Reduction of 2,4-dichlorophenol toxicity to *Pseudomonas putida* after oxidative incubation with humic substances and a biomimetic catalyst

Dittmar Hahn\(^{a,b,*}\), Annunziata Cozzolina\(^c\), Alessandro Piccoloc, Piero M. Armenante\(^a\)

\(^a\)Department of Chemical Engineering, New Jersey Institute of Technology, 323 Dr. Martin Luther King Blvd., Newark, NJ 07102, USA
\(^b\)Biology Department, Texas State University, 601 University Dr., San Marcos, TX 78666, USA
\(^c\)Dipartimento di Scienze del Suolo, dalla Pianta e dell’Ambiente, Università di Napoli Federico II, Naples, Italy

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Abstract

The effect of a synthetic iron(III)-porphyrin meso-tetra(2,6-dichloro-3-sulfonatophenyl)porphyrinate as a biomimetic catalyst in the oxidative treatment of 2,4-dichlorophenol (2,4-DCP) with humic substances and \(\text{H}_2\text{O}_2\) was evaluated in factorial design experiments conducted at different concentrations of 2,4-DCP (0–25 ppm) and different incubation treatment times (0, 24, 96, or 120 h). In the absence of this treatment, bioassays with the bacterium *Pseudomonas putida* (ATCC11250) showed decreasing specific growth rates \(m\) (used here to quantify 2,4-DCP toxicity) with increasing concentrations of 2,4-DCP. However, when 2,4-DCP was treated as mentioned above the toxicity of the resulting 2,4-DCP solution was reduced significantly. At low 2,4-DCP concentrations (up to 5 ppm) and long incubation periods (as long as 120 h), the specific growth rate \(m\) was comparable to that of cultures grown in the absence of 2,4-DCP. The reduction in toxicity was directly correlated to a decrease in the concentration of 2,4-DCP in the treated solutions, as measured by high-performance liquid chromatography. The reduced concentrations of 2,4-DCP in the treated solutions could be correctly predicted based on the relationship between the specific growth rates and the 2,4-DCP concentrations in untreated solutions. These results indicate that the oxidative coupling of 2,4-DCP to humic substances catalyzed by the synthetic iron(III)-porphyrin catalyst in the presence of \(\text{H}_2\text{O}_2\) is responsible for the removal of 2,4-DCP from solutions. This approach appears to be a promising alternative treatment to reduce 2,4-DCP bioavailability and thus toxicity in the environment.

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Keywords: Bioassay; 2, 4-DCP; Growth rate; Iron-porphyrin; Optical density; Respiration

1. Introduction

Chlorophenols are organic compounds widely used in many industrial applications and commonly found in the effluents of many industrial facilities (Kopperman et al., 1976; Landner et al., 1977). They have been used as antiseptics since the late 18th century, and some of them, such as 2,4,6-trichlorophenol and pentachlorophenol, are still used today as fungicides and preservatives (Sittig, 1981; Hägglom and Välo, 1995). Because of their toxicity and recalcitrance to aerobic biodegradation, chlorophenols have been the focus of many studies investigating their degradability in soils and their interactions with soil components (Boyd, 1982; Fathepure and Vogel, 1991; Kiyohara et al., 1992). Soils are complex mixtures of organic and inorganic components, organo-mineral complexes, ions, and other compounds that can promote a variety of reactions that contribute to transformation and availability of xenobiotic compounds (Piccolo, 1996). Humic acids (HA), for example, resemble organic compounds in soil that influence the mobility and degradation of chlorophenols and many other xenobiotic compounds (Isaacson and Frink, 1984; Senesi and Chen, 1989; Xing and Pignatello, 1998; Piccolo et al., 2000).

