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Influence of graphene oxide and biochar on anaerobic degradation of petroleum hydrocarbons

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The anaerobic degradation of petroleum is an important process in natural environments. So far, few studies have considered the response of the microbial community to nanomaterials during this process. This study explored the potential effects of graphene oxide and biochar on the anaerobic degradation of petroleum hydrocarbons in long-term experiments. Cyclic voltammetry and electrochemical impedance spectroscopy indicated that the addition of carbon-based materials promoted the electrochemical activity of anaerobic cultures that degrade petroleum hydrocarbons. The maximum degradation rates for benzene, toluene, ethylbenzene, and xylene (BTEXs) in the cultures incubated for 10 weeks with graphene oxide (0.02 mg/L) and biochar (20 mg/L) were 76.5% and 77.6%, respectively. The maximum degradation rates of *n*-alkanes in the cultures incubated for 10 weeks with graphene oxide (2 mg/L) and biochar (100 mg/L) were 70.0% and 77.8%, respectively. The 16S rDNA copy numbers in the treatments with 0.02 mg/L graphene oxide and 20 mg/L biochar were significantly higher than others during the process ($P < 0.05$). In the 2nd week, the maximum copy numbers of the *masD* and *bamA* genes in the treatments with biochar were 349 copies/mL (20 mg/L) and 422 copies/mL (20 mg/L), respectively, and in the treatments with graphene oxide were 289 copies/mL (0 mg/L) and 366 copies/mL (0.02 mg/L). The contents of carbon-based materials had slight effects on the microbial community structure, whereas the culture time had obvious effects. *Paracoccus denitrificans*, *Pseudomonas aeruginosa*, and *Hydrogenophaga caeni* were the dominant microorganisms in the culture systems under all treatments.

[**Key words:** Anaerobic degradation; Biochar; Graphene oxide; Mixed electron acceptor; Petroleum hydrocarbon]

In recent decades, industrial development has led to the widespread pollution of soil and water with petroleum hydrocarbons as a common environmental problem. Among them, many contaminated sites such as sediments and groundwater are characterized by anoxic or anaerobic conditions (1). It is difficult and expensive to degrade contaminants in these sites by providing oxygen to stimulate the growth of microorganisms. Thus, natural attenuation is the preferred method for remediating sediments and subsurface environment contaminated by petroleum hydrocarbons (2). Therefore, it is necessary to develop a low-cost and efficient technique to remove contamination by petroleum hydrocarbons from sediments and subsurface environments. Recently, it has been demonstrated that anaerobic bioremediation can potentially be applied for the remediation of contaminated subsurface environment and sediments. Studies indicate that many anaerobic microorganisms can degrade benzene, toluene, ethylbenzene, and xylene (BTEXs), alkanes, and polycyclic aromatic hydrocarbons in an efficient manner (3,4). In addition, petroleum hydrocarbons can be degraded with nitrate, sulfate, and iron as electron acceptors in anaerobic environments (5,6). Electron acceptors and nutrients are usually the limiting factors in anaerobic environment. Therefore, stimulating native microorganisms to degrade petroleum hydrocarbons is an effective situ bioremediation technique for the remediation of sediments, groundwater, and soils polluted by petroleum hydrocarbons (7). Mixed electron acceptor systems are ubiquitous in contaminated areas but very few studies have investigated them (8). During the degradation of pollutants in sediments and subsurface environments, different electron acceptors are likely to co-exist in the same area and influence the process. Therefore, this study investigated the anaerobic degradation of petroleum hydrocarbons under conditions with mixed electron acceptors.

Alkanes are the most important constituents of petroleum, and some are acutely toxic and difficult to remove by remediation. Most anaerobic alkane-degrading bacteria activate alkane degradation via the fumarate reaction (9,10). *n*-Alkanes are apparently activated by the addition of subterminal carbon to fumarate to yield (1-methylalkyl) succinates, where the most likely enzyme involved is (1-methylalkyl) succinate synthase (Mas) (11). Alkyl-substituted succinate metabolites are then degraded via carbon skeleton rearrangement, carboxylation, and β-oxidation. Therefore, the *masD* gene that encodes the catalytic subunit of this key enzyme can be used as a genetic marker to study the diversity and distribution characteristic of alkane-degrading bacteria in anaerobic environments. Using this technique, von Netzer et al. (12) and Kleindienst et al. (13) recently investigated the distribution and diversity of *masD*/*assA* genes in oil-contaminated sediments and natural hydrocarbon leaks, corroborating that sulfate-reducing bacteria were

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the key alkane anaerobic degraders at marine seeps and *masD* was the vital gene in anaerobic degradation of petroleum hydrocarbons at contaminated sites, respectively.

BTEXs are among the most common groundwater contaminants. In anaerobic microorganisms, most aromatic hydrocarbons (e.g., phenol, toluene, cresols, ethylbenzene, xylenes, and benzoate analogs) are channeled to the intermediate benzoyl-CoA prior to ring cleavage (14,15). The key enzyme that catalyzes reductive benzene ring cleavage is 6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase, which is encoded by the *bamA* gene (16). Kuntze et al. (17) showed that the benzoyl-CoA degradation pathway is present in all anaerobic bacteria that use aromatics as growth substrates. Moreover, *bamA* genes are found in most of these microorganisms. Thus, it is appropriate to use the *bamA* gene as a biomarker for evaluating the biodegradation potential of aromatic hydrocarbons by microbes.

