한국환경과학회지 제16권(제7호), 779~784, 2007
Journal of the Environmental Sciences

pH Effect on the Aerobic Biodegradation of Nitrophenolic Compound in SBR

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(Manuscript received 2 November, 2006; accepted 3 May, 2007)

Dinitrophenol is preventing cells from making energy for growth and it has been suggested that pH may be important in mitigating effects of uncouplers. The effect of pH on toxicity of dinitrophenol at high concentration was investigated, over a pH range of 5.7 to 8.7. DNP inhibition was found to be strongly dependent on mixed liquor pH. The DNP degradation rate was highest in the pH range of 7.0 to 7.8; at pH 6.0 degradation of 0.41 mM dinitrophenol was significantly inhibited; at pH <5.7, dinitrophenol degradation was completely inhibited after approximately 25% of the dinitrophenol was degraded. However no significant effect of pH variation was seen on glucose uptake by the activated sludge mixed culture.

Key Words : Dinitrophenol, pH, Biodegradation, Inhibition, Activated sludge

1. Introduction

The nitrophenols (e.g., 4-nitrophenol and 2,4-dinitrophenol) are common compounds of industrial effluents and have been detected in urban and agricultural waste. Nitrophenols are intermediates in pesticide and dye synthesis, are applied as herbicides and insecticides, and are found in urban water as a result of the tropospheric transformation of alkylbenzenes[1]. The compound dinitrophenol is an especially interesting member of the nitroaromatic chemical group because it has been shown to be mineralized by strains of bacteria and in mixed populations in sewage and activated sludge[2,3], yet dinitrophenol also is known to be acutely toxic to bacteria, acting as an "uncoupler," by interrupting cell-energy generation during metabolism[4]. One mechanism which has been suggested for this effect is that the protonated form of the phenolic molecules diffuse across the cell membrane and donates H^+ which reacts with hydroxyl ions near the membrane to destroy the transmembrane proton gradient and prevent oxidative phosphorylation. The deprotonated dinitrophenol ion will have less uncoupling activity and be less toxic[5]. Therefore the author hypothesized that reduction of inhibition can be achieved by raising the solution pH.

Mayer and Ellersiek[6] found that a decrease in pH increased the toxicity of weak acids and also reduced the toxicity of weak bases by changing the proportion of ionized to un-ionized species. Sprague[7] reported that un-ionized organic molecules may be more toxic than ionized molecules because they more readily penetrate cell membranes, and that pH is the major factor determining the extent of ionization and subsequent membrane penetration. Bacterial decomposition of dinitrophenol by reduction of the nitro groups to amino groups, followed by oxidative deamination has been reported to be optimal at pH values near neutrality[8]. Decomposition by cleavage of NO_2 from the dinitrophenol molecule by *Corynebacterium simplex* was maximal at pH 8.0[9].

The substrate dinitrophenol is oxidized by aerobic bacteria, probably ultimately via the tricarboxylic acid (TCA) cycle. The carbon in dinitrophenol is oxidized to CO_2. The electrons from the oxidation of dinitrophenol would be expected to be accepted by electron carriers such as NAD (the oxidized form of nicotinamide adenine dinucleotide), resulting in the re-

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duced form $NADH_2$. The $NADH_2$ carries electrons to the cytochromes in the electron transport chain (ETC), which are located in the cytoplasmic membrane. As the electrons are passed through the cytochromes, protons are transported across the cytoplasmic membrane to the exterior of the cell. Finally, the electrons react with molecular oxygen, the "terminal electron acceptor," and protons to produce H_2O.

The protons exported by the ETC cytochromes accumulate at the membrane exterior forming an electrochemical proton gradient. A H^+-ATP synthase enzyme then transports protons across the membrane in an energy-releasing (exergonic) process to drive the phosphorylation of ADP to ATP. The synthesis of ATP is highly dependent on the membrane proton gradient; it provides the energy for formation of the ADP-Pi bond. It has been said that the maintenance of a proton gradient and ATP synthesis are "coupled" (see Fig. 1).