Humic substances are commonly believed to consist of high-molecular-weight, highly polydisperse macromolecules (Kononova, 1961; Stevenson, 1994). Recent studies, however, indicate that they can be depicted as...
supramolecular associations of self-assembling heterogeneous and relatively small molecules that are held together by weak forces such as dispersive hydrophobic interactions (van der Waals, π–π, and CH–π bindings) and hydrogen bonds, rather than by covalent linkages as in real polymers (Piccolo, 2001; Wershaw, 2004; Peuravuori, 2005). The supramolecular association can be altered by interaction with organic acids derived from root exudates or from anthropogenic sources (Piccolo, 2002) or by oxidation (Piccolo et al., 2000). Enzymes such as peroxidases can catalyze the formation of covalent alkyl- and aryl-ether bonds between alkyl and aromatic components of humic acids, thereby increasing their conformational stability or even promoting their transformation into stable polymers (Bollag and Bollag, 1990; Piccolo et al., 2000; Cozzolino and Piccolo, 2002). Phenoloxidases have been shown to promote oligomerization and polymerization of phenols and anilines through a free-radical mechanism and hence are believed to contribute to soil detoxification from related organic contaminants (Bollag et al., 1988; Bollag, 1992; Kim et al., 1997).

Water-soluble metal-porphyrins are biologically important complexes present in many enzymes and proteins responsible for the storage and transport of electrons and oxygen. Metal-porphyrins can catalyze oxidation reactions similar to those catalyzed by enzymes such as monooxygenase, peroxidase, ligninase, or cytochrome P-450 and thus act as biomimetic catalysts (Song et al., 1997). Chlorophenols, for example, are oxidized by hydrogen peroxide (H₂O₂), as the oxidizing agent, in the presence of metal-porphyrins (Labat et al., 1990). The active site of the metal-porphyrin is a tetrapyrrolic ring to which a metal atom is coordinated. The lateral constituents are different depending on the particular porphyrin, but all prevent the formation of μ-oxo complexes between porphyrins that would inactivate them completely. In the presence of oxygen donors such as H₂O₂, metal-porphyrins can form very reactive metal-oxo components (e.g., iron(IV)-porphyrin cation radicals and oxo-iron(IV)-porphyrin) that are involved in one or two consecutive oxygen transfers oxidizing chlorophenols to quinones in the presence of metal-porphyrins (Song et al., 1997). The reactivity of such metal-oxo components can account for reactions of spontaneous polymerization, as reported for the formation of some fractions of humic substances in soils (Stevenson, 1994).

The objective of this work was to evaluate the effect of the synthetic iron(III)-porphyrin meso-tetra(2,6-dichloro-3-sulfonatophenyl)porphyrinate (Piccolo et al., 2005) as a biomimetic catalyst in the oxidative coupling of 2,4-dichlorophenol (2,4-DCP) to humic acids in the presence of H₂O₂. In a series of factorial design experiments, 2,4-DCP solutions were treated with iron(III)-porphyrin, humic acids, and H₂O₂ for different time periods with the aim of reducing the concentration of 2,4-DCP and, as a consequence, decreasing its toxicity. The toxicity of the resulting 2,4-DCP solutions after this treatment was assessed with a bioassay quantifying the specific growth rates of the bacterium Pseudomonas putida. These growth rates were compared to those obtained when the cultures were exposed to untreated 2,4-DCP solutions, which were used here as baseline data to predict the residual 2,4-DCP concentrations of treated solutions. The accuracy of these predictions was finally evaluated using HPLC analysis of 2,4-DCP concentrations, and a good match was found.

2. Material and methods

2.1. Experimental setup

HA was isolated from an oxidized coal provided by Eniricerehe SpA (Italy) and purified using the following routine procedure (Stevenson, 1994). The oxidized coal was shaken in 1 M NaOH and 0.1 M Na₂PO₄ under N₂ atmosphere for 24 h. HA was separated from alkaline extracts by lowering the pH to 1 with HCl, and it was purified extensively by repeated dissolution in 1 M NaOH and subsequent precipitation in 6 M HCl, as described elsewhere (Cozzolino and Piccolo, 2002). Purified HA, free of chloride and divalent metal salts, was resuspended in distilled water and titrated to pH 7.0 with 0.5 M NaOH. The resulting sodium humate was finally freeze-dried.

Solutions of different concentrations of 2,4-DCP (99%; Arcos Organics, Morris Plains, NJ, USA; 0.5, 1, 5, 10, 15 or 25 ppm) were prepared in purified water and incubated in the dark with 0.24 μmol of iron(III)-porphyrin meso-tetra(2,6-dichloro-3-sulfonatophenyl)porphyrinate (Piccolo et al., 2005), 0.2 mg mL⁻¹ of HA, 0.41 mmol of H₂O₂ (30%, Arcos Organics), or combinations of two or three of these components at room temperature for 0, 24, 96, or 120 h. After preparation, each series of solutions was apportioned in two series of bottles, one series for microbial analysis and the other series for reverse-phase chromatography analysis.

