Biodegradation of Complex Bacteria on Phenolic Derivatives in River Water[1]

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Objective To isolate, incubate, and identify 4-chlorophenol-degrading complex bacteria, determine the tolerance of these bacteria to phenolic derivatives and study their synergetic metabolism as well as the aboriginal microbes and co-metabolic degradation of mixed chlorophenols in river water. **Methods** Microbial community of complex bacteria was identified by plate culture observation techniques and Gram stain method. Bacterial growth inhibition test was used to determine the tolerance of complex bacteria to toxicants. Biodegradability of phenolic derivatives was determined by adding 4-chlorophenol-degrading bacteria in river water. **Results** The complex bacteria were identified as *Mycopiana*, *Alcaligenes*, *Pseudomonas*, and *Flavobacterium*. The domesticated complex bacteria were more tolerant to phenolic derivatives than the aboriginal bacteria from Qinhuai River. The biodegradability of chlorophenols, dihydroxybenzenes and nitrophenols under various aquatic conditions was determined and compared. The complex bacteria exhibited a higher metabolic efficiency on chemicals than the aboriginal microbes, and the final removal rate of phenolic derivatives was increased at least by 55% when the complex bacteria were added into river water. The metabolic relationship between dominant mixed bacteria and river bacteria was studied. **Conclusion** The complex bacteria domesticated by 4-chlorophenol can grow and be metabolized to take other chlorophenols, dihydroxybenzenes and nitrophenols as the sole carbon and energy source. There is a synergetic metabolism of most compounds between the aboriginal microbes in river water and the domesticated complex bacteria. 4-chlorophenol-degrading bacteria can co-metabolize various chlorophenols in river water.

Key words: Complex bacteria; Substituted phenols; Biodegradability; Synergetic metabolism

INTRODUCTION

A large variety of chemicals are commercially produced and newly synthesized each year. Compounds are discharged into water environment during their use and manufacture and cause great damages to aquatic organisms. Over the last decades, halogenated aromatic compounds have been used extensively as pesticides and herbicides[1], among which chlorinated phenols in waters are toxic to aquatic organisms and prone to concentrate and accumulate in these organisms. Therefore, it is difficult to eliminate their effects by degrading aborigine microorganisms in natural waters[2-3].

In treating chlorophenols, biological methods have attracted more attention than physical and chemical ones because of their relatively low cost and less secondary pollution[4]. For example, *Arthrobacter chlorophenolicus* A6, isolated from a soil slurry enriched with increasing concentrations of 4-chlorophenol (4-CP), is used to degrade unusually high concentrations of 4-CP and other *p*-substituted phenols, such as 4-nitrophenol (4-NP) and 4-bromophenol[5]. Kiyohara *et al*.[6] demonstrated that three strains of *Pseudomonas pickettii*, isolated from different mixed cultures of soil bacterial populations, can grow with 2,4,6-trichlorophenol (2,4,6-TCP) as the sole source of carbon and energy. Moreover, co-metabolism of mixed chemicals and synergetic metabolism of complex bacteria have been investigated for the degradation of chlorinated phenols[7-8].

Qinhuai River flows through the urban area of Nanjing before it joins the Yangtze River in China. Due to the rapid economic growth and high population density in the main downtown area, the water quality of Qinhuai River has been continuously worsened over the past 20 years. The current

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treatment rate of wastewater in Nanjing is only 80%, and a large amount of untreated industrial wastewater and urban sewage are released into this river. In this study, we investigated the biodegradation kinetics of chlorinated phenols, dihydroxybenzenes, and nitrophenols in Qinhuai River water using 4-CP-degrading complex bacteria from enriched mixed cultures and demonstrated the potential of co-metabolism of mixed pollutants and the synergetic effects between complex bacteria and aboriginal microorganisms in river water with a view to providing the theoretical basis for application of augmented bioremediation techniques of these compounds in urban inner rivers.

MATERIALS AND METHODS

Chemicals

Phenolic derivatives were purchased from Shanghai Chemical Reagent Co., China (analytical reagent grade). Other compounds used for preparation of medium were of chemical purity.

