Bull. Environ. Contam. Toxicol. (2002) 69:463–470

DOI: 10.1007/s00128-002-0085-7

Bulletin of Environmental Contamination and Toxicology

2,4,6-Trichlorophenol Degradation by River Sediment Exposed to Bleached Kraft Mill Discharge

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Received: 13 December 2001/Accepted: 5 July 2002

Chlorophenol compounds are toxic, persistent, bioaccumulable environment pollutants (Fewson 1988) which are secondarily produced by bleach kraft pulp mills. The bleaching processes of these types of mill can use chlorine and/or chlorine dioxide in the oxidative stage. Effluents arising from the bleaching process contain several organochlorine compounds which are discharged into the environment (Kringstad and Linstöm 1984, Earl and Reeve 1990). Some of these chlorophenols are included in the list of Toxic and Recalcitrant Compounds supplied by the US Environmental Protection Agency. In particular 2,4,6-trichlorophenol (2,4,6-TCP) is found as a major component of kraft pulp mill effluents (Andreoni et al. 1998). Despite its intrinsic toxicity, the degradation of these compounds by specific bacteria have been described by Aranda et al. (1999) and Martínez et al. (2000).

Aranda et al. (1999) and Martínez et al. (1999) detected bacteria with degradable abilities in a river area contaminated with chlorophenols. However, they did not evaluate the potential self- purification present in this environment. Also, in order to predict the behavior of these compounds, it is important to consider the kinetic biodegradation in the river; this has not been determined at this stage. The Andrew´s kinetic (1) was used to describe inhibition of 2,4,6-TCP and evaluate the inhibition constant (Gu and Korus 1995; Wang and Loh 2000; Reardon et al. 2000), considering the initial rate of the substrate degradation (r_s) for each concentration evaluated (Vidal et al. 1997).

$$r_s = -\frac{dS}{dt} = \frac{X\,\mu_m}{Y_{X/S}} \cdot \frac{S}{K_s + S + \frac{S^2}{K_i}} \qquad (1)$$

where, S is substrate concentration, X is the cell concentration, K_s is the Monod half saturation constant, K_i is an inhibition constant, μ_m is the maximum growth rate and $Y_{X/S}$ is the cell yield.

The aim of this study was to investigate the 2,4,6-TCP biodegradation by aerobic bacteria present in sediments from a contaminated river (Biobío river central Chile) that contains chlorophenols coming from bleached kraft mill effluents. Also, the kinetic model of 2,4,6-TCP degradation by isolated bacterial strains from the sediment was determined.

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MATERIALS AND METHODS

The Biobío River sediment was selected because clorophenol-containing bleached kraft mill effluents are discharged into this river (Godoy et al. 1999). Sediment was obtained with a Petite Ponar dredge (225 cm^2 dredge area) 15 meters from the right bank of the river, approximately 1,000 m downstream from a source of bleached pulp kraft mill effluent. Microorganisms in the sediment from this location were assumed to have had some exposure to chlorophenols. Various bacterial strains, able to degrade chlorophenols, were isolated in this area during a previous study. (Godoy et al. 1999; Martínez et al. 2000).

A stock solution of 2,000 mg 2,4,6-TCP/L (Sigma Company, St Louis, U.S.A.) 0.1N NaOH solution was prepared in order to expose the sediment and then isolate the strain to different concentrations of 2,4,6-TCP. Additionally, a mineral medium (MM) solution was made (MM composition: 7 g Na_2HPO_4/L, 0.8 g $(NH_4)_2SO_4$/L and 3 gKH_2PO_4/L). The MM was placed in an autoclave (20 min at 105 °C) and then 2.5 mL of 1 mol/L $MgSO_4$, 2.5 ml of 36 mmol/L $FeSO_4 \cdot 7H_2O$, and 2.5 of a trace element solution were added (Andreoni et al. 1998).