Carbon-based materials such as granular activated carbon (18), black carbon (19), biochar (20), activated carbon fibers (21), carbon nanotubes (22), and graphene (23) have been applied as redox mediators to increase the redox conversion of different contaminants in anaerobic environments. Biochar is a carbon-rich solid produced via the pyrolysis of biomass below 700°C with a limited oxygen supply. Biochar has positive effects on increasing soil fertility, reducing N_2O emissions, and promoting plant growth (24). Due to the irregular surface shape and high adsorption capacity of biochar, it can also protect microorganisms from the damage caused by high fluid shear forces in liquid environments. In addition, microbes can form biofilms on biochar surfaces to increase the contact area with contaminants and degrade pollutants more efficiently. Moreover, the electrical conductivity and redox activity of biochar may promote the transfer of electrons during the anaerobic microbial degradation of pollutants (25). Due to the aromatic and quinone structures in biochar, it has important roles in promoting the anaerobic bio-oxidation of pollutants (26,27). In addition, Tong et al. (20) demonstrated that the addition of biochar to paddy fields was beneficial for the growth of Fe(III)-reducing and dechlorinating bacteria in soil. Their results suggested that biochar is involved in electron transfer in anaerobic environments. In fact, the capacity of biochar to reversibly donate and accept electrons in an electrochemical cell was quantified by Klüpfel et al. (27).

Graphene oxide and reduced graphene oxide have abundant functional groups and large surface areas. Graphene oxide is not conductive but it can be reduced to reduced graphene oxide by many microorganisms and reduced graphene oxide exhibits conductivity (28,29). All of these features are helpful for catalyzing the degradation of pollutants by anaerobic microorganisms (30). It has been reported that partially reduced graphene oxide and graphene oxide can promote the biological and abiotic reduction of refractory pollutants, such as azo dyes, nitroaromatic compounds, and halogenated pollutants (31–33). Moreover, a recent study demonstrated the chemical transformation of iopromide using reduced graphene oxide as a redox mediator, and the importance of the physical and chemical properties of graphene oxide-based materials in the reductive process (34). However, the presence of high graphene oxide contents can damage cellular structures and the synthesis of chlorophyll in wheat (35). It has also been proposed that carbonaceous nanomaterials may aggregate on cell surfaces and hinder the hydraulic conductivity of cells and the availability of water (36), thereby reducing transpiration and affecting plant development. Carbon-based materials can mediate the redox conversion of recalcitrant pollutants in biological treatment systems, but the use of these materials for the biological transformation of petroleum hydrocarbons under anaerobic conditions needs further research.

Little information is available about the effects of carbon-based materials on the anaerobic degradation of petroleum hydrocarbons. Therefore, in this study, we investigated the possible effects of graphene oxide and biochar on the anaerobic degradation of petroleum hydrocarbons in long-term incubation experiments. We monitored the degradation rates of petroleum hydrocarbons, the electrochemical activity of anaerobic cultures, and the general physiological activities of anaerobic microorganisms. Moreover, we analyzed the microbial community and investigated the possible roles of carbon-based materials in the anaerobic degradation process.

MATERIALS AND METHODS

Anaerobic sludge and carbonaceous materials The original seed anaerobic activated sludge used as the inoculum in the anaerobic culture systems was collected from an anaerobic digester at Jinnan District life wastewater treatment plant in Jinnan district, Tianjin, China. The anaerobic sludge was stored at 4°C until use. The biochar was produced via the pyrolysis of cypress wood sawdust at 500°C under oxygen-limiting conditions for 6 h. The biochar was then ground until it passed through a 0.5 mm sieve. Graphene oxide with a multi-layer structure and 98% purity was obtained from Shanghai Yuan Ye Biological Technology Co. Ltd. (Shanghai, China).

Anaerobic culture Experiments were conducted in anaerobic glove boxes (Type C, Coy Laboratory, Grass Lake, MI, USA) with 120 mL glass serum bottles. Petroleum hydrocarbon mixtures (100 μL) containing equal concentrations of six types of *n*-alkanes (C15–C20) and four types of BTEXs were prepared as the sole carbon sources, with nitrate (10 mM) and sulfate (10 mM) as mixed electron acceptors. Different amounts of graphene oxide (0.02, 0.2, or 2 mg/L) or biochar (20, 100, or 500 mg/L) were added to the anaerobic systems. The sterile serum bottles were transferred into 0.01 g anaerobic activated sludge with 100 mL of anaerobic culture medium and crimp sealed with butyl rubber stoppers. The anaerobic culture systems were cultivated at 30°C/160 rpm in the dark. For all of the treatments, the negative controls were autoclaved at 121°C for 20 min and cultivated at 30°C/160 rpm in the dark. The degradation rates of *n*-alkanes and BTEXs in treatments were calculated relative to that of the corresponding negative controls. The experiment lasted for 10 weeks with sampling and determination in the 2nd, 4th, 6th, and 10th weeks. Physicochemical indices comprising the pH and oxidation reduction potential (ORP) (Vstar10, Thermo Fisher Scientific, Waltham, MA, USA) were measured during the process. The adhesion of microbes to each material was investigated using scanning electron microscopy (SEM) (JSM-5510, Jeol, Tokyo, Japan) at the end of the experiment.