The test organism P. putida (ATTC 11230) was grown at 30 °C in defined medium (L⁻¹: 1 g KH₂PO₄, 0.1 g MgSO₄ × H₂O, 0.01 g CaCl₂ × 2 H₂O, 0.05 g NH₄Cl, 0.005 g NaFeEDTA, pH 6.8) with 10 μL L⁻¹ of α−glucose as an easily metabolized carbon source. Aliquots (5 mL) of the liquid culture, harvested during the exponential growth phase and diluted to an optical density close to zero, as measured with a spectrophotometer (Rileysky Spectronic 1001 Plus) at a wavelength of 600 nm (OD₆00), were transferred to 30-mL serum bottles and amended with a 1-mL sample from one of the 2,4-DCP test solutions. The glucose concentration was at least three orders of magnitude higher than that of the added 2,4-DCP, which is not likely to be degraded easily by an unacclimated microbial population. The added 2,4-DCP was assumed to be noncompetitive with respect to the utilization of the primary growth substrate. A 2-mL vial that was perforated in the upper part and contained 1 mL of 50 mM NaOH was placed in the same bottle to capture the evolving CO₂. The bottles were closed with butyl rubber stoppers and incubated at 30 °C for up to 6 h while being shaken at 100 rpm. At 1-h time intervals, three bottles were harvested to determine both the OD₆00 and the CO₂ evolved and absorbed by the NaOH solution in time. The same approach was used with control solutions.

2.2. Determination of the optical density (OD₆00)

The optical density of the cultures was monitored at a wavelength of 600 nm (OD₆00) with a spectrophotometer (Rileysky Spectronic 1001 Plus) after appropriate dilution of the 1-mL samples. Samples containing HA, which interferes with these measurements, were centrifuged in an Eppendorf centrifuge at 14,000 rpm for 1 min. The HA remaining in the supernatant when the pH was neutral was discarded, and the bacterial cells in the pellet were resuspended in growth medium. Analyses of three replicates are presented as means ± SE.
2.3. Determination of CO2 evolution

The amount of CO2 evolved from a culture was measured following the method of Anderson (1982). The CO2 evolved and adsorbed in the NaOH solution was determined after precipitation with 100 μL of a 0.1 N BaCl2 solution in the stirred sample to remove the CO2. The remaining NaOH was titrated with 2.5 mM HCl after the addition of 50 μL of a 1% phenolphthalin solution that was used to indicate the change in pH from alkaline to neutral (color changed from purple to colorless). Analyses of three replicates are presented as means ± SE.

2.4. Determination of 2,4-DCP

The concentration of 2,4-DCP was determined by reverse-phase chromatography on a Waters Alliance 2690 XE Separation Module (Waters Corp., Milford, MA, USA) equipped with an automatic sampler and a UV/VIS variable wavelength detector (Waters 484 UV/VIS) set at 254 nm. The column and the guard column were Econosphere C8 (Alltech; 150 mm, 4.6 mm i.d.) inserted in a thermostast at 25 °C. The elution was performed by a variable gradient, as presented in Table 1. Eluent A was composed of methanol and glacial acetic acid (99:1), while eluent B was MilliQ water and glacial acetic acid (99:1) (Table 1). The flow rate was set at the constant value of 0.5 mL min⁻¹. The reproducibility of the analysis was within ±1.5 μm (Armenante et al., 1994). Each sample was analyzed in triplicate and the standard deviation (SD) was always less than 1%. The internal software of the Separation Module allowed the automatic elaboration of the recorded data against calibration curves. These were generated daily to check the performance of the column and were obtained by injecting solutions of 2,4-DCP of known concentration and mixtures of phenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, and penta-chlorophenol.