Enrichments of Complex Bacteria

Activated sludge sample was collected from Sewage Treatment Work of Nanjing Chemical Plant (Jiangsu province, China) and sludge content was 5-10 g/L. The sludge sample was stored at 4 ℃. Two hundred mL of sludge sample was placed in a 1000 mL breaker filled with 200 mL of distilled water. The mixture was aerated for 24 h to activate microorganisms in the sludge. The supernatant of 10 mL sludge was placed in a 250 mL flask filled with 90 mL of sterile liquid medium containing 5 g beef extract, 10 g peptone, 5 g NaCl, and 1 L distilled water. The pH of culture medium was adjusted to 7.2-7.5. The flask was shaken in a 30 ℃ incubator at 150 rpm for 60 h. The mixture was centrifuged at 4000 rpm for 10 min, the supernatant was discarded and the sediment was cleaned three times with phosphate buffer solution. The enriched complex bacteria (ECB) were suspended in a buffer solution. Bacterial count in the suspended solution was 3.0×10^7 CFU/mL determined with standard plate count techniques[9].

Domestication of Complex Bacteria

Substituted phenols-degrading bacteria were obtained with domesticating 4-CP using the selectivity culture method. The liquid medium diluted at 1:100 was used as the selectivity medium. Ninety mL of sterile selectivity medium was placed in a 250 mL flask filled with 10 mL of ECB solution. The concentration of 4-CP was 50 mg/L. The flask was shaken in a 30 ℃ incubator for 5 d. Individual colonies were selected by gradually decreasing the concentration of 4-CP to 20 mg/L and incubated. Domestication was completed in a non-carton source medium instead of in a selectivity medium by adding 4-CP from 20 mg/L to 5 mg/L. The non-carton source medium contained 3 g KH_2PO_4, 7 g $NaH_2PO_4 \cdot 2H_2O$, 0.5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 50 mg $CaCl_2$, 13 mg $FeSO_4 \cdot 7H_2O$, 10 mg $ZnSO_4 \cdot 7H_2O$, and 1 L distilled water.

Identification of DCB

The microbial community of DCB was identified by plate culture observation techniques and Gram stain method. DCB sample (0.2 mL) was coated in pure solid medium plate and incubated at 30 ℃ for 3 d. The solid medium contained 5 g beef extract, 10 g peptone, 15 g agar, 5 g NaCl, and 1 L distilled water. Four species of microorganisms were identified. Each isolated colony was stained with Gram stain method and observed under microscope at 1000 magnification. The complex bacteria consisted of *Pseudomonas*, *Alcaligenes*, *Mycoplana*, and *Flavobacterium*.

Toxicity Test

Bacterial growth inhibition test was used to determine the acute toxicity of phenols to aboriginal bacteria in natural water, enriched and domesticated complex bacteria[10]. Natural water samples were taken from the Qinhuai River in Nanjing (Jiangsu province, China), the water temperature was 23 ℃, the content of dissolved oxygen 6.4 mg/L, the chemical oxygen demand (COD_{Mn}) was 39.6 mg/L (pH 6.8), and the bacterial count was 4.3×10^4 CFU/mL. The toxicity test process has been described elsewhere[11].

Biodegradability Assay for Individual Compounds

Using the domesticated complex bacteria (DCB) as an inoculum, we determined the biodegradability of eight phenolic derivatives under various aquatic conditions as previously described[12]. One hundred and eighty mL of water sample was placed in a 500 mL flask filled with 20 mL of inoculant and a certain amount of phenolic solution. Biodegradability of each compound and each control (no inoculum) was determined twice synchronously. An example of experimental design for biodegradability is listed in Table 1. The flask was shaken in a 30 ℃ incubator at 150 r/min. Five mL of water samples was collected periodically and centrifuged at 10 000 rpm for 10 min. The supernatant was used to test the concentration of compounds. Quantitative analysis of

compounds was performed with a spectrophotometer (UV-2450).

TABLE 1

Experimental Design of Phenols

ID[a]	Water Sample	Inoculum
A	River Water	Distilled Water
B	River Water	DCB
C	Sterilized River Water	DCB
D	Distilled Water	DCB
Control	Distilled Water	Distilled Water

Note. [a]ID is a name assigned to test biodegradability of chemicals.

