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Biodegradability of Chlorinated Anilines in Waters[1]

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Objective To identify the bacteria tolerating chlorinated anilines and to study the biodegradability of *o*-chloroaniline and its coexistent compounds. **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 toxicant. Biodegradability of chlorinated anilines was determined using domesticated complex bacteria as an inoculum by shaking-flask test. **Results** The complex bacteria were identified, consisting of X*anthomonas, Bacillus alcaligenes, Acinetobacter, Pseudomonas,* and *Actinomycetaceae nocardia.* The obtained complex bacteria were more tolerant to *o*-chloroaniline than mixture bacteria in natural river waters. The effects of exposure concentration and inoculum size on the biodegradability of *o*-chloroaniline were analyzed, and the biodegradation characteristics of single *o*-chloroaniline and 2, 4-dichloroaniline were compared with the coexistent compounds. **Conclusion** The biodegradation rates can be improved by decreasing concentration of compounds and increasing inoculum size of complex bacteria. When *o*-chloroaniline coexists with aniline, the latter is biodegraded prior to the former, and as a consequence the metabolic efficiency of *o*-chloroaniline is improved with the increase of aniline concentration. Meanwhile, when *o*-chloroaniline coexists with 2,4-dichloroaniline, the metabolic efficiency of 2,4-dichloroaniline is markedly improved.

Key words: Complex bacteria; Chlorinated anilines; Biodegradability; Coexistent compounds

INTRODUCTION

Chlorinated aromatics in waters are toxic to aquatic organisms, and prone to concentrate and accumulate in these organisms[1]. Therefore, it is difficult to eliminate their effect via degrading by microorganisms in natural waters. Due to high electron negative nature and strong electron withdrawing effect of chlorine atom in benzene ring, the electron density in benzene ring is lower, and chlorinated aromatics do not readily react with electrophilic reagents. These compounds can exist in the environment. Dechlorination is the most important tool for biodegradation process of chlorinated aromatics that includes oxidation dechlorination, reduction dechlorination and cometabolism[2]. At present, much information about the biodegradability of chlorinated aromatics derives from the study on chlorophenols[3]. It has been reported that it is more difficult to biodegrade chlorinated anilines than chlorinated phenols in natural waters[4]. The biodegradation kinetics of *o*-chloroaniline and 2, 4-dichloroaniline was investigated by using the enriched and domesticated complex bacteria in order to provide the theoretical basis for the application of augmented bioremediation techniques in natural waters.

MATERIALS AND METHODS

Enrichments of Complex Bacteria

Activated sludge sample was collected from the Sewage Treatment Plant of the Yangtse Petrochemical Company, and the sludge content was 5-10 g/L. The sludge sample was stored at 4˚C. Two hundred mL sludge sample was placed in 1000 mL beaker filled with 200 mL distilled water. The mixture was aerated for 24 h to activate the microorganisms in the sludge. Ten mL supernatant of sludge was placed in 250 mL flasks filled with 90 mL sterile liquid medium. The liquid medium contained 5 g of beef extract, 10 g of peptone, 5 g of NaCl, and 1 L

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of distilled water. The pH of culture medium was adjusted to 7.2-7.5. The flasks were shaken in a 30˚C incubator for 60 h. These mixtures were centrifuged at 4000 rpm for 10 min, and then the supernatant was discarded and the sediment was cleaned three times with phosphate buffer solution. The enriched bacteria were suspended in buffer solution. Bacterial count in the suspended solution was 2.0×10^9 CFU/mL determined by standard plate count techniques[5].

