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Anaerobic degradation of nonylphenol in subtropical mangrove sediments

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ABSTRACT

Nonylphenol (NP) is known as an endocrine disruptor and has consequently drawn much environmental concern. We investigated the effects of various factors on the anaerobic degradation of NP and characterized the structures of microbial communities in mangrove sediments collected at five sites along the Tanshui River in northern Taiwan. NP anaerobic degradation rate constants (k_1) and half-lives ($t_{1/2}$) ranged from 0.008 to 0.013 1/day and 53.3 to 86.6 days, respectively. The addition of NaCl (1%, 2%), zero-valent iron (10 g/L), humic acid (0.5 g/L), cellulose (0.96 mg/L), brij 30 (55 μM) and brij 35 (91 μM) enhanced NP anaerobic degradation. However, the addition of NaCl (3%), acetate (20 mM), lactate (20 mM), pyruvate (20 mM), and humic acid (5 g/L) inhibited NP anaerobic degradation. Sulfate-reducing bacteria, methanogen, and eubacteria are involved in the degradation of NP, sulfate-reducing bacteria being a major component of the sediment. Our results also show that the addition of various substrates changed the microbial community in mangrove sediments. Also noted was the presence of 2-butyl-1-octanol, an intermediate product resulting from the anaerobic degradation of NP accumulated in the sediments.

1. Introduction

Nonylphenol (NP) has increasingly attracted attention because of its potential to mimic the action of natural hormones in vertebrates [1]. It results from the incomplete biodegradation of nonylphenol polyethoxylates (NPEOs), which have been widely used as non-ionic surfactants in industrial processes and households [2,3]. Both NP and NPEOs are discharged into the environment, mainly due to incomplete removal in wastewater treatment facilities [4]. Due to their hydrophobicity, they tend to adsorb onto surface water particles and sediments and accumulate in aquatic organisms [5,6]. Consequently, NP represents a serious environmental and human health risk. Assessing the biodegradative processes that affect the environmental fate of these pollutants is thus of considerable importance.

Mangroves, the dominant intertidal wetlands along the coastlines of tropical and subtropical regions, have been considered significant sinks for pollutants from freshwater discharges as well as from contaminated tidal water [7,8]. The Guandu and Zhuwei Mangroves are the largest mangroves in subtropical Taiwan. Both mangroves are on the bank of the Tanshui River, which is one of the most heavily contaminated rivers in northern Taiwan.

Microbial degradation is believed to be one of the major processes that clean up NP-contaminated sediments. Biodegradation of NP in sediments has been observed [5,9,10]. The biodegradation of various pollutants in mangrove sediments has also been extensively documented [11–14], but little is known about anaerobic degradation of NP in mangrove sediments.

The climatic characteristics of subtropical regions foster diverse microbial communities. Molecular-biological methods enable studies of microbial diversity in environmental samples. Denaturing gradient gel electrophoresis (DGGE) of the PCR-amplified 16S rRNA gene can be a powerful tool for characterizing the microbial community, monitoring the dominant population and identifying species from the analysis of the sequences [15]. Many studies have used the PCR-DGGE technique to examine the degradation of pollutants by microbial communities in soil, wastewater, and sediment [16–19]. However, studies applying the PCR-DGGE technique to characterize NP-degrading microbial communities in mangrove sediments are lacking.

The aims of this study were to investigate the effects of various factors on the anaerobic degradation of NP and characterize the structure of the microbial community in mangrove sediments.

2. Materials and methods

2.1. Chemicals

NP with 98.0% analytical purity was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Solvents were purchased from Mallinckrodt, Inc. (Paris, KY). All other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). Individual stock

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solutions of NP dissolved in chloroform were established at a concentration of 10,000 mg/L, and then diluted to 500 mg/L before use.

