partially, aromatic compounds that share similar structures.[6] It was found that the bacterial catabolism of aromatic compounds involved a wide variety of peripheral pathways that activated structurally

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diverse substrates into a limited number of common intermediates, such as benzoyl-CoA, resorcinol, hydroxyhydroquinone, and/or phloroglucinol, which were further cleaved and channeled into a few central pathways.[10,11] However, compared to aerobic catabolism of aromatic compounds, the biochemical and genetic bases of the anaerobic degradation of aromatic compounds are not very well established yet.

Nitrate, one of the essential nitrogen components and more thermodynamically favorable electron acceptors for anaerobic respiration, plays important roles in the bioremediation of aromatic hydrocarbon-contaminated sediments.[12,13] Increasing evidences have shown that nitrate addition could promote the degradation of aromatic compounds under anaerobic conditions.[12,14−16] More and more nitrate reducing bacteria have been demonstrated to be able to degrade aromatic compounds, and some of them have been sequenced (e.g., *Magnetospirillum magneticum* strain AMB-1, *Azoarcus* sp. strain EbN1).[17−20] Nitrate-based bioremediation has also been applied to accelerate aromatic compound degradation in contaminated sediments.[5,13] However, to our knowledge, the response of sediment aromatic-degrading microbial communities to nitrate addition has not been studied systematically.

In this study, we hypothesized (i) that the composition and structure of sediment microbial communities involved in aromatic compound degradation would be altered dramatically by the sediment nitrate concentration due to the adsorption of aromatic compounds in the sediment; (ii) that those genes involved in the degradation of aromatic compounds with weak aromatic characteristics and highly aqueous solubility would be enriched efficiently, especially those for the central catabolic pathways; and (iii) that most of the significantly stimulated genes were derived from microorganisms capable of anaerobically degrading aromatic compounds using nitrate as the electron acceptor. To test these hypotheses, we analyzed the changes of the aromatic-degrading genes in the sediment samples from a field-scale site for *in situ* bioremediation of aromatic contaminated creek in Pearl River Delta by nitrate injection using a functional gene array (GeoChip 4.0) in concert with traditional microbiological analyses, geochemical property measurements, and organic compound detections. GeoChip 4.0 contains 12831 probes targeting 132 key enzymes involved in aromatic-compound degradation and provides a facilitative tool to analyze the functional composition, structure, and dynamics of aromatic-degrading microbial communities from a variety of ecosystems.[21] Our results generally support those hypotheses, and provide new insights into our understanding of the influences of nitrate addition on aromatic-degrading microbial communities in sediments.

MATERIALS AND METHODS

Sample Preparation and Analysis. The sediment samples were collected from a field-scale system for *in situ* bioremediation by nitrate injection in a creek of Pearl River Delta, which was polluted with a variety of aromatic compounds.[2,12] This system contains three sampling sites with three subcores at each site and 4 m distances between two adjacent sites along the flow direction of the creek as described in our previous study.[12] Every month, every square meter sediment within the system was injected with calcium nitrate solution at a dose of 45.3 gN. The sediment samples were collected using a piston-column sediment sampler (4.0 cm internal diameter, XDB0204, Beijing New Landmark Soil Equipment Co., Ltd., Beijing, China) from the sampling sites before nitrate injection (0 day), and 1 day, 2 days, 4 days, 6 days, 30 days, 60 days, 105 days, 145 days, and 190 days after nitrate injection. The samples were transported, homogenized, and subpacked in anaerobic chamber at 25 °C within 2 h, and their geochemical properties were measured according to the methods as described previously.[12] Total organic carbon content (TOC) and dissolved organic carbon (DOC) were determined by a total organic carbon analyzer (Elementar Liquid TOC II, Germany). The loss on ignition (LOI) of the sediment was determined by gravimetrical methods as described previously.[22] For gas chromatograph−mass spectrometer (GC−MS) analysis, 10 g of freeze-dried sediment was extracted with 200 mL of dichloromethane (DCM) for 48 h using a Soxhlet extractor. To remove elemental sulfur, activated copper granules were added to the extraction flasks during the extraction. Concentrated extracts were cleaned and fractionated on a 10 mm i.d. silica/alumina column, packed from the bottom to top with neutral silica gel (16 cm, 3% deactivated), neutral alumina (8 cm, 3% deactivated), and anhydrous sodium sulfate (1 cm). The columns were eluted with 30 mL of *n*-hexane (HEX), *n*-hexane/dichloromethane (4/1, HD), dichloromethane (DIC), or methanol (MET), respectively. The eluates were evaporated to 500 μL, respectively, under a gentle N_2 stream and analyzed on a ThermoFinnigan Trace DSQ GC−MS. All the experiments were repeated at least two times. Aromatic hydrocarbon compounds were identified on the basis of retention times and mass spectral interpretation, using the NIST mass spectral search program and NIST/EPA/NIH mass spectral library ver. 2.0. The analytical procedures of the 16 polycyclic aromatic hydrocarbons (PAHs) listed as priority pollutants by the United States Environmental Protection Agency (US-EPA) were conducted according to previous studies.[2] The detailed analytical procedures are provided in the Supporting Information.