Biodegradability Assay for Mixtures

To test the biodegradability of coexistent compounds, binary mixtures consisting of monochlorophenols and 2,4-DCP were used at an equiconcentration of 5 mg/L. One hundred and eighty mL of river water sample was placed in a 500 mL flask filled with 20 mL of DCB and a certain amount of phenolic mixture. The flask was shaken in a 30 ℃ incubator at 150 r/min. Five mL of water samples was collected periodically and centrifuged at 10 000 rpm for 10 min. The supernatant was extracted with 5 mL of petroleum ether. The organic phases of extract were pooled and dried with anhydrous sulfate sodium. Quantitative analysis of mixtures was performed with a SP-2000B GC equipped with a FID detector. A SE-54 quartz capillary column (0.53 mm×30 m) was used and the oven temperature was 60 ℃ held for 3 min, followed by a 20 ℃/min ram-up to 240 ℃. The carrier gas (nitrogen) was held constant at 40-60 mL/min, the hydrogen was held at 20-30 mL/min, and the air was held at 400-450 mL/min.

RESULTS

Toxicity of Phenols

The concentration causing 50% of growth inhibition (IC_{50}, mol/L) was calculated using concentration-response curves. The toxicity of phenolic derivatives was expressed as the negative logarithm value of IC_{50} (Table 2).

TABLE 2

Acute Toxicity Data of Phenols

Compounds	$\log 1/IC_{50}$ (mol/L)		
	River Bacteria	ECB	DCB
2-Chlorophenol	3.79	3.60	3.23
3-Chlorophenol	3.81	3.75	3.27
4-Chlorophenol	4.19	3.85	2.97
2,4-Dichlorophenol	4.30	4.14	3.34
1,3-Benzenediol	3.13	2.87	2.61
1,4-Benzenediol	3.25	3.20	2.89
3-Nitrophenol	3.82	3.75	3.63
4-Nitrophenol	4.05	3.92	3.71

Biodegradability under Various Aquatic Conditions

The exposure concentration of chlorinated phenols and dihydroxybenzenes as well as nitrophenols was placed at 20 mg/L and at 5 mg/L, respectively, for biodegradability study. The biodegradation kinetics curves were obtained (Fig. 1).

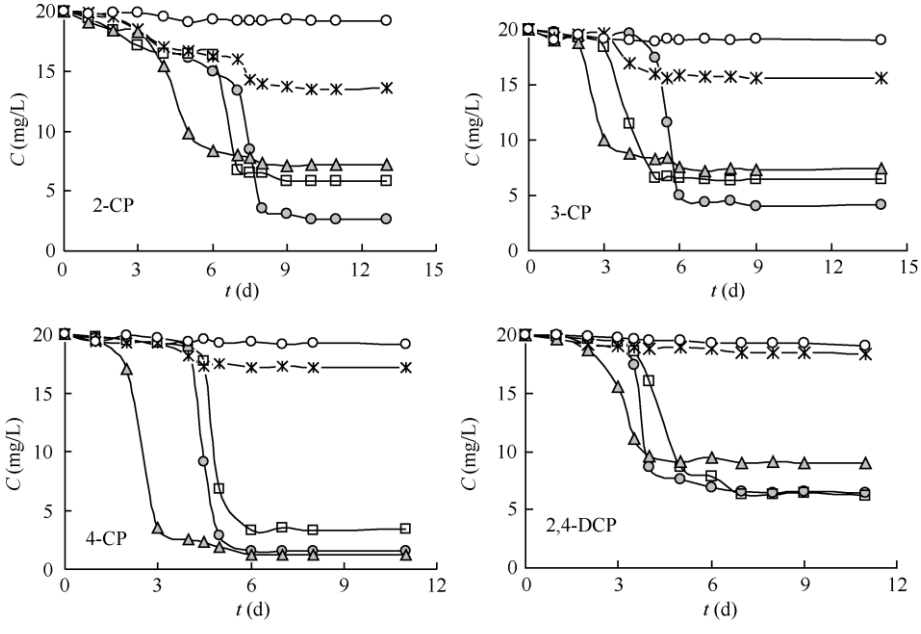

FIG. 1. Biodegradation curves for chlorinated phenols (○ control; ∗ A; ● B; □ C; △ D in Table 1).