2.2. Sample collection and medium

We selected sediment samples that were used in our previous study [19]. In July 2005, samples were taken from the Guandu and Zhuwei Mangroves in northern Taiwan. Sites A, B and C were in the Guandu Mangroves, while sites D and E were in the Zhuwei Mangroves. The deep sediments (5–20 cm) were collected during low tides using a soil core. All sediment samples were taken randomly, in triplicate, from an area of around 1 m^2 at the centre of each mangrove sediment site. Adaptation was performed by adding 100 μg/g NP to 500 g of sediment at 14-day intervals under static incubation at 30 °C without light for 6 months. In this paper, the sediment is referred to as NP-adapted sediment.

For sediment samples from sites A, B, C, D and E, the organic carbon was 1.18%, 2.54%, 1.78%, 0.89% and 1.89%, respectively; the pH, determined in sediment/water mixtures (1:1, w/v), was 7.3, 7.4, 7.1, 6.5 and 7.6, respectively; the bacterial count was 1.1×10^6, 2.4×10^7, 5.6×10^6, 1.4×10^5 and 3.1×10^6 CFU/g, respectively; the salinity was 1.3%, 1.2%, 2.0%, 2.8% and 2.1%, respectively [19].

The experimental medium consisted of (all concentrations in g/L): NH_4Cl, 2.7; $MgCl_2{\cdot}6H_2O$, 0.1; $CaCl_2{\cdot}2H_2O$, 0.1; $FeCl_2{\cdot}4H_2O$, 0.02; K_2HPO_4, 0.27; KH_2PO_4, 0.35; and resazurin, 0.001. The pH was adjusted to 7.0 following autoclaving; 0.9 mM titanium citrate was added as a reducing reagent.

2.3. Experimental design

Experiments were performed using 125 mL serum bottles containing 45 mL of medium, 5 g of mangrove sediment, and 125 μg/g of NP. NP degradation was first measured with NP-adapted or non-adapted sediment collected from sites A, B, C, D and E. The following factors were added to NP-adapted sediment collected from site B: sodium chloride (1%, 2% and 3%), humic acid (0.5 and 5 g/L), the non-ionic surfactants brij 30 and brij 35 at a concentration of 1 CMC (55 and 91 μM, respectively), zero-valent iron (10 g/L) and cellulose (0.96 mg/L); the electron donors sodium acetate (20 mM), sodium lactate (20 mM) or sodium pyruvate (20 mM); sodium hydrogen carbonate (20 mM) to create methanogenic conditions, sodium sulfate (20 mM) to create sulfate-reducing conditions, or sodium nitrate (20 mM) to create nitrate-reducing conditions; and microbial inhibitors (50 mM BESA, 50 mM vancomycin, 50 mM sodium molybdate-2-hydrate). Inoculated control samples (without sodium hydrogen carbonate, sodium sulfate or sodium nitrate), which were considered non-sterile and NP-adapted sediment, were shaken prior to incubation at 30 °C and pH 7.0 in the dark. Sterile controls were autoclaved at 121 °C for 30 min on 2 consecutive days.

All experiments were conducted in an anaerobic glove box (Forma Scientific, model 1025 S/N, USA) filled with N_2 (85%), H_2 (10%) and CO_2 (5%) gases. Bottles were capped with butyl rubber stoppers, wrapped in aluminum foil to prevent photolysis, and incubated without shaking at 30 °C in the dark. The bottle contents were periodically sampled in order to measure residual NP concentrations. We also used PCR-DGGE to analyze the microbial community in the sediment samples.

2.4. Analytical methods

NP extraction and analysis were performed as described in our previous study [10]. NP was extracted from acidified sediment samples twice by chloromethane, extracted again over 30 min at 30 °C with a Branson 5200 ultrasonic cleaner (Branson, USA), and then extracted using a C18 solid phase extraction cartridge. Extracts were analyzed with a Varian 3900 gas chromatograph connected to a Saturn 2100 ion-trap mass spectrometer (Varian, USA) and equipped with a DB-5 MS capillary column (30 m × 0.25 mm I.D., 0.25 μm film; J&W, USA). The initial column temperature was set at 80 °C for 1 min, increased at a rate of 30 °C/min to 200 °C, maintained at 200 °C for 2 min, and then increased at a rate of 10 °C/min to 300 °C, where it was held for 2 min. Injector temperatures were also maintained at 300 °C. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The splitter was opened for 30 s following injection at a split ratio of 20:1. Full-scan electron impact (EI) ionization data were collected under the following conditions: mass range 40–310 m/z, scan time 0.75 s, manifold temperature 60 °C, and emission current 4.0 μA. The average recovery was 88.2 ± 1.8%, and the detection limit was 50 μg/L. Measurements of sulfate concentrations were made using procedures described by the American Public Health Association [20].