The basic inorganic salt medium used for cultivation was 1.0 L of inorganic medium containing: NaCl 0.5 g, $MgC1_2{\cdot}6H_2O$ 0.5 g, $CaC1_2{\cdot}2H_2O$ 0.1 g, NH_4Cl 0.3 g, KH_2PO_4 0.2 g, KCl 0.5 g, $NaHCO_3$ 2.5 g, trace elements 1.0 mL, vitamins 1.0 mL, resazurin 0.1 mg, electron acceptor Na_2SO_4 1.42 g, KNO_3 1.65 g, and $Na_2S{\cdot}9H_2O$ 0.25 g. The trace elements in 1.0 L of the solution comprised: $CoCl_2{\cdot}6H_2O$ 0.5 g, $CuC1_2{\cdot}2H_2O$ 0.1 g, $FeC1_2{\cdot}4H_2O$ 7.5 g, H_3BO_3 1.0 g, $MnC1_2{\cdot}4H_2O$ 0.5 g, $Na_2MoO_4{\cdot}2H_2O$ 0.1 g, $NiC1_2{\cdot}6H_2O$ 0.1 g, and $ZnCl_2{\cdot}6H_2O$ 0.5 g. Vitamins in 1.0 L of the solution comprised vitamin B_{12} 10 mg, vitamin H 20 mg, folic acid 20 mg, nicotinic acid 50 mg, pantothenic acid 50 mg, aminobenzoic acid 50 mg, vitamin B_6 100 mg, riboflavin 50 mg, vitamin B_l 50 mg, and lipoic acid 50 mg.

Electrochemical analysis Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) analyses were performed using a 100 mL glass bottle, which was connected to a 0.2 cm^2 glassy carbon electrode as the working electrode. An Ag/AgCl (KCl salt) electrode and a 1.0 cm^2 platinum plate were used as the reference and counter electrodes, respectively. CV and EIS analyses of the culture systems were conducted using an electrochemical measurement system HZ-7000 (Hokuto Denko, Tokyo, Japan) according to the method described by Yoshida et al. (28). CV was conducted at a scan rate of 0.2 mV/s in the potential range from −400 to 600 mV (versus. Ag/AgCl). EIS was performed over a frequency range of 100 kHz–500 kHz at 200 mV, and an amplitude of 20 mV was applied for the alternating current signal. Nyquist plots were analyzed using ZSimpWin software (Princeton Applied Research, Oak Ridge, TN, USA).

Determination of *n*-alkanes and BTEXs The concentrations of BTEXs and *n*-alkanes were determined using a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP mass spectrometer (Agilent, Santa Clara, CA, USA). The gas chromatography-mass spectrometry (GC-MS) system was equipped with a Thermo DB-5MS capillary column (30 m × 0.25 mm, i.d. 0.25 μm film thickness; Agilent), which operated with helium (99.99% purity) as the carrier gas at a constant flow of 1.0 mL/min. A mixture of four types of BTEXs and 33 types of *n*-alkanes (C8–C40) was used as an external standard to determine the amounts of BTEXs and *n*-alkanes in the samples. In order to extract the *n*-alkanes, 20 mL of the medium was transferred to a separating funnel and mixed with 10 mL of *n*-hexane. The mixture was inverted and mixed for 30 min. Then, the upper organic

layer was separated and medium was extracted three more times. The extracts were merged and filtered through a 0.25-μm organic membrane and 1.0 μL aliquots of the extracts were injected into the GC-MS system, which was operated at 280°C in the pulsed and splitless mode (1.0 min followed by a split ratio of 1:50 until the end of analysis). The GC oven temperature was maintained at 60°C for 3 min and then increased from 60°C to 180°C at a rate of 15°C/min, before increasing from 180°C to 300°C at a rate of 6°C/min, and finally holding at 300°C for 10 min. The process employed for extracting the BTEXs was the same as that for the *n*-alkanes, except the extraction solution was changed from *n*-hexane to carbon disulfide (CS_2). The extracts were then filtered through a 0.25-μm organic membrane and analyzed by GC-MS. To determine the BTEXs, the column temperature was initially held at 40°C for 5 min and then increased from 40°C to 100°C at a rate of 12°C/min, before increasing from 100°C to 200°C at a rate of 50°C/min, and finally holding at 200°C for 2 min.