DNA Extraction and Analysis. The total DNA of each sediment sample was extracted according to the modified procedures described previously.[23] The purified DNA was quantified with Quant-IT PicoGreen kit (Invitrogen). The changes of the microbial communities from different sampling time points were first quickly scanned by PCR-DGGE according to our previous study.[24] For GeoChip analysis, DNA labeling with cyanine-5 and the purification of labeled DNA were carried out as described previously.[25] The GeoChip 4.0[21] used in this study was synthesized by NimbleGen (Madison, WI, USA). After hybridization, arrays were scanned with a NimbleGen Ms 200 Microarray Scanner (Roche NimbleGen) and scanned images were gridded by NimbleScan software using the gridding file containing GeoChip 4.0 probes and NimbleGen control probes to obtain the signal intensity or each probe. The hybridization and scanning conditions and data analysis procedures were conducted according to our previous study.[12,26] Probe spots with coefficient of variance (CV) > 0.8 were removed and the signal intensities for each probe were normalized based on the mean signal intensities from all spiked common oligonucleotide reference standard (CORS) probes as described by Tu et al.[21]

Statistical Analysis. The matrices of microarray data resulting from our pipeline were further analyzed with different statistical methods. Hierarchical clustering was used to compare the microbial community structure and composition. Detrended correspondence analysis (DCA), combined with analysis of similarities (ANOSIM), nonparametric multivariate analysis of variance (Adonis) and multi-response permutation

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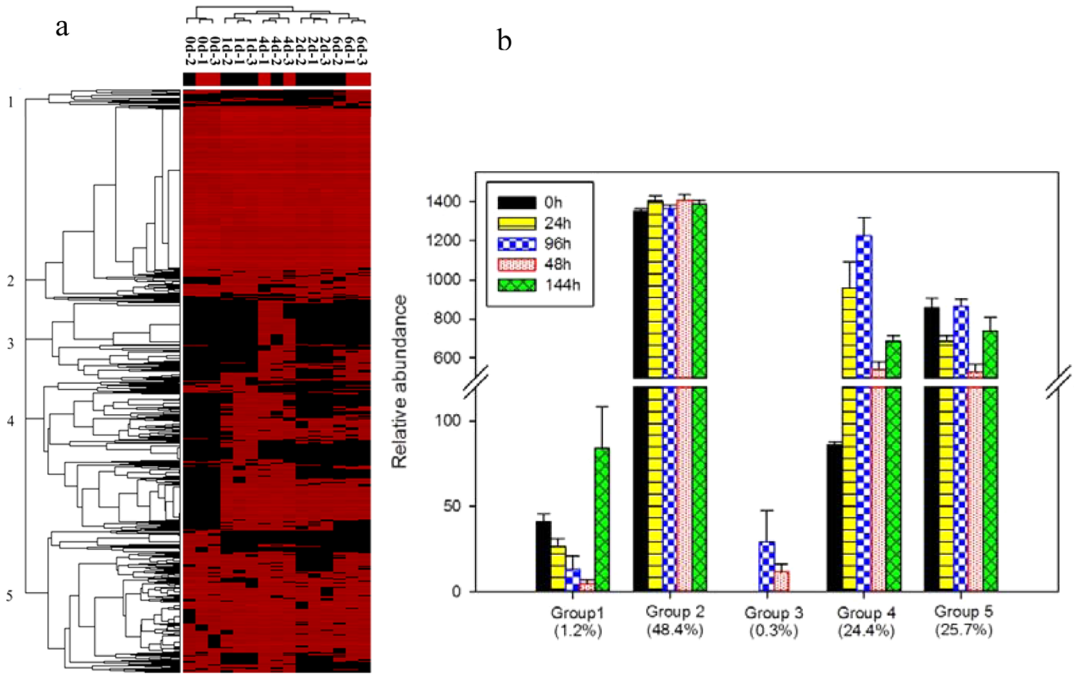

Figure 1. Clustering analysis of aromatic degradation genes detected using GeoChip 4.0. (a) The figure was generated using CLUSTER and visualized in TREEVIEW. Black indicates signal intensities below the threshold value and red indicates a positive hybridization signal. The color intensity indicates differences in signal intensity. The samples from different sampling points were clearly separated. Five different gene patterns were observed and indicated by numbers in the tree (a), and they were represented as group 1 to 5 on axis *x* and the relative abundances of the gene signal intensity contained in different groups were presented on axis *y* in the graphs (b).

procedure (MRPP), was employed to determine the overall functional changes in the microbial communities. Significant Pearson's linear correlation (r) analysis and analyses of variance (ANOVA) were applied to analyze the correlations between genes and/or environmental variables. Percentage changes by nitrate addition were calculated using the following formula: $(T_i - T_0) \times 100/T_0$, where T_0 and T_i were the average signal intensities of genes detected by GeoChip 4.0 in the samples collected before or after i days nitrate treatment as described by Xu et al.[12] Mantel test and redundancy analysis (RDA) as well as their partial analyses were used to link the aromatic-degrading communities with environmental variables. The detailed analytical procedures for the Mantel test and RDA are provided in the Supporting Information.