2.5. DNA extraction and PCR-DGGE analysis

DNA extraction and PCR-DGGE analysis were performed as described in our previous study [19]. DNA was extracted from sediment samples using the PowerSoil DNA kit. The partial sequence fragment of the 16S rRNA gene was PCR-amplified with the primers FGC968 (*E. coli* position 968-983, 5′-GCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3′) and R1401 (*E. coli* position 1401-1385, 5′-CGGTGTGTACAAGACCC-3′) for DGGE analysis.

The PCR solution consisted of 76 μL of sterile water; 2 μL of 10 × buffer with $MgCl_2$; 0.2 mM of each of the primers; 2 μL of a deoxynucleotide triphosphate mixture [2.5 mM (each) dATP, dCTP, dGTP and dTTP]; 0.2 μL of Taq; and 1 μL of template DNA solution, all combined in a sterile 0.2 mL tube. The cycling parameters were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of 45 s at 95 °C, 45 s at 55 °C, and 1 min at 72 °C; and final extension at 72 °C for 10 min. PCR products were analyzed by electrophoresis in 1.2% (w/v) nusieve 3:1 agarose gels containing ethidium bromide (1 μg/mL).

DGGE was performed as a D-gene and D-code system (Bio-Rad Laboratories, Hercules, CA, USA) with a 1 mm gel width. Approximately 500 ng of PCR product was applied directly to each 6% polyacrylamide gel in 1 × TAE buffer (40 mM tris, 20 mM acetic acid and 1 mM EDTA; pH 8.3) with denaturing gradients formed with 6% acrylamide stock solutions containing 35% and 70% denaturant (5.6 M urea). Electrophoresis was run in 1 × TAE buffer at a voltage of 200 V and a temperature of 60 °C for 3 h. After electrophoresis, the gels were incubated for silver staining. Gels were scanned using a video system.

The gels were also stained for 30 min with SYBR Green solution and visualized on a UV transilluminator. The bands of interest were excised and soaked in elution buffer (0.3 M NaCl, 0.3 mM EDTA, 30 mM tris, pH 7.6) overnight at 37 °C. The DNA was reamplified with the primers FGC968 and R1401 as for the initial PCR reaction. The reamplified products were again purified and were sequenced with an ABI-Prism automatic sequencer. Sequence comparisons were performed using the BLAST facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.Gov/BLAST).

2.6. Data analysis

The NP biodegradation data collected for this study fit well with first-order kinetics: $S = S_0\exp(-k_1 t)$, $t_{1/2} = \ln 2/k_1$, where S_0 is the initial concentration, S the substrate concentration, t the time period, and k_1 the biodegradation rate constant. Analysis of variance (ANOVA) of the regression equations was calculated using

Table 1
NP concentrations in mangrove sediment collected from five sampling sites.

Sampling site	NP concentration (ng/g)
A	445.2
B	571.9
C	521.1
D	ND
E	373.2

ND: not detected. All NP data were conducted on triplicate samples, with standard deviation within 5%.

the Statistical Package for Social Sciences software (SPSS 10.0 for Windows, SPSS Inc. IL, USA).

The DGGE profiles were analyzed by UPGMA (unweighted pair-group method using arithmetic averages) and similarities were calculated by the coefficient of DICE, using the Phoretix 1D Professional software (Nonlinear Dynamics, UK). The UPGMA method was used to reveal changes in the microbial community over different treatments.