DNA extraction and qPCR analysis of anaerobic degradation genes DNA was extracted from the anaerobic culture systems using a Bacterial Genomic DNA Extraction Kit (Tiangen, Beijing, China). The 16S rDNA, *masD*, and *bamA* genes were quantified with a Bio-Rad CFX96 fluorescence quantitative PCR system (Bio-Rad, Hercules, CA, USA) using a Quantitect SYBR Green PCR Kit (Trans Gen Biotech, Beijing, China). The primer pair comprising 1369F (5′-CGG TGA ATA CGT TCY CGG -3′) and 1492R (5′-GGW TAC CTT GTT ACG ACT T-3′) was used to quantify the 16S rDNA (37). The primer pair comprising 570F (5′-KGA YTT TGA GSA SCT TTT CS-3′) and 960R (5′-TCG TCC ACR TAR TCG TCG TC-3′) was used to quantify the *masD* gene (38). The primer pair comprising 177F (5′-GCA GTA CAA YTC CTA CAC SAC YGA BAT GGT-3′) and 505R (5′-CCR TGC TTS GGR CCV GCC TGV CCG AA-3′) was used for quantifying the *bamA* gene (39). The reaction mixtures contained 12.5 μL 2 × Trans Start Top Green qPCR Super Mix, 9.5 μL double-distilled H_2O, 1.0 μL template DNA, and 0.1 μL of the forward and reverse primers. The real-time quantitative polymerase chain reaction (qPCR) procedure was as follows. Initially, the temperature was held at 94°C for 30 s, before 40 cycles of denaturing at 94°C for 5 s, annealing at 55°C for 15 s (16S rDNA gene), and extension at 72°C for 10 s. Finally, the melting curve was obtained by increasing the temperature from 55°C to 95°C (at a rate of 4°C/s). The extension temperatures for the *masD* and *bamA* genes were 52°C and 55°C, respectively. Each DNA template was prepared with three replicates to reduce detection errors. The melting curve was used to determine nonspecific amplification and the formation of primer dimers during the amplification process. The data were analyzed with CF Manager Software version 2.1 (Bio-Rad). The template DNA at a known concentration was diluted repeatedly by 10 times and quantified by qPCR to establish the standard curve for each gene.

Analysis of the microbial community by polymerase chain reaction-denaturing gradient gel electrophoresis Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) was employed to assess the microbial communities in the anaerobic culture systems. The primer set for targeting the V3 region of bacterial 16S rDNA comprised GC-338f (5′-GC clamp- CAC GGG GGG ACT CCT ACG GGA GGC AGC AG-3′; GC clamp = CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) and 518r (5′-ATT ACC GCG GCT GCT GG-3′). The PCR conditions were set as reported previously by Liu et al. (40). The polyacrylamide gel concentration used for loading PCR amplicons was 8% (acrylamide:bisacrylamide = 37.5:1) with a denaturing gradient from 40% to 60% (100% of the denaturant comprised 7 M urea and 40% formamide, v/v). DGGE analysis was performed with a DCode Universal Mutation Detection System (Bio-Rad) at 150 V and 60°C for 4 h. After electrophoresis, the gel was stained with ethidium bromide and observed with an ultraviolet transmission instrument (Sagecreation, Beijing, China). The band brightness and cluster analysis between lanes in the DGGE electrophoresis images were quantified with Quantity One 4.6 (Bio-Rad). Dendrograms were created using the un-weighted pair-group method with the arithmetic averages (UPGMA) (41).

RESULTS AND DISCUSSION

Electrochemical and physicochemical analyses The catalytic currents and charge transfer resistance (R_{ct}) in autoclaved treatments with 2 mg/L graphene oxide and 500 mg/L biochar did not change during the culture process (Fig. 1). The CV curves obtained for the culture systems with carbon-based materials all had larger closed areas than those for the autoclaved controls, and they increased gradually from 2 weeks to 10 weeks. After culture for 10 weeks, the catalytic currents in the culture systems with 2 mg/L graphene oxide at 600 mV vs. Ag/AgCl increased from 214 mA/m^2 (in the 2nd week) to 221 mA/m^2. The catalytic currents in the culture systems with 500 mg/L biochar at 600 mV vs. Ag/AgCl increased from 158 mA/m^2 (in the 2nd week) to 163 mA/m^2. The R_{ct} was represented as the diameter of the semicircles. During the process, the R_{ct} values for the culture systems with carbon-based materials were all smaller than those for the autoclaved control and they decreased gradually from 2 weeks to 10 weeks. After culture for 10 weeks, the R_{ct} values for the culture systems with 2 mg/L graphene oxide decreased from 549 Ω/mL (in the 2nd week) to 446 Ω/mL. The values for the