Domestication of Complex Bacteria

Chlorinated anilines-degrading bacteria were obtained by domesticating *o*-chloroaniline using the selectivity culture method. The above liquid medium diluted 1:100 was used as the selectivity medium. Ninety mL sterile selectivity medium was placed in 250 mL flasks filled with 10 mL enriched bacteria solution, and the concentration of *o*-chloroaniline was 5 mg/L. The flasks were shaken in a 30℃ incubator for 3 d. Individual colonies were selected by gradually increasing the concentration of *o*-chloroaniline to 20 mg/L and incubated. Domestication was completed in non-carton source medium instead of selectivity medium and adding *o*-chloroaniline from 10 mg/L to 5 mg/L. The non-carton source medium contained 3 g of KH_2PO_4, 7 g of $NaH_2PO_4{\cdot}2H_2O$, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4{\cdot}7H_2O$, 50 mg of $CaCl_2$, 13 mg of $FeSO_4{\cdot}7H_2O$, 10 mg of $ZnSO_4{\cdot}7H_2O$, and 1 L of distilled water. The number of domesticated complex bacteria was determined to be 7.8×10^6 CFU/mL.

Identification of Complex Bacteria

The microbial community of complex bacteria was identified by plate culture observation techniques and Gram stain method. Complex bacteria sample (0.2 mL) was coated in pure solid medium plate and incubated at 30℃ for 3 d. The solid medium contained 5 g of beef extract, 10 g of peptone, 15 g of agar, 5 g of NaCl, and 1 L of distilled water. Five 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 *Xanthomonas, Bacillus alcaligenes, Acinetobacter, Pseudomonas,* and *Actinomycetaceae nocardia.*

Tolerance of Complex Bacteria to O-chloroanilines

Bacterial growth inhibition test was used to determine the acute toxicity of *o*-chloroanilines to mixture bacteria in natural waters and domesticated complex bacteria[6]. Natural water sample was taken from the Nanjing section of the Yangtze River (Jiangsu Province, China), and the toxicity test process has been described elsewhere[7].

Biodegradation Experiment

Using domesticated complex bacteria as an inoculum, the biodegradability of chlorinated anilines was determined by shaking-flask test[8]. Ninety mL sterile non-carton source medium was placed in 250 mL flasks filled with 10 mL inoculant and a certain amount of chlorinated aniline solution. Biodegradability was determined twice for each compound and each control (no inoculum). The flasks were shaken in a 30℃ incubator at 150 r/min. Two mL water samples was collected periodically, filtrated by 0.45 μm organic filtration membrane, and placed in a 10 mL tube with plug. All water samples were stored at 4℃. Quantitative analysis of the compounds was performed with a Waters1525 HPLC equipped with a Waters 2487 diode array UV detector. A Lichrospher 5-C18 octadecyl column (4.6 mm × 200 mm) was used and column temperature was 40℃. The mobile phase was planned as 70% methanol with 30% water at a flow rate of 1.0 mL/min. UV absorbance was measured at 248 nm.

RESULTS

Toxicity of o-chloroaniline

The concentrations causing 50% growth inhibition (IC_{50}) were calculated using concentration-response curves. The IC_{50} values of *o*-chloroaniline for mixture bacteria in natural waters and domesticated complex bacteria were 0.21 and 1.20 mmol/L, respectively.

Effect of Exposure Concentration on Biodegradability

The exposure concentration of *o*-chloroaniline was planned for biodegradability study at 5, 10, and 20 mg/L respectively. The biodegradation kinetics curves were obtained (Fig. 1).

Biodegradability was expressed as the first-order kinetics rate constant (K) according to the traditional Monod equation:

$$\frac{dc}{dt} = -Kc \quad (1), \quad \text{integral: } \int_{C0}^{C} \frac{dc}{dc} = -K\int_0^t dt \quad (2), \quad \text{hence,}$$

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

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. The values of K can be calculated from the slope of the straight line

obtained by plotting $\ln c/c_0$ versus time t_d, $t_{½}$ which can be obtained according to formula $t_{½}=0.693/K$ (Table 1).