3. Results and discussion

3.1. Comparison of NP concentrations in mangrove sediment from five sampling sites

As shown in Table 1, NP concentrations were 445.2, 571.9, 521.1, ND (not detected), and 373.2 ng/g for sediment samples from sites A, B, C, D, and E, respectively. Other studies on river sediment found NP concentrations ranging from 190 to 13,100 ng/g [5] and from 38.4 to 863.0 ng/g [21]. Our results show that NP concentrations in the river sediments were not higher than those in other countries. The results also indicate that the sediment samples from site B had the highest NP concentrations. The sediment samples from site B were collected from the Guandu Mangrove and had probably been affected by vehicle exhaust deposition and the discharge of industrial, agricultural, and household waste and wastewater for several decades [19]. Liu et al. [22] measured concentrations of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and phthalate esters (PAEs) at the same sampling site and found it to be one of the most heavily contaminated sampling sites in the river.

3.2. Comparison of anaerobic NP degradation in mangrove sediments from five sampling sites

We compared anaerobic degradation rates in mangrove sediment samples collected from five sampling sites (Table 2). NP anaerobic degradation half-lives were 57.8, 53.3, 69.3, 77.0, and 86.6 days with non-NP-adapted sediment from sites A, B, C, D, and E, respectively. The half-lives of NP degradation in sediment in our experiments were higher than those reported in our previous study in Erren river sediment [9], which recorded half-lives ranging from 46.2 to 69.3 days. Different ecosystems may present different microbial communities and have different NP-degrading capabilities. The results indicate that the sediment from site B had a higher NP anaerobic degradation rate than the samples from the other sites. Sediment samples from site B had higher organic carbon levels, bacterial counts and NP concentration than those from the other sampling sites. Microorganisms adapt to site-specific conditions, resulting in varied but optimal biodegradation capacities. This result is similar to that reported by Yuan et al. [23], who studied the occurrence and microbial degradation of PAEs in river sediments.

We also found that NP anaerobic degradation half-lives were 28.9, 21.7, 34.7, 40.8 and 49.5 days with NP-adapted sediment from sites A, B, C, D and E, respectively. The results show that the adaptation process enhanced NP anaerobic degradation. The results are similar to those from our previous study of the effects of adaptation on NP anaerobic degradation in sediment [9]. We will restrict our discussion to results from the NP-adapted sediment sample from site B in the next experiments.

Table 3
Effects of the addition of various substrates on NP anaerobic degradation rate constants (k_1) and half-lives ($t_{1/2}$) in the mangrove sediments.

Treatment	k_1 (1/day)	$t_{1/2}$ (days)	r[a]
Inoculated control[b]	0.032	21.7	0.91
NaCl (1%)	0.053	13.1	0.89
NaCl (2%)	0.040	17.3	0.87
NaCl (3%)	0.024	28.9	0.97
Humic acid (0.5 g/L)	0.035	19.8	0.91
Humic acid (5 g/L)	0.019	36.5	0.90
Brij 30 (55 μM)	0.043	16.1	0.89
Brij 35 (91 μM)	0.053	13.1	0.91
Zero-valent iron (10 g/L)	0.069	10.0	0.96
Cellulose (0.96 mg/L)	0.038	18.2	0.95
Acetate (20 mM)	0.014	49.5	0.92
Lactate (20 mM)	0.017	40.8	0.91
Pyruvate (20 mM)	0.019	36.5	0.98

Each figure represents the mean of three measurements. Each treatment was different significantly from the inoculated control at $p < 0.05$.

[a] r = Correlation coefficient.

[b] Inoculated control: 30 °C, pH 7.0, NP 125 μg/g.

3.3. Effects of various factors on NP anaerobic degradation

The effects of the addition of various substrates on NP anaerobic degradation in the mangrove sediment are presented in Table 3. The results show that NP anaerobic degradation was enhanced by the addition of 1% and 2% sodium chloride, but was inhibited by addition of 3% sodium chloride. Tam et al. [11] studied biodegradation of phenanthrene from mangrove sediments and found that although the optimum salinity for the biodegradation was around 1–2%, high salinity (3.5%) had an inhibitory effect on biodegradation. NP biodegradation was enhanced by addition of 0.5 g/L humic

Table 2
Anaerobic degradation rate constants (k_1) and half-lives ($t_{1/2}$) of NP in mangrove sediments collected from five sampling sites.