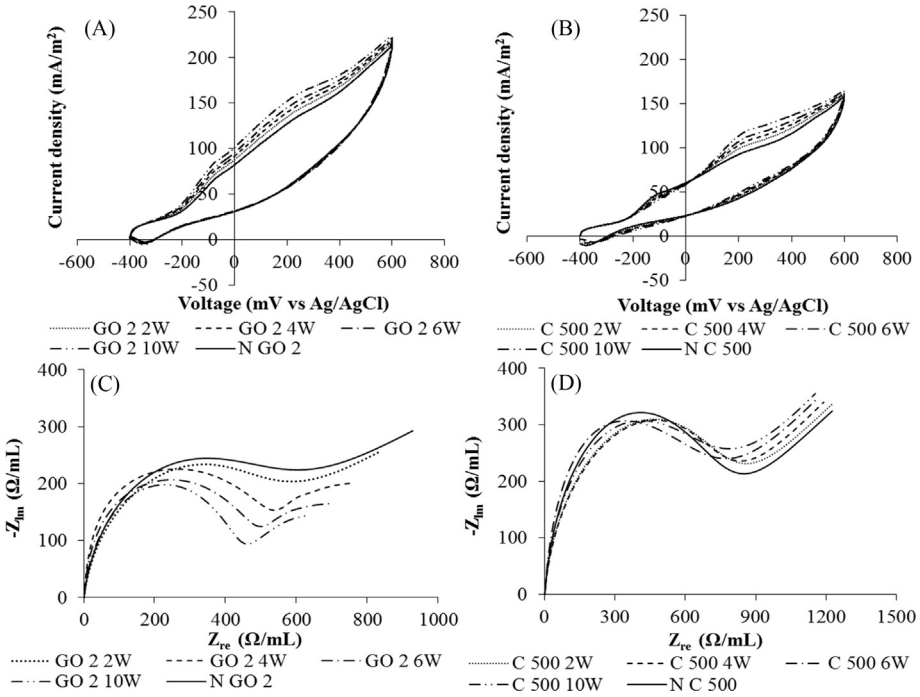

FIG. 1. Cyclic voltammetry curves and electrochemical impedance spectroscopy results for the culture systems with graphene oxide (A, C) and biochar (B, D). GO or C indicates that graphene oxide or biochar was added to the treatments, respectively. 2 and 500 denote the amounts of graphene oxide or biochar in mg/L. 2W, 4W, 6W, and 10W denote the culture time in week. N GO 2 and N C 500 denote the data for the treatments in the 10th week because the electrochemical properties of the sterilized control did not change during the culture process.

culture systems with 500 mg/L biochar decreased from 801 Ω/mL (in the 2nd week) to 761 Ω/mL. The CV curves and EIS results obtained for the culture systems with different carbon-based materials all indicated greater electrochemical activity than those for the autoclaved control, especially the culture systems containing graphene oxide. The CV curves and EIS results indicated that biofilms formed on the surfaces of the two carbonaceous materials after the anaerobic incubation period, as confirmed by the SEM results (Fig. S1).

The microbial degradation of petroleum hydrocarbons assisted by electron acceptors in an anaerobic environment comprises a series of redox reactions. Biochar and graphene oxide can be used as electron acceptors to promote anaerobic respiration by microorganisms (19,28). By changing their redox state, carbon-based materials can transfer the reducing equivalent from the contaminants to the electron acceptors, thereby catalyzing the anaerobic biodegradation of contaminants (42). We found that the improvement of electrochemical performance in the treatments with graphene oxide was greater than that in the treatments with biochar. We suggest that some of the graphene oxide may have been transformed into reduced graphene oxide and the conductivity could have improved due to the action of microorganisms, as shown in a previous study (43). The biofilm and reduced graphene oxide both promoted the transfer of electron in these reactions (28,29).

The pH values determined for the culture systems containing different concentrations of graphene oxide ranged from 7.16 to 7.64, and pH values of the culture systems with biochar ranged from 7.21 to 7.56 (Figs. S2A and B). As the culture time increased, the pH of the culture systems increased gradually, resulting from the consumption of H^+ by nitrate or sulfate reducing bacteria during the anaerobic degradation of petroleum hydrocarbons (44). In addition, the pH values in the culture systems containing biochar were lower than those with graphene oxide, probably because the protonation/deprotonation of biochar increased the pH buffering performance of the culture systems (45). The ORP values for the culture systems containing different concentrations of graphene oxide ranged from −77 mV to −43 mV, and those for the culture systems with biochar ranged from −101 mV to −55 mV (Figs. S2C and D). As the culture time increased, the ORP values for the culture systems containing different concentrations of graphene oxide all decreased gradually. Anaerobic microorganisms that use nitrate as an electron acceptor grow preferentially in culture systems with mixed electron acceptors according to Cunningham et al. (46). Furthermore, The ORP values for the culture systems ranged from −101 mV to −43 mV, which were suitable for the growth of denitrifying bacteria (47). The decrease of ORP during the process was probably due to that the anaerobic degradation process of petroleum hydrocarbons consumed oxidizing substances (e.g., NO_3^-, SO_4^{2-}), accompanied by the formation of reducing substances (e.g., S^{2-}) (44).

Degradation rates of *n*-alkanes and BTEXs The degradation rates of *n*-alkanes and BTEXs compared with the autoclaved controls are shown in Fig. 2, and their concentrations are presented in Fig. S3. After culture for 10 weeks, the degradation rates for *n*-alkanes reached up to 70.0% and 77.8% with 2 mg/L of graphene oxide and 100 mg/L of biochar, respectively (Fig. 2A, B). In the first 6 weeks, the degradation rates of *n*-alkanes in the culture systems with graphene oxide were all lower than those in the control groups, except for the treatment with 2 mg/L graphene oxide. In the first 6 weeks, the degradation rates of *n*-alkanes in the culture systems with biochar were not significantly different from those in the control groups. However, after culture for 10 weeks, the addition of different concentration of graphene oxide or biochar all significantly promoted the degradation of *n*-alkanes ($P < 0.05$). After culture for 10 weeks, the degradation rates of BTEXs were up to 76.5% and 77.6% with the addition of 0.02 mg/L graphene oxide and 20 mg/L biochar, respectively (Fig. 2C, D). The degradation rates of BTEXs in all of the treatments with different concentrations of carbon-based materials were significantly higher compared with the control ($P < 0.05$). The addition of the two types of carbon-based materials increased the degradation rates of *n*-alkanes in