RESULTS

Nitrate Addition Accelerated the Removal of Aromatic Compounds. After 30 days of nitrate addition, the percentages of TOC in the sediment significantly decreased from 5.23 ± 0.03% to 3.45 ± 0.03% ($P < 0.005$) and then to 2.49 ± 0.01% ($P < 0.005$) after 60 days, whereas no significant change was observed in the control sites (Supporting Information Figure S1). The concentrations of organic compounds in the sediment were dramatically reduced and the removal rates of organosilicon compounds, PAHs, benzene homologues, and heterocyclic compounds were 46.73%, 36.25%, 23.19%, and 35.92%, respectively, after 60-day treatment. The concentrations of 16 priority PAHs decreased from an initial 2986.89 ± 0.09 $\mu g \cdot kg^{-1}$ to 222.77 ± 0.02 $\mu g \cdot kg^{-1}$ after 190-day treatment (Figure S2a). Within the 16 PAHs, the relative removal rates of high-ring (≥4) PAHs were higher than those of the low-ring (<4) PAHs (Figure S2b). After 30-day nitrate treatment, almost all of the high-ring PAHs (except the four-ring chrysene and six-ring benzo[*g*,*h*,*i*]-perylene) showed significant ($P < 0.05$) degradation, and only one low PAH (naphthalene) showed significant ($P < 0.05$) degradation. After 60-day treatment, almost all of the 16 PAHs were significantly ($P < 0.05$) degraded, although some negatively relative removal rates were observed during the treatment processes, especially for the low-ring PAHs, suggesting that some new low-ring PAHs were produced through the degradations of high-ring PAHs. These results indicated that the degradation of the aromatic compounds, especially those with high molecular weight, was enhanced after nitrate injection.

Overall Review of Aromatic-Degrading Microbial Community Responses to Nitrate Addition. To assess the response of microbial communities to nitrate addition, all DNA samples were first analyzed by PCR-DGGE, showing obvious changes in DGGE profiles for those from day 0, 1, 2, 4, 6 (Figure S3), which were further analyzed by GeoChip 4.0 to examine key functional genes involved in aromatic compound degradation. As revealed by three complementary nonparametric multivariate statistical tests (analysis of similarity, permutational multivariate analysis of variance using distance matrices, and response permutation procedure analysis), the microbial community functional structure involved in aromatic hydrocarbon remediation at the treatment stage significantly ($P < 0.001$) shifted from the initial (Table S1). The gene number, abundance, diversity (H' and $1/D$) and evenness of the aromatic compound degradation genes detected significantly ($P < 0.05$) increased in all treatment time points, peaking at day 4 (Table S2). Although ~50% of the aromatic-degrading genes detected were significantly ($P < 0.05$) increased at day 1 after

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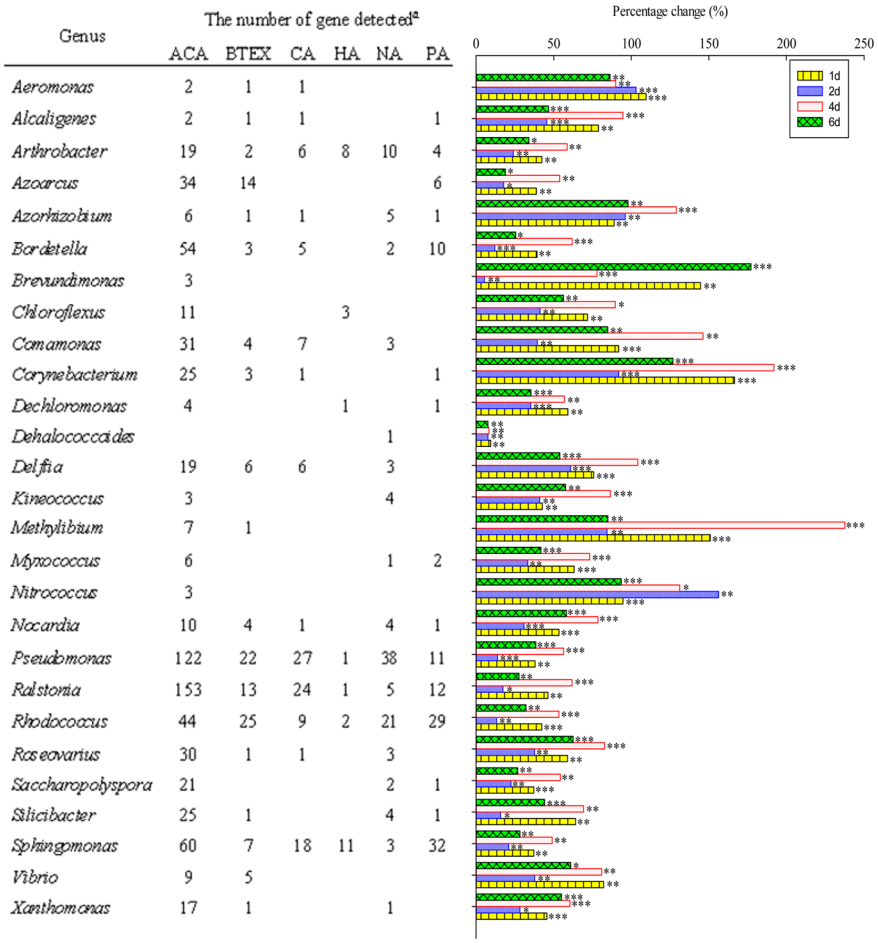

Figure 2. Commonly significantly increased functional genes from the versatile microorganisms in aromatic degradation. (a) The number of genes detected for aromatic carboxylic acid (ACA), BTEX and related aromatic (BTEX), chlorinated aromatic (CA), heterocyclic aromatic (HA), nitroaromatic (NA), PAH (PA) degradation. The calculation of percentage change was based on the average signal intensity of each microorganism. Significances between the samples from the initial stage and different time points following nitrate amendment were performed by the Student *t*-test. (***) $p < 0.01$; (**) $p < 0.05$; (*) $p < 0.1$.