Dinitrophenol is one of a group of uncoupler compounds. Dinitrophenol is a weak acid with a pK_a value of 4.03 (20 ℃). Even at neutral pH values, a small fraction of the molecule will be protonated (0.1% at pH=7). The protonated species, Dinitrophenol-H, can diffuse across the membrane fairly easily since it is hydrophobic, as is the inside of the cytoplasmic membrane. Once Dinitrophenol-H is in the cytoplasm, it can donate a proton to react with an OH-ion, diminishing the proton gradient. Then, while substrate degradation and ETC transport of electrons continues, the synthesis of ATP and production of energy stops (see Fig. 2). As the intracellular reserves of ATP decline as a result of uncoupling, cell metabolism of the substrate and transport of electrons to oxygen increases, presumably because the cell speeds up metabolism in a futile attempt to make more ATP. In bacteria, the observed result of uncoupling at first is increased respiration, followed by low cell growth and, eventually, by complete cessation of growth. This effect has been reported by several investigators with pure strains of bacteria[10~12], and in activated sludge by Okey and Stensel[13].

The mechanism of uncoupling by dinitrophenol resulted in two areas of investigation. First was to test the toxicity of dinitrophenol to activated sludge bacteria at various pH values. Second, if bacteria in activated sludge were found to resist dinitrophenol toxicity, it was hypothesized that substitution of a proton gradient to support a H^+-dependent ATP synthase might be one mechanism to allow growth in the presence of this uncoupler.

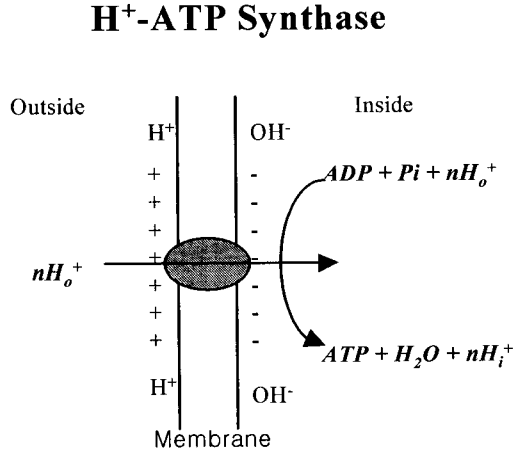

Fig. 1. Schematic diagram of the coupling of electron transport and ATP synthesis by the generation of a proton electrochemical gradient across the cytoplasmic membrane. H^+ is pumped out of the membrane during electron transport and its exergonic return powers the synthesis of ATP.

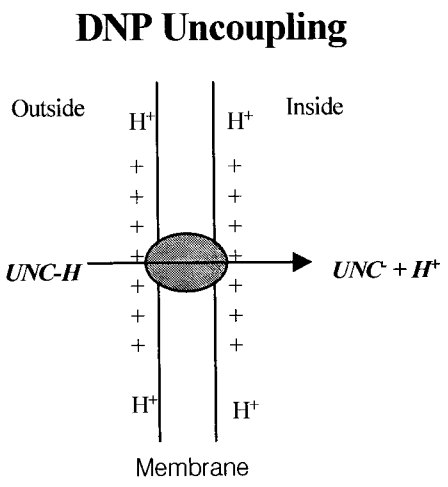

Fig. 2. The protonated DNP diffuses across cytoplasmic membrane thereby discharging the electrochemical gradient generated by the electron transport chain.

2. Materials and Methods

Using SBR(Sequencing Batch Reactor) with a 4-liter working volume, activated sludge was acclimated to degradation of dinitrophenol and glucose as carbon and energy substrates, and KNO_3 as a nitrogen source for the low concentration. The SBR activated sludge culture was used as the inoculum for flask experiments on the effect of pH on degradation of relatively high concentrations of dinitrophenol.