Biodegradability was expressed as the first-order kinetics rate constant (K) according to the traditional Monod equation (on the assumption that the amount of biomass was invariable during the experiment):

$$\frac{dc}{dt} = -Kc \quad (1), \text{ integral: } \int_{C_0}^{C} \frac{dc}{c} = -K\int_0^t dt \quad (2),$$

$$\text{hence, } \ln\frac{c}{c_0} = -Kt \quad (3).$$

Where c_0 is the initial concentration of compounds, c is the residual concentration at time t_d, t is the biodegradation time and K is the biodegradation rate constant. When there was an obvious lag period in biodegradation course, the biodegradation rate constant was obtained according to Thomas's amendment formula: $\ln\frac{c}{c_0} = -K\ (t - t_0)$, t_0 is the lag period. The values of biodegradation rate constant (K), the half-life ($t_{½}$) and the removal rate at degradation balance were obtained (Table 3).

Biodegradability for Mixtures

After DCB was added into the river water samples, the degradability of binary mixtures of 4-CP and 2-CP, 4-CP and 2,4-DCP was determined. The biodegradation curves for coexistent compounds and individual compounds (Figs. 2 and 3). The biodegradation rate, the half-life and the removal rate of single and mixed conditions are shown in Table 4.

TABLE 3

Biodegradability of Chemicals under Different Conditions

Compounds	K (mg/L d)				$t_{½}$ (d)				Removal Rate (%)			
	A	B	C	D	A	B	C	D	A	B	C	D
2-Chlorophenol	0.056	0.251	0.139	0.129	14.38	3.26	5.49	5.87	32	87	71	64
3-Chlorophenol	0.102	0.503	0.357	0.163	9.79	5.38	4.94	6.25	25	80	68	63
4-Chlorophenol	0.071	0.644	0.445	0.589	13.76	5.08	4.56	2.18	16	93	83	94
2,4-Dichlorophenol	0.010	0.269	0.308	0.266	72.30	5.58	5.25	4.61	8.0	69	68	55
1,3-Benzenediol	0.075	0.817	0.550	0.533	11.74	1.35	3.26	3.30	27	93	86	91
1,4-Benzenediol	0.055	0.284	0.260	0.402	14.60	2.94	3.17	3.22	18	81	62	86
3-Nitrophenol	0.017	0.737	0.509	0.377	41.77	1.44	2.36	2.34	12	94	87	90
4-Nitrophenol	0.012	0.312	0.214	0.257	58.75	2.72	4.24	3.20	6.4	88	68	81

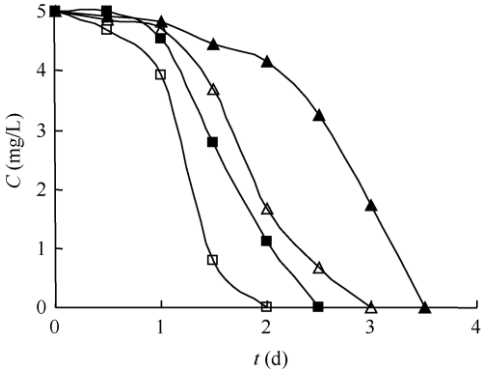

FIG. 2. Biodegradation curves for 4-CP coexisting with 2-CP. □ 4-CP (Individual), ■ 4-CP (Coexistent), Δ 2-CP (Individual), ▲ 2-CP (Coexistent).

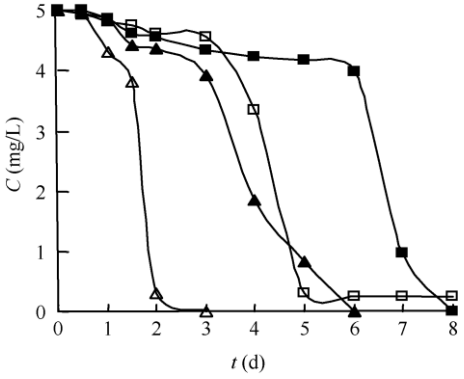

FIG. 3. Biodegradation curves for 4-CP coexisting with 2,4-DCP. Δ 4-CP (Individual), ▲ 4-CP (Coexistent), □ 2,4-DCP (Individual), ■ 2,4-DCP (Coexistent).

TABLE 4

Biodegradability of Single and Mixed Conditions

Biodegradability	4-CP		2-CP		4-CP		2,4-DCP	
	Single	Mixed	Single	Mixed	Single	Mixed	Single	Mixed
K_b (mg/L d)	2.407	2.197	2.159	1.967	2.422	1.378	1.341	1.633
$t_{½}$ (d)	0.788	1.315	1.321	1.852	0.786	3.003	3.520	6.425
Removal Rate (%)	100	100	100	100	100	100	94.8	100

DISCUSSION

Phenols can show inhibitory or lethal effects on microorganisms. To find out whether the degradability of phenols is related with the tolerance of microorganisms to phenols, $\log 1/IC_{50}$ of phenols against DCB was compared with those against ECB and aboriginal river bacteria.