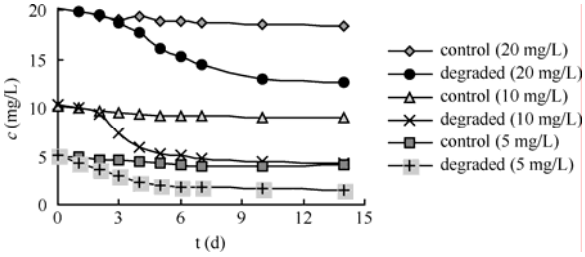

FIG. 1. Biodegradation curves of *o*-chloroaniline at different exposure concentrations.

TABLE 1

Biodegradability Data of *o*-chloroaniline

Concentration (mg/L)	K_b (mg/L·d)	$t_{½}$ (d^{-1})	Removal Rate (%)
5	0.18	3.85	69.8%
10	0.10	6.93	58.3%
20	0.04	17.3	37.5%

Influence of Inoculum Size on Degradability

The influence of complex bacteria concentration on the degradability of *o*-chloroaniline was determined according to the changes in inoculum size (Fig. 2). The final removal rate of *o*-chloroaniline increased from 58% to 68% when the inoculum volume changed from 10 mL to 60 mL.

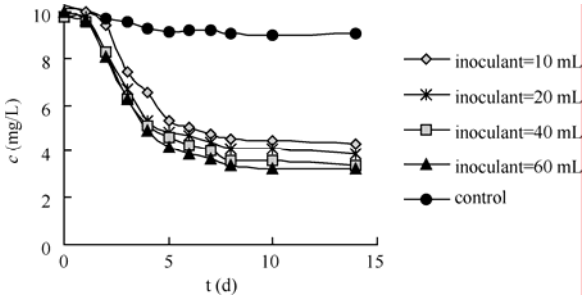

FIG. 2. Biodegradation curves of *o*-chloroaniline at different inoculum volumes.

Biodegradability of O-chloroaniline *Coexisted With Aniline*

The concentration of aniline was planned at 5, 10, 15, and 20 mg/L, respectively, while the concentration of *o*-chloroaniline was planned at 10 mg/L and filled with 10 mL complex bacteria in order to investigate the biodegradability of coexistent compounds. The biodegradation curves of *o*-chloroaniline are shown in Fig. 3.

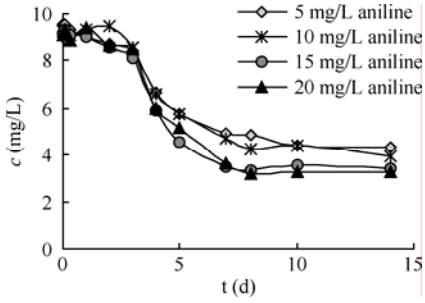

FIG. 3. Biodegradation curves of *o*-chloroaniline coexisted with anline.

Biodegradability of Coexistent o-chloroaniline and 2,4-dichloroaniline

The concentration of *o*-chloroaniline was planned at 5 mg/L by adding 3.5 mg/L of 2,4-dichloroanline and filled with 10 mL complex bacteria in order to investigate the biodegradability of the two coexistent chloroanilines. The biodegradation curve of the two coexistent chlorinated anilines was compared with that of single compound in Fig. 4. The biodegradation rate and the removal rate of single and coexistent conditions are shown in Table 3.

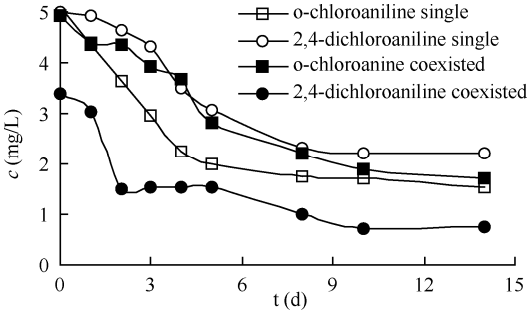

FIG. 4. Biodegradation curves of *o*-chloroaniline coexisted with 2,4-dichloroanline.