Sites	Non-NP-adapted sediment			NP-adapted sediment		
	k_1 (1/day)	$t_{1/2}$ (days)	r[a]	k_1 (1/day)	$t_{1/2}$ (days)	r[a]
A	0.012	57.8	0.85	0.024	28.9	0.94
B	0.013	53.3	0.93	0.032	21.7	0.91
C	0.010	69.3	0.96	0.020	34.7	0.86
D	0.009	77.0	0.92	0.017	40.8	0.91
E	0.008	86.6	0.97	0.014	49.5	0.92

All treatment figures were significantly different at $p < 0.05$. Each figure represents the mean of three measurements. The sediment samples from five sampling sites were incubated at 30 °C and pH 7.0 in the dark.

[a] r = Correlation coefficient.

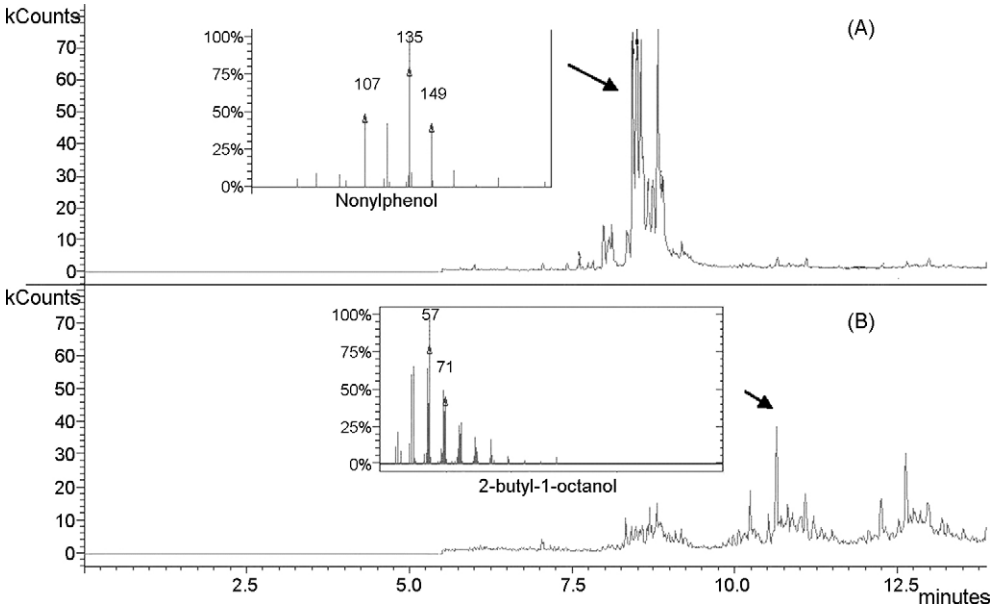

Fig. 1. GC/MS chromatograms at day 0 (A) and day 28 (B) of NP incubated and biotransformed in mangrove sediments.

acid, but inhibited by addition of 5 g/L humic acid. Humic acid showed a higher reducing capacity in deeper layers, probably due to reduction by humic-acid-reducing microorganisms [24]. Higher concentrations of humic acid enhance the sorption and binding of organic pollutants, thus inhibiting NP degradation [25,26]. The addition of cellulose enhanced the biodegradation of NP because it stimulates both aerobic and anaerobic respiration [27].

As is also shown in Table 3, addition of brij 30 and brij 35 enhanced NP biodegradation. NP partitioned into a surfactant's micellar phase is directly available to be metabolized by a microorganism, and this clearly enhances its degradation [28]. NP degradation rates were higher in the presence of brij 35 compared with brij 30. The compounds contain identical alkyl chains, but their polyoxyethylene chains differ significantly in length (23 for brij 35 and 4 for brij 30). The observed difference in bioavailability in the micellar phase is probably due to the difference in polyoxyethylene chain length, which defines the micelles' surface characteristics [29]. The addition of zero-valent iron enhanced the anaerobic degradation of NP. Zero-valent iron can serve as an electron donor and is capable of efficiently removing PAH [30]. We also noted an inhibition of NP degradation as a result of the addition of acetate, lactate or pyruvate. It may be that acetate, lactate and pyruvate do not function as electron donors in this experiment.