The biodegradation of 2,4,6-TCP by sediment was carried out in batch reactors. 50g of sediment (S) and 50mL of sterile de-ionized water was placed in Erlenmeyer flasks (250 ml). The bacterial degradation capability of the river water was evaluated. River water (100 mL) was inoculated in a Erlenmeyer flasks (250 mL). In order to evaluate the 2,4,6-TCP adsorption phenomena, sediment was treated in two different ways: autoclaved (AS) and calcinated (CS). For AS, the sediment was sterilized in an autoclave (20 min at 105°C) and in the CS case, the sediment was calcinated (3 hr at 550°C) and allowed determination total of organic content of sediment as in Mills (1978). MM was added to each of the three assays (with concentration of 10, 20 and 40 mg/L of 2,4,6-TCP respectively). All of the assays were carried out in triplicate. The flasks were incubated in a shaker at 150 r.p.m., in the dark, at 20 ± 2 °C. The variation of the 2,4,6-TCP concentration was analyzed according to the procedure described in this section, during a period of 3 days, until total mineralization occured (case of biodegradation assays using sediment).

In order to isolated bacterial strains with 2,4,6-TCP degradation ability present in S batch reactor, samples obtained after 3 days incubation were placed on R2A agar plates and incubated for 48 hr at 30°C. Bacterial colonies with different morphological characteristics were selected and frequently checked for purity in R2A agar plates and then, characterize for Gram-strain type. Gram negative bacilli were characterized for oxidative or fermentative capacities in glucose H_2S medium (Ward et al. 1986).

The isolated cells were incubated in Erlenmeyer flasks (250 mL) and incubated at 20 °C with constant shaking (150 r.p.m.) in the same way as in Godoy et al. (1999). MM was added to each of the five Erlenmeyer flasks (containing respectively 20, 40, 100, 175 and 260 mg/L of 2,4,6-TCP)-- the MM and 2,4,6-TCP being the sole carbon and energy source. On the other hand, we investigated the biodegradation of 2,4,6-TCP using R2A broth in order to evaluate the behavior of bacterial strain growth. For this propose, 250 mL Erlenmeyer flasks

containing (100 mL) R2A broth diluted at 1/4 supplemented with 20 mg/L 2,4,6 TCP and assays without chlorophenol, were used as a control. The 2,4,6-TCP biodegradation and bacterial-growth samples from each Erlenmeyer flask were analyzed after 0, 12, 20, 36, 60, 84, 108, 132 and 156 hours. The 2,4,6-TCP concentration was calculated according to the analytical techniques described later. Bacterial viability was determined in R2A agar plates and incubated at 25 °C for 5 days (Herbert 1990; Godoy et al. 1999; Martínez et al. 1999). All experiments were made in triplicate.

The 2,4,6-TCP concentration was determined in the liquid phase for each assays by spectrophometry according to the process described by Aranda et al. (1999). The sample extraction was carried out according to the instructions of Veith and Kiwus (1977). The complete mineralization of the 2,4,6-TCP was confirmed by high performance liquid chromatography (HPLC) as in Andreoni et al. (1998).

RESULTS AND DISCUSSION

The kinetic biodegradation of 2,4,6-TCP by sediment coming from the Biobío River is shown in Figure 1. After 3 days of incubation, results show a total 2,4,6-TCP removal by the sediment's microcosm between 10, 20 or 40 mg/L of these compounds. The maximum degradation rate (mg/L·hr), calculated by the slope of each kinetic curve, indicates that they increased from 0.21 to 1.25 mg/L·hr in the same manner as the initial concentration assays (10 to 40 mg/L of 2,4,6 TCP). The maximum degradation rate values indicate that 40 mg/L of 2,4,6-TCP does not have inhibitory effect. In this way, the contaminated river sediment concentration of chlorophenols is no higher than 3.7 µg/kg (Wegman and van den Broek, 1983). Also, we detected an increase in the bacterial population in 3 log-cycles, after 3 days of incubation. These results may be explained by the fact that the studied sediment receives clorophenol-containing bleached kraft mill effluents and that the bacterial population is able to tolerate and degrade 2,4,6-TCP compounds. This has been previously described by Godoy et al. (1999) and Martínez et al. (2000). A characterization of the sediment shows that a large fraction of the sediment consists of inorganic compounds, while only 2.2% in fact corresponds to organic matter. This indicate a low content of organic matter associated with this kind of sediment (Gao et al. 1998). Münster and Chróst (1990) and Aranda et al. (1999) indicated that the easily metabolizable substrate present in water or sediment is an important factor for the autodepurative capacity of a polluted environment because of its influence on xenobiotic degradation.