2.5. Calculation of specific microbial growth rates

The specific microbial growth rate, μ, was calculated for each set of experiments using both the OD600 data and the CO2 evolution data. Assuming direct proportionality between the OD600 values and the logarithm of the biomass concentration (X), it is

\[ \frac{dX}{dt} = \mu X \Rightarrow \mu = \frac{d(lnX)}{dt} = \frac{d(OD_{600})}{dt}. \]  

(1)

The OD600 values were plotted against time (t) and regressed to calculate μ (in h⁻¹) from the slope. Only the OD data collected between t = 1 h and 6 h were used (data not shown). During this time period the glucose concentration did not appreciably change from the initial concentration (as indicated by the CO2 evolution data described below) and the lag phase was completed, thus ensuring that all points used in the determination of μ belonged to the exponential growth phase. Linear relationships between OD600 and t were obtained for all experiments, with R² values being always above 0.98.

Table 1

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (mL min⁻¹)</th>
<th>% Eluent A</th>
<th>% Eluent B</th>
<th>Flow variation mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5</td>
<td>30</td>
<td>70</td>
<td>Constant</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>30</td>
<td>70</td>
<td>Linear</td>
</tr>
<tr>
<td>15</td>
<td>0.5</td>
<td>80</td>
<td>20</td>
<td>Linear</td>
</tr>
<tr>
<td>20</td>
<td>0.5</td>
<td>30</td>
<td>70</td>
<td>Step</td>
</tr>
<tr>
<td>23</td>
<td>0.5</td>
<td>30</td>
<td>70</td>
<td>Constant</td>
</tr>
</tbody>
</table>

*Eluent A: methanol/glacial acetic acid (99:1).
*Eluent B: MilliQ water/glacial acetic acid (99:1).

The biomass concentration, X, at a generic time during the experiment is also given by the mass balance

\[ X = X_0 + Y_{CO2}(C_{CO2} - [CO2]). \]  

(2)

where Y_{CO2} is the biomass yield as a function of CO2 production (i.e., the amount of biomass produced in a microbial process per unit of CO2 generated), and the subscript 0 denotes concentrations at time t = 0. Thus, μ can be calculated as

\[ \mu = \frac{d\ln X}{dt} = \frac{d\ln X_0 + Y_{CO2}(C_{CO2} - [CO2])}{dt}. \]  

(3)

If the initial biomass concentration, X₀, is very small compared to that at any other time (included in the data analysis) and if Y_{CO2} is constant then

\[ \mu \approx \frac{d\ln X}{d(C_{CO2} - [CO2])} = \frac{d\ln ACO2}{d(C_{CO2} - [CO2])} \]  

(4)

Hence, the logarithmic values of the evolved CO2 were plotted against time (t) and regressed to calculate μ (in h⁻¹) from the slope. Linear relationships between [ACO2] and t, with R² values always above 0.97 (typically >0.99), were obtained for all experiments. Analyses of three replicates are presented as means ± SE.

2.6. Functional relationship between microbial growth rate and 2,4-DCP concentration

To determine the functionality between the microbial growth rate and the concentration of 2,4-DCP it was postulated that the presence of 2,4-DCP resulted in a noncompetitive inhibition of the utilization rate of the primary substrate, i.e., glucose, by P. putida. This was assumed to be the case since (a) 2,4-DCP is a recalcitrant compound not likely to be utilized as a carbon source by an unacclimated microbial population and (b) 2,4-DCP is actually a toxic compound likely to inhibit microbial growth. The effect of noncompetitive inhibition on the microbial growth rate is typically represented mathematically by an equation of the following type (Grady et al., 1999; Rittman and McCarty, 2001),

\[ \mu = \mu_{max} \left( \frac{K_I}{K_I + C_I} \right) \left( \frac{C_S}{K_S + C_S} \right) \]  

(5)

where μ is the specific growth rate, C_I is the concentration of noncompetitive inhibitor, K_I is the inhibition coefficient for the inhibitor, C_S is the concentration of the primary growth-rate-limiting substrate, K_S is the half-saturation coefficient for the primary substrate, and \( \mu_{max} \) is the maximum specific growth rate. Equations of this type have been used to describe other noncompetitive processes, such as the inhibition effect of oxygen on denitrification by Pseudomonas cultures in the presence of organic substrates and nitrates (Barker and Dold, 1997; Tchobanoglous et al., 2003) or the effect of organic inhibitors on nitrification by Nitrosomonas and Nitrobacter (Oslislo and Lewandowski, 1985; Grady et al., 1999). With regard to our work, Eq. (5) can be rewritten as

\[ \mu = \mu_{max} \left( \frac{K_I}{K_I + [2,4-DCP]} \right) \left( \frac{[\text{glucose}]}{K_S + [\text{glucose}]} \right) \]  