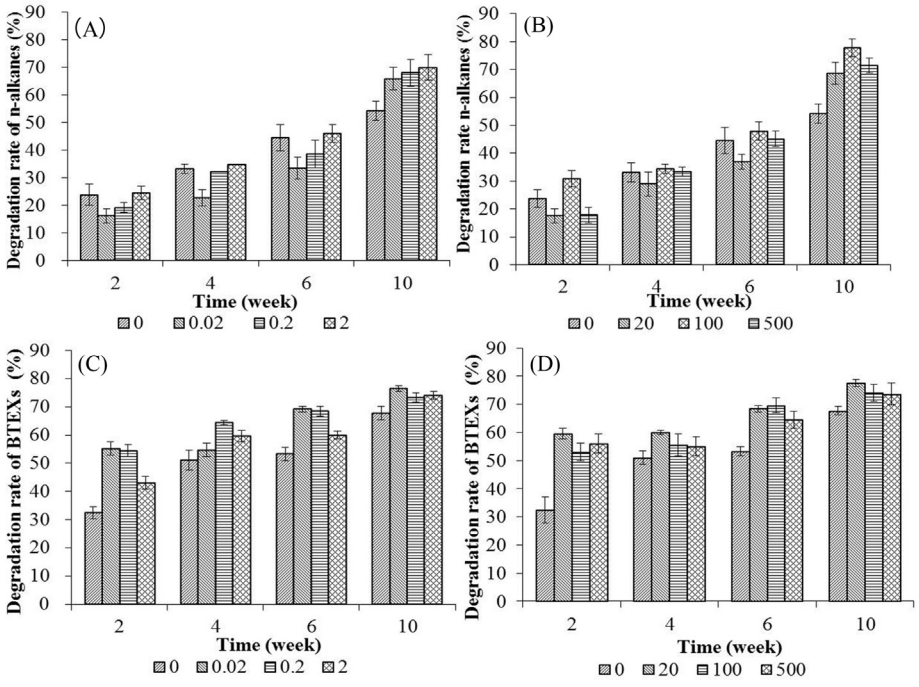

FIG. 2. Degradation rates of *n*-alkanes and BTEXs with different concentrations of graphene oxide (A, C) and biochar (B, D). The amounts of graphene oxide or biochar are 0, 0.02, 0.2, 2, 20, 100, and 500 mg/L.

the culture systems during the late stage of cultivation (in the 10th week), and enhanced the degradation rates of BTEXs in the culture systems throughout the overall process.

The growth of methanogenic consortia and the degradation of *n*-alkanes and BTEXs are slow in anaerobic environments without electron acceptors (48,49). Many anaerobic microorganisms have been isolated that can use various electron acceptors to efficiently degrade *n*-alkanes and BTEXs (10,50). Moreover, the addition of carbon-based materials enhances these degradation processes further (23) because the addition of carbon-based materials can provide a large specific surface area for the growth of microorganisms and promote the electron transfer process, thereby improving the anaerobic degradation of petroleum hydrocarbons (19,51). In addition, compared with alkanes (C15–C20), BTEXs were preferentially degraded by anaerobic microorganisms (52) and the degradations of BTEXs were promoted by carbon materials more remarkably.

16S rDNA, *masD*, and *bamA* gene copy number As the culture time increased, the 16S rDNA copy numbers in the culture systems with different concentrations of carbon-based materials all increased gradually during the first 6 weeks, and then decreased slightly subsequently, except for the treatments with 2 mg/L graphene oxide or 500 mg/L biochar (Fig. 3A, B). The 16S rDNA copy numbers in the treatments with different concentrations of graphene oxide ranged from 2.68×10^3 copies/mL (2 mg/L, in the 2nd week) to 5.91×10^4 copies/mL (0.02 mg/L, in the 6th week). Except for the 10th week, 16S rDNA copy numbers in the culture systems with 0.02 mg/L graphene oxide were always highest followed by treatments with 0.2 mg/L, 0 mg/L and 2 mg/L graphene oxide. The 16S rDNA copy numbers in the treatments with different concentrations of biochar ranged from 2.67×10^3 copies/mL (500 mg/L, in the 2nd week) to 4.54×10^4 copies/mL (20 mg/L, in the 6th week). The treatments with 20 mg/L biochar exhibited the highest 16S rDNA copy numbers all over the experiment, while the ones with 500 mg/L biochar exhibited the lowest.

The 16S rDNA copy numbers determined in the treatments indicated that low concentrations of carbon-based materials promoted the growth of anaerobic microorganisms (graphene oxide $\leq$0.2 mg/L; biochar $<$100 mg/L), whereas high concentrations of materials inhibited microbial growth. This was mainly because the low concentration of carbon-based materials provided a large surface area for the growth of microorganisms (Fig. S1), whereas the high concentration may have inhibited the uptake of nutrients by microbes or mechanically damaged the microorganisms (53,54).

As the culture time increased, the *masD* gene copy numbers in the culture systems with different concentrations of carbon-based materials all decreased gradually during the first six weeks, but then increased slightly subsequently (Fig. 3C, D). The *masD* gene copy numbers in all treatments did not differ significantly, except in the treatments with 20 mg/L of biochar in the 2nd and 4th weeks. With different concentrations of graphene oxide, the *masD* copy numbers in the treatments ranged from 97 copies/mL (0.2 mg/L, in the 6th week) to 289 copies/mL (0 mg/L, in the 2nd week). The *masD* gene copy numbers in treatments with different concentrations of biochar ranged from 119 copies/mL (500 mg/L, in the 6th week) to 349 copies/mL (20 mg/L, in the 2nd week). However, after culture for 10 weeks, the addition of different concentration of