On the other hand, no consumption of 2,4,6-TCP was detected in the batches performed with river water (W). These results suggest that the importance of the sediment bacterial population's ability to degrade chlorophenol compounds like 2,4,6-TCP. These results can be explained by the fact that, in sediment, bacterial cells are denser and more protected than in water by action of toxic compounds(Ascon-Cabrera et al. 1995).

From the adsorption experience in which AS is used as inoculum, it can be seen that 27.63 % of the total 2,4,6 TCP is absorbed. On the other hand, only a 6.6 % of the total 2,4,6-TCP is adsorbed in the assay with CS as inoculum. These results suggest that the low organic fraction of the sediment plays an important role in

the adsorption of the chlorophenols (21%, approximately). Similar results are found by Gao et al. (1998) and Fall et al. (2001).

It can therefore be concluded that the main 2,4,6-TCP degradation is due to the bacterial activity, as is supported by previous studies (Godoy et al. 1999; Martínez et al. 1999 and 2000). This way, Gram negative nonfermentative bacilli was isolated, similar to those results reported by Ruckdeschel et al (1987), Godoy et al. (1999) and Martínez et al. (2000). Moreover, it was determined that the strains are able to degrade the chlorophenols as a sole carbon source and with the presence of another carbon source.

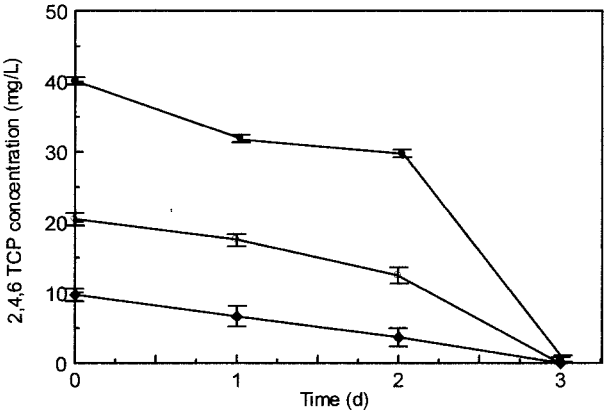

Figure 1. Degradation of 2,4,6-TCP in batch system inoculated with Biobío river sediment. Initial concentrations: 40 (•), 20 (o) and 10 (♦) mg 2,4,6-TCP/L.

In fact, Figure 2 shows biodegradation of 20 mg/L 2,4,6-TCP and viability of Gram negative bacilli isolated from sediment in R2A broth 1/4 and MM. In both experiments (Figure 2a and 2b), no lag phase was found. In both cases, chlorophenols were completely mineralized by Gram negative bacilli after 20 hours.

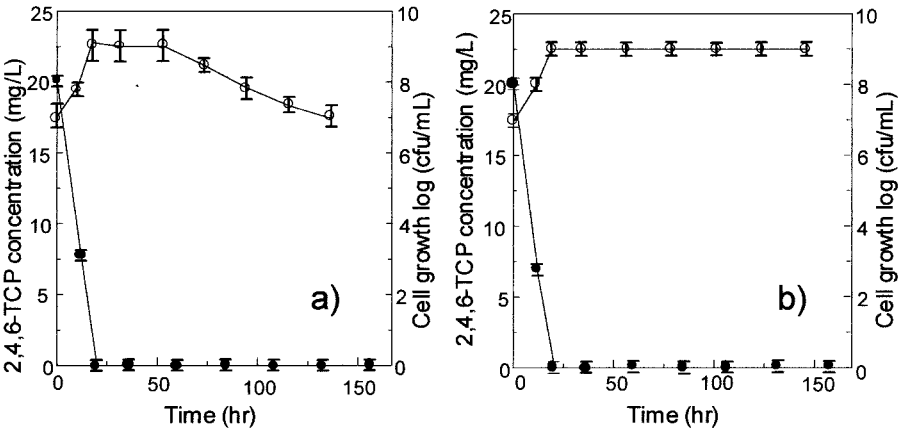

Figure 2. Degradation of 2,4,6-TCP (•) and cells growth (o) at 20 mg/L with a) MM as the sole carbon and energy source and b) R2A broth ¼.