nitrate addition, less increases in BTEX genes and no significant ($P > 0.05$) change in PAH degradation genes were observed (Figure S4). The percentage of unique genes from the treatment samples after nitrate amendment (ranging from 21.9% to 36.9%) was significantly ($P < 0.05$) higher than that from the initial stage (day 0) ranging from 7.1% to 16.8% (Table S2). Hierarchical clustering analysis showed that all samples treated with nitrate were clustered together and were well separated from the initial stage samples (Figure 1a). Five major gene groups could be visualized in the color tree and considerable variability in functional gene distribution was also observed among different sampling times. In addition, a number of aromatic-degrading genes were found to be enriched after nitrate addition, especially at day 2 and 4. For example, all genes in group 3 were detected at day 2 and 4 (Figure 1b), and 75.6% of these genes were involved in aromatic carboxylic acid degradation (Table S3). Group 4 with 24.4% of all aromatic-degrading genes detected, largely belonged to the nitrate treatment samples, although some variations were observed (Figure 1b).

In total, the genes detected by GeoChip were based on the probes from 305 known microbial genera, 33 of which showed significant increases after nitrate addition and almost maintained at all time points. Within those 33 significantly increased genera, 27 were bacterial origin and showed versatility in aromatic compound degradation including several important nitrate-reducing bacteria (Figure 2). For example, the genes detected from the important denitrifier *Azoarcus* were involved in aromatic carboxylic acid-, BTEX-, PAH-, and related aromatic-degradation, whereas those from the genus *Dechloromonas* were involved in aromatic carboxylic acid, heterocyclic aromatic, and PAH degradation. The genes from *Arthrobacter*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, and *Sphingomonas* were involved in all of the degradations of six aromatic compounds. Accordingly, the average functional gene abundances from these bacteria increased between 5.30% and 177.24% after nitrate addition (Figure 2). Consistently, most of the enriched nitrate reducing genes were from these genera (Table S4). All these results indicated that the functional characteristics of sediment microbial communities for aromatic compound degradation were significantly altered, and that the functional genes from the nitrate-reducing microorganisms with versatile aromatic degradation functions were more efficiently enriched after nitrate addition.

Nitrate as a Predominant Factor Shaping Aromatic-Degrading Microbial Communities. The most significant environmental variables shaping microbial community structure were evaluated by the Mantel test or partial Mantel tests and RDA. On the basis of variance in inflation factors, five variables were selected: the concentrations of ammonium (NH_4^+–N-

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Table 1. Relationships between the Aromatic-Degrading Genes Detected and the Environmental Factors Selected[a] by RDA

functional process	first canonical axis			sum of all canonical axes		
	eigenvalue	*F*-value	*P*-value	eigenvalue	*F*-value	*P*-value
all	0.226	2.629	0.045	0.587	2.563	0.001
aromatic carboxylic acids	0.222	2.575	0.051	0.586	2.551	0.001
BTEX and related aromatics	0.215	2.463	0.109	0.586	2.553	0.001
polycyclic aromatics	0.221	2.551	0.061	0.591	2.600	0.001
chlorinated aromatics	0.249	2.982	0.016	0.593	2.618	0.001
nitroaromatics	0.236	2.786	0.017	0.582	2.504	0.001
heterocyclic aromatics	0.315	4.140	0.001	0.638	3.178	0.001

[a]The five environmental factors selected include four interstitial water factors (NH_4^+–N-W, TN-W, TP-W, SO_4^{2-}-W) and one sediment factor (NO_3^--S).