We also studied NP biotransformation in the mangrove sediments. GC–MS chromatograms taken on days 0 and 28 of incubation are shown in Fig. 1. We found that NP concentration decreased as incubation time increased from day 0 to day 28. No intermediate products were observed on day 0, but a single intermediate product, identified as 2-butyl-1-octanol, appeared after day 28.

3.4. Comparison among various reducing conditions and inhibitors of microbial NP anaerobic degradation

Table 4 indicates that the anaerobic degradation of NP was enhanced under methanogenic and sulfate-reducing conditions and inhibited under nitrate-reducing conditions. The order of rates for anaerobic degradation of NP in mangrove sediments under these conditions was observed to be sulfate-reducing conditions > methanogenic conditions > nitrate-reducing conditions. After 35 days of incubation, sulfate concentration decreased from 165 to 2 mg/L under sulfate-reducing conditions. It may be that the sulfate-reducing bacteria grew rapidly and subsequently reduced the sulfate concentrations in the sediment samples. We also found from GC–MS chromatograms that sulfur and cyclic octaatomic sulfur products appeared within 35 days of incubation. These observations indicate that NP degradation is closely related to sulfate reduction.

Table 5 shows that the anaerobic degradation of NP was inhibited by the addition of molybdate (a selective inhibitor of sulfate-reducing bacteria), BESA (a selective inhibitor of methanogens) or vancomycin (a selective inhibitor of eubacteria) [31,32]. The results obtained under the three anaerobic conditions and in the presence of the three microbial inhibitors indicate that sulfate-reducing bacteria, methanogens and eubacteria are

Table 4
Comparison among the NP anaerobic degradation rate constants (k_1) and half-lives ($t_{1/2}$) in mangrove sediments under three reducing conditions.

Treatment	k_1 (1/day)	$t_{1/2}$ (days)	r[a]
Inoculated control[b]	0.032	21.7	0.91
Nitrate-reducing conditions	0.024	28.9	0.90
Sulfate-reducing conditions	0.055	12.6	0.92
Methanogenic conditions	0.043	16.1	0.87

Each figure represents the mean of three measurements. Each treatment was different significantly from the inoculated control at $p < 0.05$.

[a] r = Correlation coefficient.

[b] Inoculated control: 30 °C, pH 7.0, NP 125 μg/g.

Table 5
Comparison among the NP anaerobic degradation rate constants (k_1) and half-lives ($t_{1/2}$) in mangrove sediments following the addition of three microbial inhibitors.

Treatment	k_1 (1/day)	$t_{1/2}$ (days)	r[a]
Inoculated control[b]	0.032	21.7	0.91
Vancomycin	0.018	38.5	0.91
BESA	0.014	49.5	0.96
Molybdate	0.011	63.0	0.89

Each figure represents the mean of three measurements. Each treatment was different significantly from the inoculated control at $p < 0.05$.

[a] r = Correlation coefficient.

[b] Inoculated control: 30 °C, pH 7.0, NP 125 μg/g.

involved in NP degradation. Sulfate-reducing bacteria may play the largest microbial role, but methanogens and eubacteria are also involved. This result is similar to that reported by Chang et al. [9], who studied the anaerobic degradation of NP in river sediments.