In assays using 2,4,6-TCP as the sole carbon source (Figure 2 a), the initial degradation rate was 0.776 mg/L·hr and the viable bacterial counts decrease 2 lag cycles after 50 hours of incubation. However, in the R2A (Figure 2b) broth, viable bacterial counts were constant at 1.0 X 10^9 cfu/L after 20 hours of incubation whereas a fast initial degradation rate was 1,333 mg/L·hr. These results can be explained by an improvement in the metabolic state, protecting cells from the toxic effect of chlorophenol (Aranda *et al.* 1999).

Figure 3 shows the capacity of the bacteria to degrade 40, 100, 175 mg 2,4,6-TCP/L until mineralization at 20, 36, 84 and 156 hr, respectively. 2,4,6-TCP concentration was supplied as the sole carbon and energy source in these assays. Concentrations of 2,4,6-TCP higher than 260 mg/L were not degraded. Assays with initial concentration of 2,4,6-TCP between 40 mg/L (Figure 3a) and 100 mg/L (Figure 3b) show a complete degradation of the chlorophenol at 36 and 60 hours respectively. After that, biomass could grow to 1.0 X 10^9 cfu/mL. The increases in biomass after the complete degradation of 2,4,6-TCP has been described as an intracellular dynamic that is often associated with phenol metabolism (Kim and Hao 1999). A different behavior was shown in assays with 175 mg 2,4,6-TCP/L (Figure 3c). A lag phase of about 50 hours was observed. This indicates the toxic effect of the chlorophenols and its inhibitory effect on the specific enzymatic activities. Due to this, no biodegradation was observed until 60 hr. Also, from 36 to 60 hr the population density decreased until 1.0 X 10^4 cfu/mL.

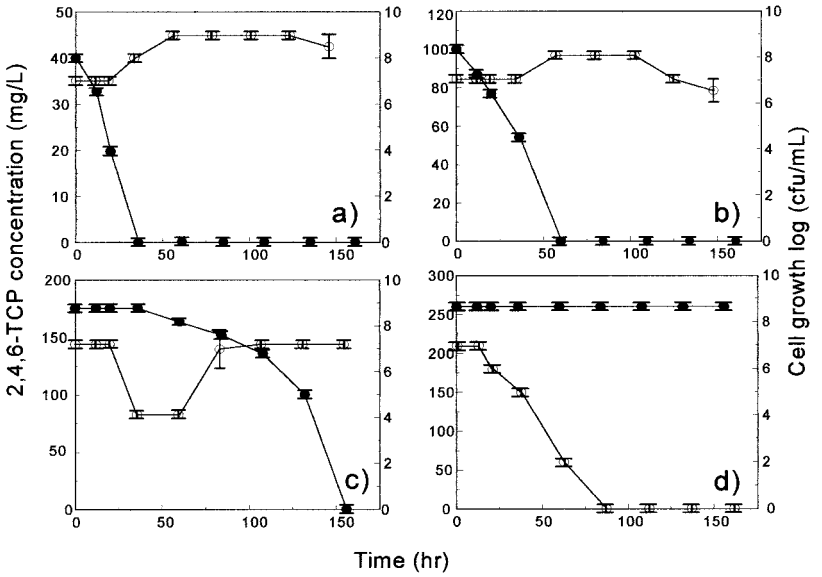

Figure 3. Degradation of 2,4,6-TCP with MM (•) and cells growth (o). Initial concentration of 2,4,6-TCP in each assays: a) 40 mg/L b) 100 mg/L, c) 175 mg/L and d) 260 mg/L.

However, adaptation was observed and 175mg 2,4,6-TCP/L was completely degraded at 156 hours. A maximum degradation rate (4.4 mg/L·hr) and cell density (1.0 X 10^7 cfu/mL) were observed in the period between 132 to 156 hr.