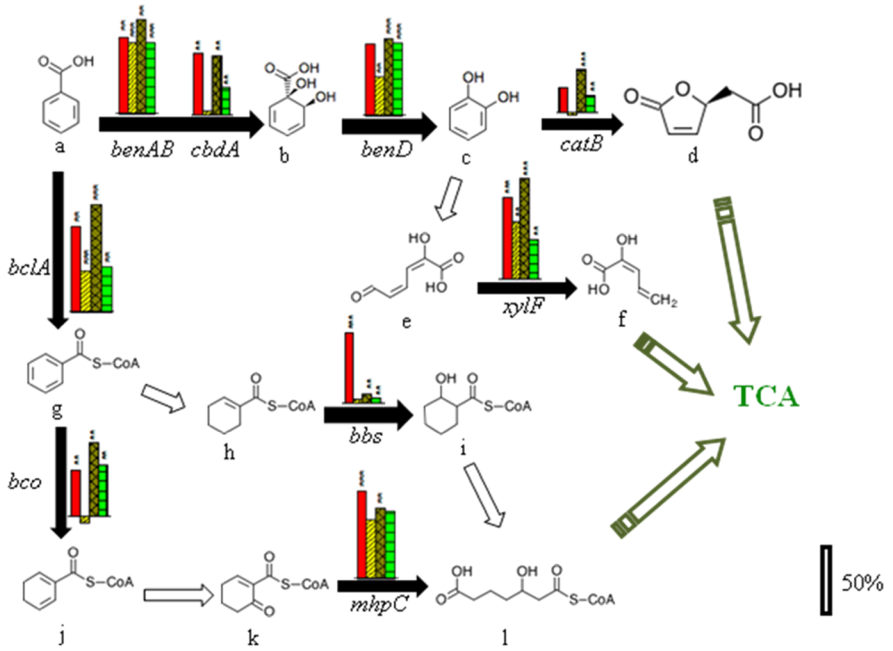

Figure 3. Effects of nitrate addition on abundances of the key genes involved in the degradation pathway of benzoyl-CoA. The metabolites are as follows: benzoic acid (a), 1,6-dihydroxycyclohexa-2,4-dienecarboxylic acid (b), pyrocatechol (c), 2-((S)-2,5-dihydro-5-oxofuran-2-yl) acetic acid (d), (2*E*,4*Z*)-5-formyl-2-hydroxypenta-2,4-dienoic acid (e), (*E*)-2-hydroxypenta-2,4-dienoic acid (f), S-COA benzothioate (g), S-COA cyclohex-1-enecarbothioate (h), S-COA 2-hydroxycyclohexanecarbothioate (i), S-COA cyclohexa-1,5-dienecarbothioate (j), S-COA 2-oxocyclohexanecarbothioate (k), 6-((S-COA)carbonyl)-5-hydroxyhexanoic acid (l). The upward bars presented the positive percentage changes, while the downward bars presented the negative percentage changes. Significances between the samples from the initial stage and different time points following nitrate amendment were performed by the Student *t*-test. (***) $p < 0.01$; (**) $p < 0.05$; (*) $p < 0.1$.

W), total nitrogen (TN-W), total phosphorus (TP-W), sulfate (SO_4^{2-}-W) in the porewater, and nitrate in the sediment (NO_3^--S). The specified RDA model was significant (P = 0.001), and 58.7% of the total variance could be explained by all canonical axes (F = 2.563, P = 0.001) with the first axis explained 22.6% (F = 2.629, P = 0.045) for all of the detected aromatic-degrading genes (Table 1, Figure S5). Significant (P = 0.001) correlations were also detected between the genes subcategories for aromatic carboxylic acid-, BTEX-, PAH-, chlorinated aromatic-, nitroaromatic-, and heterocyclic aromatic-degrading genes, and those five selected environmental factors with 58.6%, 58.6%, 59.1%, 59.3%, 58.2%, and 63.8% explanations, respectively (Table 1). Additionally, the sediment nitrate concentration was identified as the first environmental factor for shaping the aromatic-degrading microbial community structure at day 1, 2, and 6 after nitrate addition, while the sediment TN concentration was the main factor at day 4 (Table S5). These results indicated that sediment nitrate concentration could be a dominant factor shaping the aromatic-degrading microbial community structure and potentially regulating associated microbial functional processes.

Responses of Key Functional Genes Involved in Aromatic-Degrading Processes. *Aromatic Carboxylic Acid Degradation Genes.* There were 2679 aromatic carboxylic acid degradation genes detected in 39 gene families with 27 encoding oxidoreductase. Within the gene families detected, 17 of them increased significantly ($P < 0.05$) at day 1 with the highest for *pobMO* encoding phenoxybenzoate dioxygenase alpha subunit, and six of them (*bclA* for benzoate-CoA ligase, *benAB* for beta subunit of hydroxylase component of benzoate 1,2-dioxygenase, *gCoADH* for glutaryl-CoA dehydrogenase, *hmgA* for homogentisate dioxygenase, *nagI* for gentisate 1,2-dioxygenase, and *pobA* for *p*-hydroxybenzoate hydroxylase) maintained at all the time points after nitrate addition. Interestingly, significant ($P < 0.05$) increases were observed in most genes involved in the degradation pathway of benzoyl-CoA, the most common intermediate in the anaerobic degradation of aromatic compounds (Figure 3).

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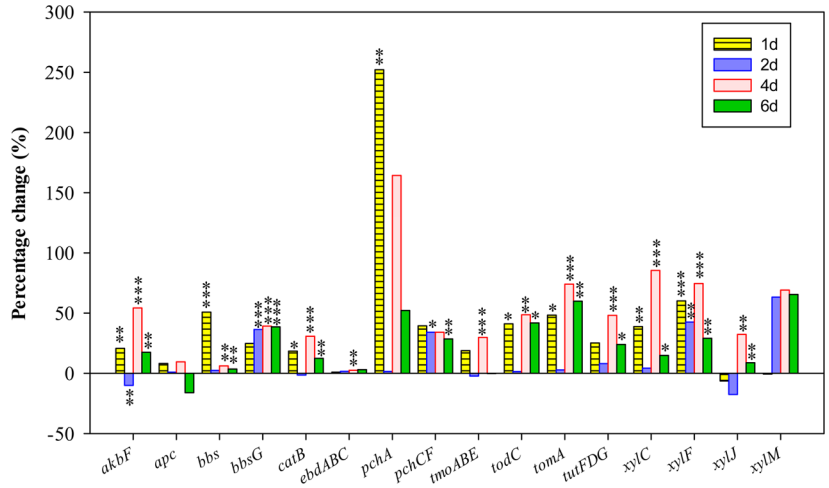

Figure 4. Effects of nitrate addition on abundances of functional genes involved in BTEX and related aromatics degradation. Significances between the samples from the initial stage and different time points following nitrate amendment were performed by the Student *t*-test. (***) $p < 0.01$; (**) $p < 0.05$; (*) $p < 0.1$.