(6)

This equation shows that the specific growth rate of the microbial population is highest (and equal to \( \mu_{max} \)) when the glucose concentration is much higher than \( K_S \) and the concentration of the inhibitor, 2,4-DCP in our case, is zero. All experiments in this study were conducted at a sufficiently high initial glucose concentration to insure that [glucose] > \( K_S \), implying that the second term in parentheses on the right-hand side of Eq. (6) is equal to 1. Furthermore, the value of μ was experimentally obtained using only the initial portion of the microbial growth curve when the glucose concentration had not appreciably changed from the initial concentration but the lag phase, if any, had ended. Since 2,4-DCP acts as an inhibitor, its concentration was not likely to change to any meaningful extent during an experiment. Under these conditions, the semilogarithmic plot of the biomass concentration vs time is linear (Eq. (1)), and the slope
is constant (Eq. (6)) and is given by
\[
\mu = \frac{\mu_{\text{max}}}{1 + ([2,4-\text{DCP}]/K_1)}.
\]
(7)
This equation was used here to determine the values of \(K_1\) from experimental \(\mu\) vs [2,4-DCP] data. Although this equation could be rearranged as
\[
\frac{1}{\mu} = \frac{1}{\mu_{\text{max}}} + \frac{1}{\mu_{\text{max}} K_1 [2,4-\text{DCP}]}
\]
(8)
to determine both \(\mu_{\text{max}}\) and \(K_1\) from a simple linear regression, in this work the value of \(\mu_{\text{max}}\) was not allowed to float but was set equal to the average triplicate value obtained in the experiments with no 2,4-DCP. This approach is more rigorous since it requires that both \(\mu_{\text{max}}\) and \(K_1\) be determined independently from those for \(K_1\) obtained in the presence of the inhibitor. In other terms, \(\mu_{\text{max}}\) was obtained here under normal physiological conditions, whereas \(K_1\) was determined independently while the microbial population was under stress. In principle, allowing both \(\mu_{\text{max}}\) and \(K_1\) to float could produce a better fit between the experimental data and the regression curve but also an unrealistic value of \(\mu_{\text{max}}\), an easily measured, independent parameter of microbial growth that cannot and should not be a function of the inhibitory effects of 2,4-DCP on the population.

3. Results

Fig. 1 presents the relationship between the OD600 and the corresponding concentration of CO2 evolved. Both data sets correlate very well with each other, with typical \(R^2\) values larger than 0.99 within the same treatment and for all treatments (data not shown). This implies that \(Y_{\text{CO2}}\) was indeed constant and that both methods resulted in similar reliable values of the microbial biomass concentration, which could in turn be used to determine the growth rate \(\mu\).

The specific growth rates \(\mu\) (obtained using data regressed with Eq. (1)) for the control cultures of \(P.\ putida\) with no added 2,4-DCP resulted in a mean value of 0.45 h\(^{-1}\) (±0.01 h\(^{-1}\) as SE). This value was not appreciably different when either iron(III)-porphyrin alone (\(\mu = 0.43 \pm 0.02\) h\(^{-1}\)) or \(\text{H}_2\text{O}_2\) alone (\(\mu = 0.44 \pm 0.01\) h\(^{-1}\)) was also present in the test solution. The presence of humic substances, however, decreased the growth rate significantly (\(\mu = 0.26 \pm 0.01\) h\(^{-1}\)). Since the growth rate was linear over time even in the presence of HA (\(R^2 = 0.99\), methodological artifacts caused by the additional centrifugation and dilution steps could be excluded. It was concluded that humic acids have a toxic effect on \(P.\ putida\). Growth inhibition was not detected when humic substances were mixed with iron(III)-porphyrin and \(\text{H}_2\text{O}_2\) and then incubated for 0 h (\(\mu = 0.42 \pm 0.01\) h\(^{-1}\)), 24 h (\(\mu = 0.42 \pm 0.01\) h\(^{-1}\)), or 80 h (\(\mu = 0.45 \pm 0.01\) h\(^{-1}\)). Since our major goal was to analyze the effect of this mixture and different incubation times on 2,4-DCP availability and thus on toxicity of 2,4-DCP, the lack of any toxic effects of the mixture on \(P.\ putida\) provided the basis for the reliable determination of the toxicity effects caused by 2,4-DCP.