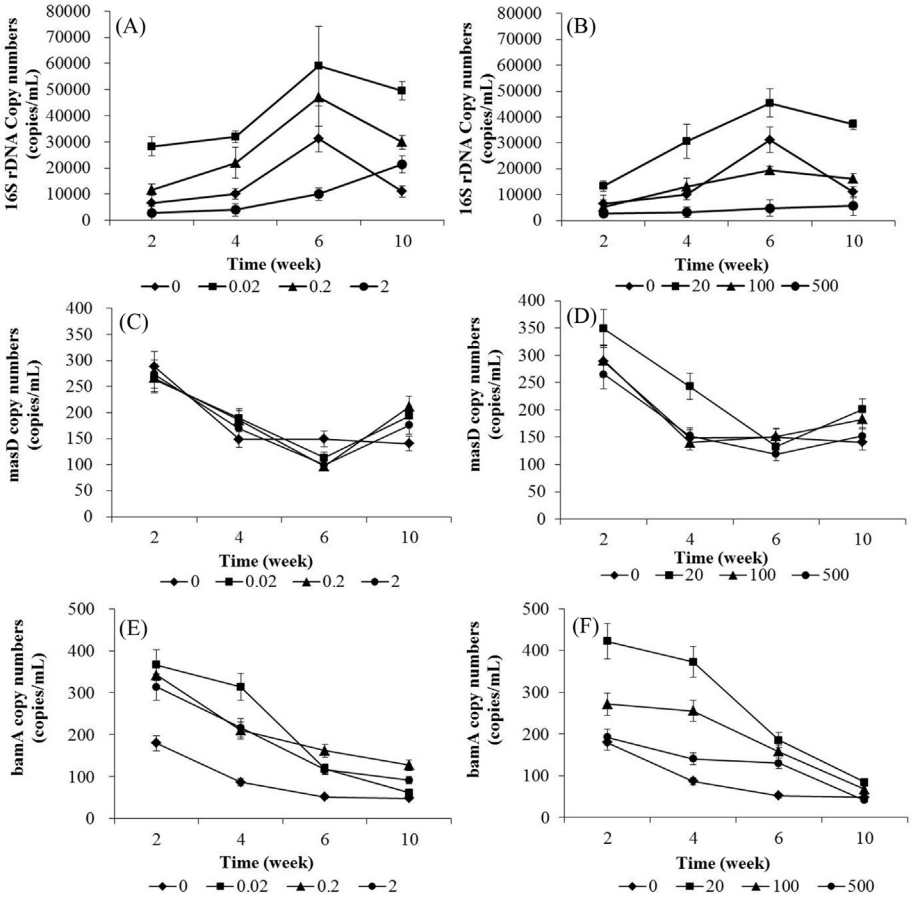

FIG. 3. Copy numbers of 16S rDNA, *masD*, and *bamA* genes in the culture systems with different concentrations of graphene oxide (A, C, E) and biochar (B, D, F).

graphene oxide or biochar all increased the *masD* gene copy numbers ($P < 0.05$).

As the culture time increased, the *bamA* gene copy numbers in the culture systems with different concentrations of carbon-based materials all gradually decreased throughout the process (Fig. 3E, F) but they were all significantly higher ($P < 0.05$) than those in control, except in the 10th week for the treatment with 500 mg/L biochar. The *bamA* gene copy numbers in the treatments with different concentrations of graphene oxide ranged from 48 copies/mL (0 mg/L, in the 10th week) to 366 copies/mL (0.02 mg/L, in the 2nd week). With different concentrations of biochar, the *bamA* gene copy numbers in the treatments ranged from 41 copies/mL (500 mg/L, in the 10th week) to 422 copies/mL (20 mg/L, in the 2nd week). Except for the 10th week, the *bamA* gene copy numbers in the treatments with 20 mg/L biochar were always the highest followed by treatments with 100 mg/L, 500 mg/L and 0 mg/L biochar.

The copy numbers of the two degradation genes were very high in the 2nd week when the amount of microorganisms began to increase, mainly because the microorganisms used petroleum hydrocarbons for their growth and metabolism (9). As the culture time increased, the number of microorganisms increased but the copy numbers of the two degradation genes decreased gradually, possibly because the products from the degradation of petroleum hydrocarbons promoted the growth of other microbes in the community. In addition, the increase in the pH and the decrease in the ORP value may have prevented the further growth of degrading microbes (47). In addition, compared with *masD*, the addition of carbonaceous material remarkably increased the *bamA* gene copy numbers. This confirmed that the carbonaceous material promoted the degradation of BTEXs effectively.

DGGE electrophoresis, clustering analysis, and phylogenetic relationships DGGE electrophoresis analysis and the phylogenetic relationships in the microbial community indicated that the dominant microorganisms in the culture systems under all of the treatments comprised *Pseudomonas aeruginosa* (band GO1, C1), *Paracoccus denitrificans* (band GO2, C2), and *Hydrogenophaga caeni* (band GO3, C3) (Fig. 4). These three kinds of microorganisms appeared in most of the culture systems. In the late stage of cultivation, *Bacillus* sp. appeared in the culture systems with graphene oxide, whereas *Thiobacillus* sp. appeared in the culture systems with biochar. The Shannon indices determined for the treatments containing different concentrations of carbon-based materials ranged from 2.25 to 3.21 (Fig. S4A, B). When different concentrations of graphene oxide were added, the maximum Shannon indices for all treatments occurred in the 4th week, whereas the maximum indices were determined in the 6th week without graphene oxide. In addition, at the early stage (in the first 4th weeks) carbon materials remarkably improved the microbial community diversity in the anaerobic culture system. The clustering analysis results indicated that the culture time had significant effects on the microbial community structures, while the concentration of carbon-based materials had the 2nd highest effect (Fig. S4C, D).