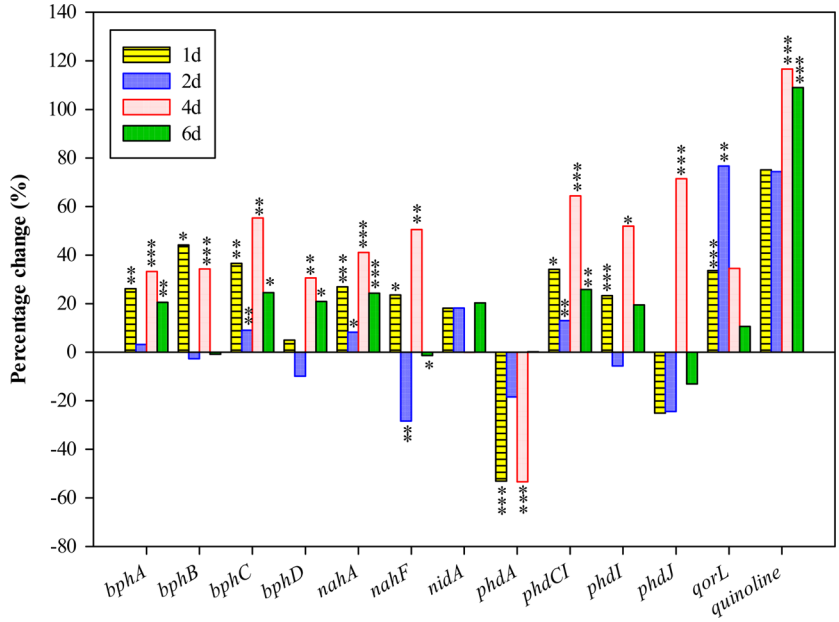

Figure 5. Effects of nitrate addition on the abundance of functional genes involved in PAH degradation. Significances between the initial stage samples and different time points after nitrate addition were performed by the Student *t*-test. (***) $p < 0.01$; (**) $p < 0.05$; (*) $p < 0.1$.

Furthermore, most of the significantly ($P < 0.05$) increased genes were from microorganisms which have the ability to anaerobically degrade aromatic compounds under nitrate reduction conditions. Two (23664434 and 56478449) of four significantly ($P < 0.05$) increased *bclA* genes were from denitrifying *Azoarcus* species, and one (91779182) of the two significantly ($P < 0.05$) increased *benAB* genes was from the versatile hydrocarbon-degrading denitrifier *Burkholderia xenovorans* LB400.[6] The *tphA* gene (110825109) from *Rhodococcus jostii* RHA1 encoding terephthalate 1,2-dioxygenase beta subunit was unique to the treatment samples. Only one gene, *dfbA* encoding phthalate 4,5-dioxygenase large subunit, showed significant ($P < 0.05$) decrease at all the sampling time points after nitrate addition (Figure S6). Totally, 704 genes from 158 genera involved in aromatic carboxylic acid degradation were unique to nitrate treatment samples, while only 219 genes from 91 genera were unique to the initial samples. Among the increased genes, ~ 90% were bacterial and most of them were from nitrate-reducing bacteria, such as *Azoarcus* sp., *Burkholderia* sp., *Bradyrhizobium* sp., *Pseudomonas* sp., *Ralstonia* sp., and *Sphingomonas* sp. (Table S6). These results indicated that nitrate addition might enhance the degradation of aromatic central intermediates formed during anaerobic catabolism of cyclic aromatic compounds by enriching their associated genes, especially those from nitrate-reducing bacteria.

BTEX and Related Aromatics Degradation Genes. Among 388 genes from 16 gene families detected for BTEX and related aromatics degradation, 99 genes from 15 families were unique to nitrate treatment samples, 38 genes from 10 families were unique to the initial samples, and 43 significantly ($P < 0.05$) changed with 14 genes from 9 increased families and 23 genes from 7 decreased families in all the time points after nitrate addition. Within 16 gene families detected, five of them (*akbF* encoding 4-hydroxy-2-oxovalerate aldolase, *bbs* encoding E-

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phenylitaconyl-CoA hydratase, *pchA* encoding hydroxybenzaldehyde dehydrogenase, *xylC* encoding betaine aldehyde dehydrogenase and *xylF* encoding 2-hydroxy-6-oxohepta-2, 4-dienoate hydrolase) showed a significantly ($P < 0.05$) higher abundance after 1-day nitrate injection with the highest in *pchA*. No significant ($P > 0.05$) decrease was observed in all gene families for BTEX and related aromatic degradation, except *akbF* gene at day 2 after nitrate addition (Figure 4). Almost all unique or significantly ($P < 0.05$) increased genes after nitrate addition were from bacteria, especially the important xenobiotic compound degrading denitrifiers. For example, all three significantly ($P < 0.05$) increased *akbF* genes (111022764, 226243761 and 226362830) were from the denitrifying *Rhodococcus* species and both significantly ($P < 0.05$) increased *apc* (56312803) and *bbsG* (56312526) were from the important denitrifier, *Azoarcus* sp. EbN1. The *bbs* gene (9622538) from the denitrifying bacterium, *Thauera aromatic*, also showed a significant ($P < 0.05$) increase and maintained at all time points after nitrate addition (Figure S7). These results indicated that the functional genes for BTEX and related aromatic degradation in sediment were stimulated and remained relatively stable in response to nitrate addition, suggesting that the functional potential for degrading those compounds could be enhanced.