Short-term flask experiments were used to measure dinitrophenol degradation by the SBR-acclimated activated sludge under different pH. Each 250 mL flask contained 200 mL of activated sludge mixed liquor with a selected initial concentration of dinitrophenol (75 mg/L), and inorganic salts for nutrients. Flask biomass concentration was increased from 1,200 to 1,600 mg/L MLSS. Table 1 is a summary of flask experimental conditions. Inorganic salts were added to the flasks along with the activated sludge inoculum to insure balanced growth of activated sludge bacteria, along with high concentrations of dinitrophenol, 0.41 mM (75 mg/L). The flask liquid contents were aerated and mixed using a Gyrotory® Model G2 shaker operating at 200 revolutions per minute and allowed to react at room temperature, 22±2℃.

To investigate the effect of pH on dinitrophenol biodegradation by activated sludge, experimental pH values varied from nominal values of 5 to 9. The pH range of 5 to 9 was chosen because other tolerance effects might limit the activity of the dinitrophenol degrading bacteria below pH 5 and minerals required for balanced growth, such as magnesium and calcium, might form precipitates and be unavailable to microorganisms above pH 9. The pH of mixed liquor was maintained (0.1 unit) during tests by the addition of buffer (0.4 M Na_2HPO_4, 0.4 M NaH_2PO_4), acid (0.2 M citric acid, 0.4 M HCl), and base (0.4 M Tris) and was adjusted before tests. The dissociation constant (pK_a) of each buffer is given in Table 2. The phosphate buffer used in the experiment would not have been effective in controlling the pH values higher than 8 or lower than 6. Citrate and phosphate buffer were added to satisfy the pH 5, and Tris and HCl were added to get the pH 9.

Table 1. Activated sludge flask reactor conditions for pH experiment

Variable	Concentration
Dinitrophenol (mM)	0.41
Dinitrophenol (mg/L)	75
pH	5.7~8.7
Reaction period (hr)	36~66
C:N ratio	6:1
$CaCl_2$ and $MgSO_4$ (mg/L)	7.5
MLSS (mg/L)	1,200 ~ 1,600

Activated sludge activity in the experimental range of pH values in the presence of glucose substrate was used as a control to detect the effect of pH on activated sludge without dinitrophenol. Glucose was measured as dissolved organic carbon (DOC) using a Shimadzu Model TOC-5000 total organic carbon analyzer (Shimadzu Corp., Kyoto, Japan). It was found that measured DOC values at the beginning of the reaction closely matched theoretical calculated values for glucose concentration in the flasks. During flask experiments to obtain profiles of glucose uptake in the range of pH values tested, 5 mL samples were taken from each flask using a syringe every 10 minutes until the glucose was consumed. DOC samples were filtered through a 0.2 μm polycarbonate membrane filters and acidified with two drops of 2N HCl before DOC analysis.

Initial concentration of dinitrophenol was used in the single-washed activated sludge cultures: 75 mg/L (0.41 mM). This concentration seems to be close to the level where dinitrophenol is inhibitory even to acclimated bacteria.

2.1. Analyses

Samples were taken from the flask reactors every 6 hours with a 16-cm syringe fitted with a 20-gauge stainless steel needle. The samples were immediately

Table 2. pK_a values and concentrations of buffer compounds used in the pH variation experiments with activated sludge. All pK_a values are for 25℃. HCl was used to adjust final flask pH for each experimental value of 8.68

Buffering agent	pK_a	Concentration (M)
Tris	8.0	0.2
Monobasic phosphate	7.2	0.2
Dibasic phosphate	12.0	0.2
Citrate	3.1, 4.8, 6.4	0.1

vacuum filtered through a 0.2 μm polycarbonate filter (Nucleopore Corp., Pleasanton, CA, USA) and stored at 4 ℃ to eliminate bacterial activity.