Martínez et al. (1999) demonstrated that bacteria with high degradative properties (as in this study) are not always the most tolerant. Tolerance is related to changes in cellular structure with unspecific mechanisms preventing the entry of these compounds into the cell (Martínez et al. 2000). In fact, this isolated strain tolerance to 2,4,6-TCP was observed below 260 mg 2,4,6-TCP/L. Above this concentration, isolated strains were not able grow on 2,4,6-TCP. Moreover, after 80 hr of 260 mg 2,4,6-TCP/L in the biodegradation assays, biomass was complete killed off (see Figure 3d). Moreover, Martínez et al. (2000) demonstrated that tolerance and degradation abilities are independent properties, in fact bacteria, tolerant to chlorophenols, do not necessarily have chlorophenol degradative abilities.

The Andrews model was considered in order to obtain the main kinetic constant (saturation and inhibitory constant) of the isolated strain in the presence of 2,4,6-TCP. Model parameters were determined by minimizing the deviations between experimental data and model predictions of the degradation rate (Figure 4). Thus, 107 mg/L and 155 mg/L were determined as saturation (K_s) and inhibition (K_i) constants. Figure 4 shows the model fitted to the experimental data.

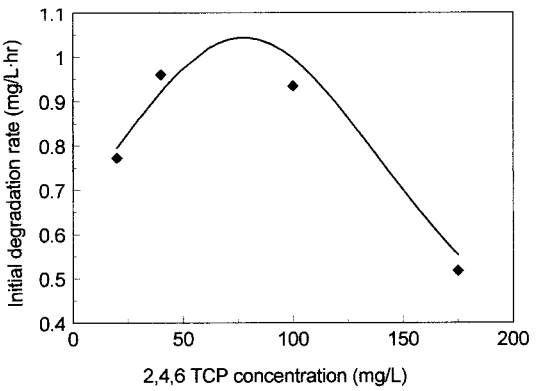

Figure 4. Initial degradation rate of 2,4,6-TCP. Experimental data (♦) and prediction by Andrews model (—).

The different model parameters for the degradation of 2,4,6-TCP (Table 1) could been explained as the difference in substrate transport across the different cells' membrane. Buttom (1991) used a two-stage substrate uptake model (transport across the cell membrane followed by metabolism) to demonstrate the differences in enzymatic reaction rate in determining relative substrate uptake kinetics. Little is known about how aromatic and other hydrophobic compounds are transported into cells; although diffusion is the most-cited mechanism (Reardon et al. 2000).

Table 1 shows a high value of K_s in this study compared with Gu and Korus (1995) and Vidal et al. (1997). It could be explained by the high substrate

concentrations that may cause inhibitions in some enzymatic reactions and therefore may reduce reaction rates.

In conclusion, sediments from this sector of the Biobío River (central Chile) contain specific bacteria with 2,4,6-TCP degradative properties. So, this study together with other authors (Aranda et al. 1999; Godoy et al. 1999; Martínez et al. 2000) strongly suggests that these bacteria participate in the river's self-purification processes. On the other hand, Andrews kinetic shows that the inhibition concentration for bacteria biodegradation is 156 mg 2,4,6-TCP/L and the saturation constant is 107 mg 2,4,6-TCP/L. The kinetic saturation constant is higher than that of the reference, however it is important to consider that the bleach kraft mill effluent contains many other chlorophenols and resinic-acid compounds that may affect the biodegradation of specific 2,4,6-TCP. In the future, it is necessary to study how the mixed compounds affect the bacterial biodegradation.

Table 1. Kinetic constants determined by Andrews model in the 2,4,6-TCP degradation.

Cells source	K_s (mg/L)	K_i (mg/L)	Reference
Sediments from Biobío river	107	156	This study
Flavobacterium sp. ATCC 39723	14	21	Gu and Korus (1995)
Sludge from aerated lagoon treat pulp kraft mill effluent	48.1	N.D.*	Vidal et al. (1997)

*Not Determined

Acknowledgments. This work was supported by grant 200.091.028-1.0, Dirección de Investigación Universidad de Concepción, Chile.

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