DISCUSSION

The toxicity test showed that the complex bacteria domesticated by *o*-chloroaniline and selectivity

TABLE 2

Biodegradation Data of Coexistent *o*-chloroaniline and 2,4-dichloroaniline

Biodegradability	*o*-chloroaniline		2,4-dichloroaniline	
	Single	Coexisting	Single	Coexisting
K_b (mg/L·d)	0.18	0.10	0.09	0.14
Removal Rate (%)	69.8	65.0	55.6	77.7

medium were much more tolerant to toxicant than mixture bacteria in natural river waters, and the complex bacteria could metabolize chloroanilines more efficiently.

The effects of exposure concentration and inoculum volume on the biodegradability of *o*-chloroaniline were analyzed. As shown in Fig.1, the biodegradability of *o*-chloroaniline was related to its increased concentration in water. The time reached degradation balance and the lag period was extended with the increased concentration of *o*-chloroaniline in water, while the biodegradation rate constant and final removal rate decreased accordingly (Table 1). Microorganisms need a longer adaptive period to produce enough enzymes for metabolizing the target compound when the exposure concentration of a pollutant is increased. In addition, the higher concentration of a pollutant may strengthen toxicity to microorganisms, decrease metabolic activity and degradation rate. The other factor may be related to lower inoculum volume of the complex bacteria and deficient biomass in system.

From Fig. 2, we can find that the degradation rate and final removal rate of *o*-chloroaniline gradually increased with the inoculum volume ranged between 10 and 60 mL. The more the effective biomass in water and the more the yielded enzyme or enzyme system, the more target compounds are metabolized by microorganisms.

When *o*-chloroaniline coexists with aniline, the complex bacteria utilize aniline first for growth metabolism, and aniline of 5-20 mg/L is almost entirely consumed within 24 h. As shown in Fig. 3, a longer lag period was found on the degradation curves of *o*-chloroaniline, and the metabolic efficiency of *o*-chloroaniline improved with the increased aniline concentration. Aniline can be utilized by microorganisms as growth matrix, and is more degradable than *o*-chloroaniline. The presence of aniline could provide carbon and energy source for growth of microorganisms, and accordingly the biomass in system is increased and the metabolic activity is enhanced. As a result, the degradability of coexistent *o*-chloroaniline is improved.

Tongpim and Michael studied the co-metabolism of phenanthrene by mycobacterium growing on anthracene and found that microorganisms have self-resuming function against toxic reaction in the course of co-metabolism, and the activity of microorganisms can be resumed by increasing the concentration of growth matrix or decreasing the concentration of target pollutants[9].

From Fig. 4, we can find that the complex bacteria domesticated by *o*-chloroaniline could utilize 2,4-dichloroanline as carbon and energy source and the degraded rate of 2,4-dichloroanline was more than 50% within 8 days. By comparing the degradation curve of coexistent compounds with that of the single ones, we could find that the presence of 2,4-dichloroanline inhibited the degradation rate of *o*-chloroaniline in the initial stages due to the increased total toxicant concentration, but exerted no obvious influence on its final removal rate. However, the metabolic efficiency of 2,4-dichloroaniline was markedly improved when more readily degradable *o*-chloroaniline coexisted, and the removal rate of 2,4-dichloroaniline was increased by 22%, and the degradation rate constant was increased by more than 50% (Table 2).

CONCLUSION

Domesticated complex bacteria are more tolerant of *o*-chloroaniline. The biodegradation rate of *o*-chloroaniline can be influenced by the exposure concentration of pollutant and inoculum volume of complex bacteria. Therefore, it is necessary to increase the number of complex bacteria when the concentration of pollutants in waters is high. When *o*-chloroaniline coexists with aniline, aniline is degraded prior to *o*-chloroaniline, and the metabolic efficiency of *o*-chloroaniline is improved with increased aniline concentration. When *o*-chloroaniline coexists with 2,4-dichloroaniline, the metabolic efficiency of 2,4-dichloroaniline is markedly improved.

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