3.5. Microbial community analysis

Fig. 2A shows the microbial community changes in the DGGE resulting from various treatments within 35 days of incubation. The results show that the DGGE profile consisted of at least six bands, and the number of bands was changed by the addition of various treatments. Upon comparison of the NP-adapted and non-NP-adapted treatments, we found that the microbial community had become simpler after the NP-adaptation process, but the two communities were more similar to each other than to those from other treatments. The similarities in the DGGE patterns of 16S rDNA fragments after various treatments are shown in Fig. 2B. We found that the microbial communities changed significantly when substrates were added. This is consistent with our previous finding that treatment with different substrates changes the microbial communities in soil samples [19]. We also found highly similar microbial communities in samples receiving various treatments such as NaCl (1% and 2%), humic acid (0.5 g/L), cellulose, zero-valent iron, and brij 30 and brij 35, which can all enhance NP degradation. The microbial communities were especially similar under the same kind of additive, such as NaCl (1%) and NaCl (2%), or brij 30 and brij 35. However, microbial communities had higher similarities under treatments that can inhibit NP degradation, such as NaCl (3%), humic acid (5 g/L), acetate, lactate and pyruvate.

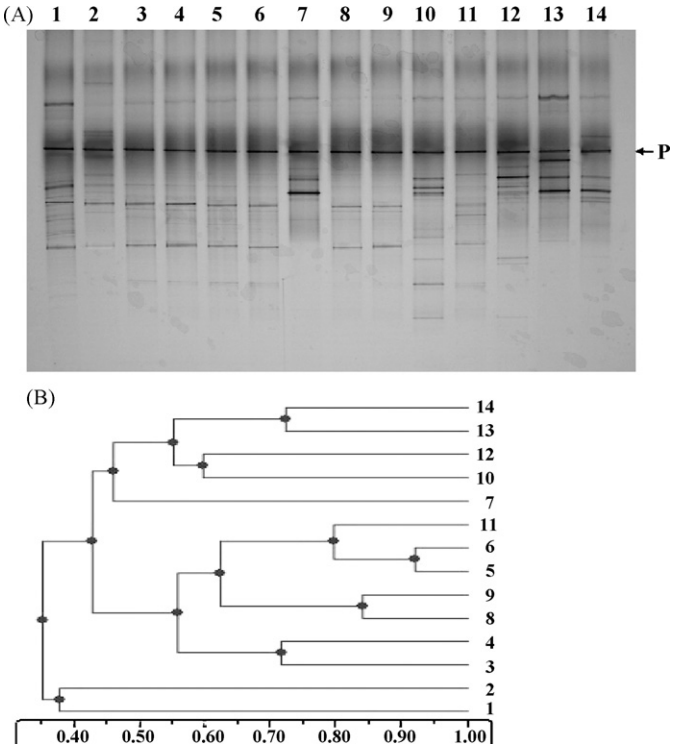

Fig. 2. Changes in the DGGE band profiles (A) and similarities of DGGE patterns of 16S rDNA fragments (B) following the addition of various substrates within 35 days of incubation. Sample designations: lane 1 represents the sample without addition of substrates and without NP-adaptation; line 2 represents the sample without addition of substrates and with NP-adaptation; lanes 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 respectively represent the addition of humic acid (0.5 g/L), cellulose (0.96 mg/L), brij 30 (55 μM), brij 35 (91 μM), sodium chloride (3%), sodium chloride (2%), sodium chloride (1%), humic acid (5 g/L), zero-valent iron (10 g/L), acetate (20 mM), lactate (20 mM) and pyruvate (20 mM).

We also excised a portion of band P from the DGGE band profiles and found that band P matched the genomic sequence of *Clostridium aminovalericum*, with a similarity of 99.0%. This strain, an obligatory anaerobe, grows normally after growth conditions are changed from anoxic to micro-oxic, when the cells consume oxygen efficiently [33]. *C. aminovalericum* was the consistently dominant bacterium in the mangrove sediments under the various treatments applied to enhance or inhibit NP degradation.

4. Conclusions

Microbial degradation of NP is a major process that results in the decontamination of mangrove sediments. The addition of NaCl, humic acid, zero-valent iron, brij 30, brij 35, acetate, lactate, and pyruvate can influence NP anaerobic degradation and change the diversity of the microbial community in mangrove sediments. The results support the feasibility of the removal NP in mangrove sediment by anaerobic degradation.

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