The enriched and isolated complex bacteria in this study were more tolerant to phenolic derivatives than aboriginal bacteria in river water, and the complex bacteria domesticated by 4-CP and selective medium were more tolerant to toxicants, especially to 4-CP (Table 2). The toxicity of chemicals was significantly related to the aboriginal bacteria and ECB. The square of the correlation coefficient was 0.939. Nevertheless, the toxicity of phenols to DCB was not well correlated either to river bacteria or to ECB.

The complex bacteria domesticated by 4-CP can grow and be metabolized to take other substituted phenols as a sole carbon and energy source. In our study, however, 4-CP was still most easily degraded, the final removal rate for the compounds was over 50% when DCB was added into water samples, the degradation rate for aboriginal microbes in natural water to all target compounds was inadequate, and the biodegradability of compounds was greatly improved when DCB was added into river water.

It was reported that diverse microflora are often more efficient than pure culture to degrade toxic and persistent contaminants due to their synergetic effects[13-14]. The metabolic relationship between the dominant mixed bacteria and river bacteria was investigated in this study. DCB was quickly acclimated to distilled water with a short lag period, and the time reaching degradation balance was only 3-6 d (Fig. 1). However, DCB added into river water needed a longer acclimation period, and both the lag period and the time reaching degradation balance were extended. Microorganisms utilize organic substances in river water firstly for growth metabolism and the biomass increases and produces more enzymes. Consequently, the biodegradation and removal rates for most compounds are increased when DCB is added into river water. These findings suggest that aboriginal microbes and DCB have synergetic effects on most compounds. The suspend and organic substances can also affect the growth, propagation and metabolism of mixed bacteria.

Bacteria that are able to grow on biphenyl usually have the ability to co-metabolize various PCB congeners[15]. 2-chloroaniline-degrading bacteria can co-metabolize other chlorinated anilines[16]. Similar results were obtained in the present study. As shown in Fig. 2, 5 mg/L of 4-CP or 2-CP were degraded quickly in the river water after addition of DCB, and the removal rate for 4-CP and 2-CP reached 100% within 48 h and 72 h, respectively. When 4-CP and 2-CP coexist in river water due to increased total concentration of pollutants, microorganisms need a longer acclimation period to produce enough enzymes for metabolizing target compounds, so the degradation rate decreases and the half-life of both 4-CP and for 2-CP is prolonged. Since the complex bacteria are obtained by domesticating 4-CP, DCB utilizes 4-CP firstly for growth metabolism, and about 80% of 4-CP is consumed at 48 h. After a lag period of 48 h, 2-CP is quickly degraded. 4-CP and 2-CP are entirely degraded at 36 h and at 60 h, respectively.

The lag period of mixed components was longer than that of individual ones (Fig. 3). The coexistent 4-CP was entirely degraded on day 6, and 2, 4-DCP was quickly degraded when 4-CP was almost exhausted. The degradation course of coexistent components could be divided into two stages. 4-CP could be utilized by microorganisms firstly as a growth matrix, provide carbon and energy source for the growth of microorganisms. Accordingly, the biomass in the system was increased and the metabolic activity was enhanced. As a result, the metabolic efficiency of 2, 4-DCP was improved, and the removal rate was increased by 5.2% and 2,4-DCP was entirely degraded within 8 d.

In conclusion, 4-CP-domesticated complex bacteria isolated from activated sludge are more tolerant to phenolic derivatives than aboriginal bacteria from the Qinhuai River. The complex bacteria can grow and be metabolized to take chlorophenols, dihydroxybenzenes and nitrophenols as a sole carbon and energy source. The complex bacteria have a higher metabolic efficiency on chemicals than river aboriginal bacteria. The final removal rate for chemicals in water can be increased at least by 55% when the complex bacteria are added into river water samples. The aboriginal microbe and the domesticated complex bacteria are synergetically metabolized. When 4-CP coexists with 2,4-DCP in river water after DCB is added, 4-CP is degraded prior to 2,4-DCP, and the metabolic efficiency of 2,4-DCP is improved. The degradation course of coexistent compounds is divided into two stages.

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