The dinitrophenol content of samples from both the activated sludge was measured by UV light absorbance at 260 nm in a spectrophotometer (Model UV 160V, Shimadzu Co., Kyoto, Japan). Biomass density was measured as mixed liquor suspended solids (MLSS) using the membrane filter technique in Standard Methods[14]. The pH of flask contents was measured at each sampling time using an Orion Research expandable Ionanalyzer (Model EA920, Orion Corp., Boston, MA, USA) and Orion pH probe (Model 910600) which was calibrated daily. Cell concentration in flasks was determined by filtration of a 20-mL sample aliquot through an 0.2 μm polycarbonate filter (Nucleopore Corp., Pleasanton, CA, USA). The filter was dried at 80℃ for 20 hours before weighing on an analytical balance.

3. Results and Discussion

Aerobic biodegradation of nitroaromatic compounds is a well-documented phenomenon. However, the effects of wastewater treatment system operating parameters such as pH have received little attention despite their potential impact on degradation of uncoupling compounds by biological processes. Because the existence of dinitrophenol-degrading bacteria is well documented, the focus of these experiments was on the effect of the environmental factor pH, on enhancing the biodegradation of dinitrophenol by an already acclimated activated sludge culture. It was hypothesized that biodegradation of levels of dinitrophenol near reported toxicity levels at neutral pH would be enhanced at higher pH values. Range of pH was selected to bracket tolerance ranges suggested for activated sludge cultures, 5<pH<9. Inhibition was detected as either a lag, reduction in rate, or complete loss of dinitrophenol degradation activity by acclimated activated sludge. The effect of pH on dinitrophenol toxicity to activated sludge cultures were investigated at dinitrophenol concentration of 0.41(75 mg/L). Other investigators have observed that bacteria can mineralize dinitrophenol in a concentration as high as 0.5 mM (92 mg/L)[2], but inhibition occurs quickly at higher dinitrophenol concentration, even for acclimated bacteria.

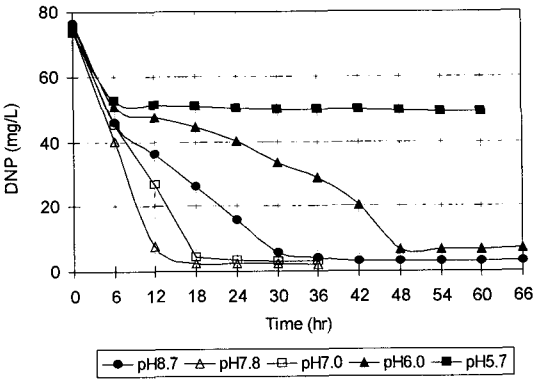

Fig. 3. Dinitrophenol concentration profiles from first flask experiments to investigate effect of pH on DNP de gradation by acclimated activated sludge. Initial dini trophenol concentration was 0.41 mM (75 mg/L), an d flask activated sludge solids concentration was 1, 292 mg/L MLSS.

Fig. 3 and 4 are dinitrophenol profiles for duplicate experiments, each with duplicate flask activated sludge reactors buffered at experimental pH values: 5.7, 6.0, 7.0, 7.8, and 8.7. The initial dinitrophenol concentration in all the flasks was 0.41 mM (75 mg/L). In both experiments, the maximum dinitrophenol degradation rate was observed in the pH range of 7.0 to 7.9.

Fig. 5 contains the control DOC profiles for the flask reactors containing only the glucose carbon/energy substrate with pH values 5.7, 7.0 and 7.9. As with the activated sludge experiments, each profile data point represents an average of samples from duplicate flasks.

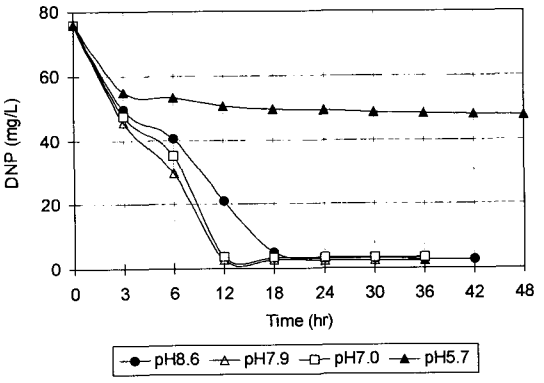

Fig. 4. Dinitrophenol concentration profiles from flask exper iments duplicating conditions in Figure 4.