PAH Degradation Genes. The changes in abundance of most PAH degradation genes were similar to those for BTEX and related aromatic degradation genes with a general increase for nitrate treatment samples. Within the 15 gene families involved in PAH degradation, two of them (*nahB* encoding *cis*-naphthalene dihydrodiol dehydrogenase and *phdG* encoding dihydrodipicolinate synthetase) were unique to the treatment samples, and five of them (*bphA* encoding biphenyl dioxygenase large subunit, *bphC* encoding biphenyl-2,3-diol 1,2-dioxygenase, *nahA* encoding aromatic ring-hydroxylating dioxygenase, *phdI* encoding 1-hydroxy-2-naphthoate dioxygenase, *qorL* encoding quinoline 2-oxidoreductase) were significantly ($P < 0.05$) increased, while only one gene family, *phdA* encoding the iron−sulfur protein large subunit of the ring-hydroxylating dioxygenase, showed a significant ($P < 0.05$) decrease after 1-day nitrate addition (Figure 5). A small drop was observed in the samples collected at day 2, in which only four gene families showed significant ($P < 0.05$) changes: an increase for *bphC*, *phdCI*, *qorL*, and a decrease for *nahF*. After 4 days, changes of the gene families rebounded with nine of them showing significant increases (Figure 5). Among 197 commonly detected genes in all samples, 38 of them were significantly ($P < 0.05$) changed and most of them belonged to bacteria which could degrade PAHs under nitrate reduction conditions. Within 16 significantly increased genes, eight of them were *nahA* from six genera (*Azoarcus* sp., *Burkholderia* sp., *Dyella* sp., *Polaromonas* sp., *Sphingobium* sp., *Sphingomonas* sp.) and two were uncultured bacteria, respectively, with the highest increase (219563170) from an intertidal sediment uncultured bacterium (Figure S8). These results suggested that PAH degradation in the sediment could be accelerated through the stimulation of the bacterial functional genes by nitrate addition, and the genes for aromatic ring oxygenation from nitrate-reducing bacteria might play key roles.

Overall, the abundances of most genes involved in the degradation of aromatic carboxylic acid, BTEX, and PAHs were significantly increased after nitrate addition, and the changing trends of the genes were related with the aromatic compound characteristics. The abundances of those genes involved in aromatic carboxylic acid degradation, especially those for the central catabolic pathways of aromatic compound degradation, were more easier enriched than those for the degradation of BTEX and PAH compounds, which usually contain stronger aromatic characteristics and lower aqueous solubility than aromatic carboxylic acids. Moreover, most of the significantly increased aromatic-degrading genes were derived from nitrate-reducing microorganisms.

DISCUSSION

The widespread contamination of harmful and persistent aromatic compounds in sediment has caused a major environmental problem. Nitrate injection has been considered as a promising approach to accelerate aromatic compound degradation in anaerobic sediments.[5,12,13] Understanding the responses of sediment aromatic-degrading microbial communities to nitrate addition is critical for designing bioremediation strategies. In this study, we examined the functional composition and structure of a sediment aromatic-degrading microbial community before nitrate injection (0 day), and 1 day, 2 days, 4 days, and 6 days after nitrate injection. Our results indicated that the genes involved in the degradation of several kinds of aromatic compounds were significantly enriched after nitrate injection, especially those involved in central catabolic pathways of aromatic compound degradation, and most of the enriched genes were derived from nitrate-reducing microorganisms, resulting in the acceleration of aromatic compound degradation and nitrate reduction in aromatic compound-contaminated sediments.

One of our hypotheses is that the microbial functional genes in the sediment would be increased due to nitrate addition, which could serve as an important nutrient and electron acceptor for microorganisms, especially denitrifiers. The degradation of aromatic substances is generally dominated by aerobic and anaerobic bacteria and aerobic fungi, and the crucial step in the degradation of aromatic compounds is overcoming the resonance energy that stabilizes the ring structure.[6] Because of the limited chemical reactivity of aromatic compounds, the distribution of aromatic metabolism pathways depends on the availability of electron acceptors for microbial respiration. Essential to anaerobic aromatic metabolism is the replacement of all oxygen-dependent steps by an alternative set of reactions. More and more oxygen-independent strategies that cope with the problem of cleaving the aromatic ring under low dissolved oxygen levels or even oxygen-free conditions have been identified and most of these strategies have been performed by denitrifying bacteria. Increasing evidences showed that denitrifying bacteria were able to use a variety of electron acceptors for respiration when coupled with the degradation of organic compounds and survival under the fluctuating redox environments when nitrate is unavailable.[17,27−30] In this study, the diversity and abundance of functional genes for aromatic compound degradation were increased and most of the significantly enriched genes were from the nitrate-reducing bacteria with versatile aromatic-degrading capabilities. For example, the *tphA* gene for aromatic carboxylic acid degradation and those aromatic-degrading genes from the typical denitrifying betaproteobacteria *Azoarcus* spp. were significantly enriched after nitrate addition, and most of nitrate-reducing microorganisms, such as *Dechloromonas*, *Pseudomonas*, *Ralstonia*, and *Sphingomonas*, were found to be involved in a variety of aromatic-degrading pathways. These results well support the hypothesis and suggest that the

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aromatic-degrading nitrate reducers could make great contributions to nitrate-based bioremediation of aromatic-contaminated sediments.

Our second hypothesis is that sediment nitrate concentration would play important roles in shaping the composition and structure of sediment microbial communities involved in aromatic compound degradation due to the adsorption and accumulation of aromatic compounds in the sediment. Previous studies indicated that the sediment microbial community structure exhibited a considerable shift over the remediation phases with the addition of electron acceptors or donors.[12,31,32] However, the water content of sediment significantly influenced the diffusion coefficient of electron acceptors added, and different microenvironmental conditions could be formed to regulate the microbial community structure and function due to the mass transfer rate of electron acceptor and the spatial separation in the sediment matrix.[12,25,33] Increasing evidences showed that introducing nitrate into contaminated sediments could enhance *in situ* biodegradation of organic contaminants, as well as the hydrophobic aromatic compounds, due to the high solubility and great energy conservation of nitrate.[11,20,34] In this study, the compositions and structures of aromatic-degrading microbial communities were significantly changed after nitrate addition, and the sediment nitrate concentration was identified as the most important factor for shaping the microbial community structure although four porewater parameters (NH_4^+−N-W, TN-W, TP-W, SO_4^{2-}-W) were also selected as the significant environmental variables.