An important characteristic of dominant bacteria *P. denitrificans* is that it can reduce NO_3^- to N_2 under anaerobic growth conditions (55). *P. denitrificans* is a metabolically versatile strain that can

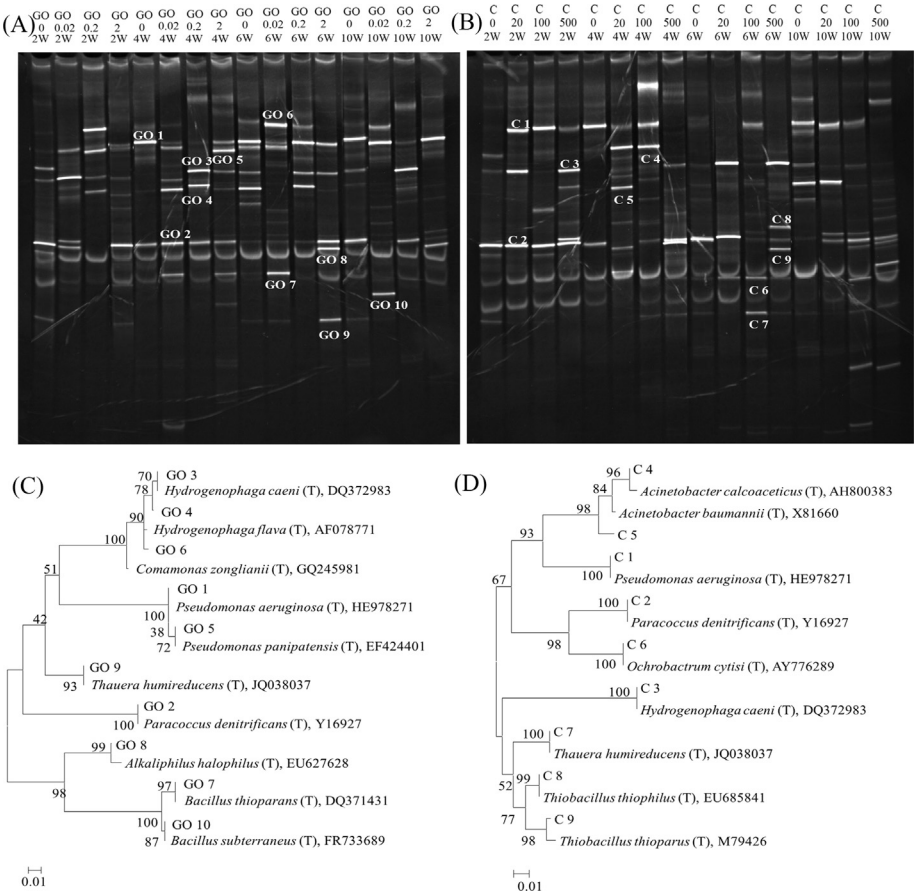

FIG. 4. DGGE electrophoresis results and phylogenetic relationships of microbial communities with different concentrations of graphene oxide (A, C) and biochar (B, D). GO or C indicates that graphene oxide or biochar was added to the treatments, respectively. The amounts of graphene oxide or biochar are 0, 0.02, 0.2, 2, 20, 100, and 500 mg/L. 2W, 4W, 6W, and 10W denote the culture time in week.

obtain energy by degrading organic compounds and by converting inorganic compounds chemolithotrophically, such as via sulfur oxidation (55). Yang et al. (56) demonstrated that *P. denitrificans* strain M-1 isolated from petroleum-contaminated sediment could degrade pyrene under denitrifying conditions. Another dominant bacteria *P. aeruginosa* can also generate energy in the absence of oxygen via denitrification (57), where molecular oxygen is replaced by nitrate as the terminal electron acceptor during this process. Grishchenkov et al. (58) found that a facultatively anaerobic *Pseudomonas* sp. isolated from petroleum-contaminated soil could degrade petroleum hydrocarbons under anaerobic conditions. The biodegradation of benzene, toluene, ethylbenzene, and *o*-xylene by a co-culture of *Pseudomonas* was demonstrated in a fibrous-bed bioreactor (59). Moreover, studies suggest that *H. caeni* can degrade many components of petroleum hydrocarbons (60,61). Golby et al. (62) indicated that *Hydrogenophaga* strains were the most abundant bacteria (19.5%) in anaerobic biofilms from an oil sand tailings pond and they could also survive in aerobic environments. Therefore, in mixed electron acceptor culture conditions in the presence of nitrate, we found that facultative bacteria were dominant, especially denitrifying bacteria, as shown in previous studies (46,63).

In conclusion, our results showed that the addition of carbon-based materials increased the electrochemical activity of the culture system and promoted the anaerobic degradation of petroleum hydrocarbons. The 16S rDNA, *masD*, and *bamA* gene copy numbers all increased by varying degrees in the treatments with suitable carbon-based materials. The contents of carbon-based materials slightly influenced the microbial community structure, whereas the cultivation time had a major effect.

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