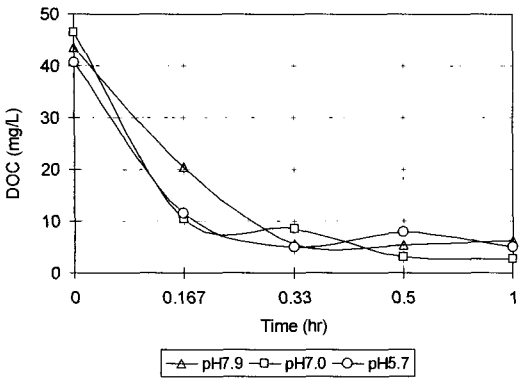

Fig. 5. Dissolved organic carbon concentration profiles from control activated sludge flask reactors at various pH values. Initial glucose concentration was 40 mg/L as dissolved organic carbon (DOC) and the flask activated sludge concentration was 1,600 mg/L MLSS. The measured background DOC of the activated sludge mixed liquor has been subtracted from each data point.

A zero-order rate model was fitted to the four profiles in the pH range of 7.0~7.9, and the results are shown in Table 3. There was no significant difference (p=0.05) in a t-test between the maximum dinitrophenol degradation rate coefficients. Significant inhibition of dinitrophenol degradation was seen in the pH range of 5.7 to 6.0. Also dinitrophenol degradation appears to be somewhat slower at the high pH range of 8.6~8.7. As can be seen from the DOC profiles in Fig. 5, in the control experiment there was no change in glucose degradation rates with pH between 5.7 and 7.9. However, tests at pH 8.7 were not conducted so that the decrease in dinitrophenol degradation at pH 8.7 cannot be definitively attributed to general decreased bio-activity at this pH. The reasons that pH range for glucose control was smaller than dinitrophenol are the citrate and Tris which were added to satisfy the pH 5 and pH 9, respectively, were detected as DOC in huge amount and made trouble. The results of the pH experiments indicate that dinitrophenol is more toxic at low pH values (5 to 6) than at higher pH values of 7 to 8. One explanation for the pH effect is that dinitrophenol is a weak acid with a pK_a value of 4.09 and is increasingly ionized as pH increases. Sprague[7] has contended that un-ionized organic molecules are more toxic because they more readily penetrate cell membranes, which supports the observation that dinitrophenol toxicity decreases with increasing pH. A similar pH effect on glucose degradation was not found.

Table 3. Zero-order rate constant for pH 7.0, 7.8 and 7.9 profiles in Fig. 3 and 4

Experiment	pH	Maximum specific degradation rate coefficient (mg/mgMLSS/hr)	Rate constant (mg/L/hr)
Experiment 1	7.0	$0.006\pm1.2 \times 10^{-4}$	7.60±0.16
	7.8	$0.006\pm3.1 \times 10^{-5}$	8.01±0.04
Experiment 2	7.0	$0.006\pm3.9 \times 10^{-5}$	7.50±0.05
	7.9	$0.007\pm7.9 \times 10^{-5}$	8.90±0.10

4. Conclusions

Substrate inhibition of dinitrophenol biodegradation was investigated using activated sludge which had been adapted to mineralize dinitrophenol. After acclimation of the activated sludge, the effect of pH on toxicity of dinitrophenol at high concentrations (0.41 mM) were investigated, over a pH range of 5 to 9. Dinitrophenol inhibition was found to be strongly dependent on mixed liquor pH. Biodegradation of dinitrophenol by acclimated activated sludge cultures was affected by pH. Biodegradation of 0.41 mM (75 mg/L) dinitrophenol was fastest in the pH range of 7.0 to 7.9 dinitrophenol degradation was severely inhibited at pH values less than 6.0, although no inhibition of glucose degradation by the same activated sludge culture was observed at pH 5.7.

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