We also predicted that the abundance of those genes involved in the degradation of aromatic compounds with weak aromatic characteristics and high aqueous solubility would more easily be enriched than those with strong aromatic characteristics and low aqueous solubility. Aromatic compounds have different aromatic characteristics and aqueous solubility, which are highly related to their biodegradability. Because of the fully developed aromatic characteristics of the benzene ring, the unsubstituted BTEX and PAH compounds are less aqueous soluble and more recalcitrant for biodegradation than the ring-substituted aromatics.[35] Although several novel biochemical processes were discovered and characterized in the anaerobic metabolism in recent years, only a few pure bacteria or enrichment cultures showed the ability to anaerobiclly degrade BTEX and PAHs, and the pathways involved remain essentially unknown.[11,20,36] In this study, higher removal rates in the high-ring PAHs were detected with some low-ring PAHs produced after nitrate addition, indicating an increased aromatic compound degradation in the sediment. Although the abundance and diversity of almost all of the aromatic compound degradation genes detected were significantly ($P < 0.05$) increased at all of the time points, a smaller increase in the abundance of BTEX and PAH degradation genes, or even no significant ($P > 0.05$) change was observed in those involved in PAH degradation at day 1 after nitrate addition. It was revealed that a limited number of common intermediates, which are converted from a multitude of structurally diverse aromatic substrates via different peripheral pathways, serves as electron donors for the corresponding dearomatizing enzymes.[10,11,37] The key intermediates formed during the anaerobic catabolism of aromatic compounds with a benzene-based structure (homocyclic aromatics) are benzoyl-CoA, phloroglucinol, hydroxyhydroquinone (HHQ), and resorcinol. Within these four key intermediates, the anaerobic degradation of benzoyl-CoA is more mechanistically difficult to achieve than others and requires a complex enzyme system coupled to an input of energy.[35,38] In this study, several important genes encoding the central catabolic pathways, such as *bclA* for benzoate-CoA ligase, *bco* for A-subunit of benzoyl-CoA reductase, *benAB* for beta subunit of hydroxylase component of benzoate 1,2-dioxygenase, *gCoADH* for glutaryl-CoA dehydrogenase, and *pimF* for enoyl-CoA hydratase, increased after nitrate addition, which could be due to the enrichment of the aromatic degradation genes and accumulation of key intermediates (e.g., benzoyl-CoA). These results confirm the hypothesis and provide some details about the responses of key genes involved in the degradation of aromatic compounds.

In summary, the results allow us to form a conceptual model for further understanding the responses of sediment aromatic-degrading microbial communities to nitrate addition (Figure 6).

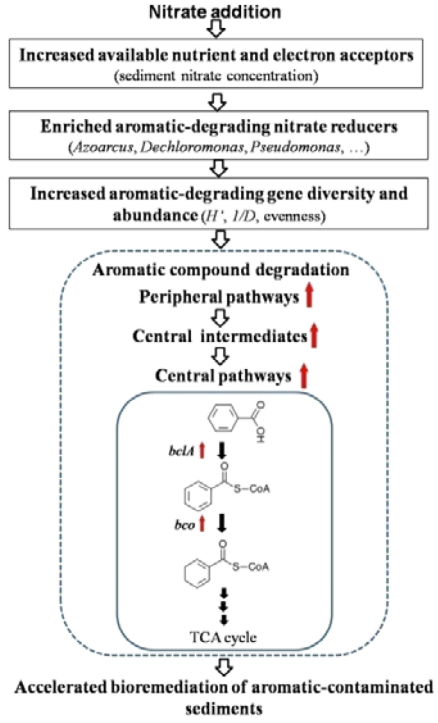

Figure 6. A concept model for examining the effect of nitrate on sediment aromatic-degrading microbial communities.

First, the sediment microbial community involved in aromatic compound degradation was sensitive to elevated nitrate concentrations, and the abundance and diversity of sediment aromatic-degrading nitrate reducers increased dramatically after nitrate addition. Second, higher abundances were observed in the aromatic-degrading genes involved in the degradation of aromatic compounds with weak aromatic characteristics and high aqueous solubility, such as aromatic carboxylic acid. In addition, the abundance of key genes involved in the central catabolic pathways (for example, benzoyl-CoA degradation) significantly increased, such as *bclA*, *bco*, *benAB*, *gCoADH*, and *pimF*. Finally, all those changes might enhance the degradation of aromatic compounds in the sediment, including recalcitrant BTEX and PAH compounds. However, to further understand and evaluate the potential impacts of elevated nitrate on bioremediation of aromatic-compound contaminated sediments, an integrated and comprehensive monitoring program is necessary to track the dynamics of aromatic-degrading

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microbial communities associated with the degradation of aromatic compounds in sediments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b03442.

Detailed analytical procedures; additional information, tables and figures as noted in the text (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +86-20-87683656; fax: +86-20-87684471; e-mail: xumy@gdim.cn.

Notes

The authors declare no competing financial interest.

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