Addition of 2,4-DCP to the growth medium alone reduced the specific growth rate \(\mu\) of \(P.\ putida\) significantly (Table 2, Fig. 2). Even at 2,4-DCP concentrations as low as 0.5 ppm, a 33% reduction of the specific growth rate \(\mu\) was observed compared to that of the control cultures without 2,4-DCP. These results corroborate those of other investigations (Reinke et al., 1995; Gellert, 2000) and demonstrate the validity of our test system. Since chlorophenols are classic cytochrome uncouplers (Weinbach, 1957) and 2,4-DCP has been shown to interfere with oxidative phosphorylation (Stockdale and Selwyn, 1971), increasing

![Fig. 1. Regression line depicting the relationship between optical density (OD600) and CO2 evolution (respiration) data within the same treatment. This example presents data on the effect of the addition of iron(III)-porphyrin, HA, and \(\text{H}_2\text{O}_2\), but without 2,4-DCP, on \(\mu_{\text{max}}\) and respiration of \(P.\ putida\) (reaction time = 120 h).](image-url)

Table 2
Specific growth rate \(\mu\) of \(P.\ putida\) (h\(^{-1}\)) in growth media containing different concentrations of 2,4-DCP (ppm) that had been pretreated with individual compounds or a complete mixture of iron(III)-porphyrin, HA, and \(\text{H}_2\text{O}_2\) for different times (0, 24, 96, and 120 h).

<table>
<thead>
<tr>
<th>Addition of 2,4-DCP (ppm)</th>
<th>HA*</th>
<th>Mixture of iron-porphyrin, HA, and (\text{H}_2\text{O}_2) incubated for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>24 h</td>
</tr>
<tr>
<td>0.5</td>
<td>0.29 (0.02)</td>
<td>0.32 (0.01)</td>
</tr>
<tr>
<td>1</td>
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<td>0.29 (0.01)</td>
</tr>
<tr>
<td>5</td>
<td>0.24 (0.01)</td>
<td>0.26 (0.01)</td>
</tr>
<tr>
<td>10</td>
<td>0.16 (0.01)</td>
<td>0.22 (0.01)</td>
</tr>
<tr>
<td>15</td>
<td>0.14 (0.01)</td>
<td>0.19 (0.01)</td>
</tr>
<tr>
<td>25</td>
<td>0.10 (0.01)</td>
<td>0.18 (0.01)</td>
</tr>
</tbody>
</table>

*Values are those after 120 h of incubation and are representative for those of all other incubations without or with individual compounds only, at different incubation times. Data represent means ± standard error (SE) (n = 3).
concentrations of 2,4-DCP were anticipated to decrease the specific growth rate \( \mu \).

Our results are consistent with this assumption, as evidenced by the decreasing growth rates with increasing 2,4-DCP concentration (Table 2). The lowest growth rate \( (\mu = 0.1 \pm 0.01 \, \text{h}^{-1}) \) was obtained at the highest concentration of 2,4-DCP (25 ppm) (Table 2), which constitutes a reduction in growth rate of about 77% compared to that of cultures grown without 2,4-DCP.

The inhibitory effect of 2,4-DCP on the specific growth rate of \( P. \ putida \) was substantially removed when 2,4-DCP solutions underwent the above-mentioned treatment with iron(III)-porphyrin, HA, and \( \text{H}_2\text{O}_2 \) prior to inoculation with \( P. \ putida \) (Table 2, Fig. 2). This effect was dependent on treatment incubation time (with longer incubation times between 96 and 120 h resulting in higher growth rates) and on the initial concentration of 2,4-DCP (with lower concentrations producing higher growth rates; Table 2). Growth rates that essentially resembled those of controls without 2,4-DCP addition were obtained for low 2,4-DCP concentrations (0.5–5 ppm) and long incubation treatment times (96 and 120 h; Table 2). At higher 2,4-DCP concentrations, e.g., 10 ppm, the values of the specific growth rate \( \mu \) increased by about 25% compared to values without initial treatment and to about 55% after 120 h of treatment. These results suggest a reduction of 2,4-DCP availability, and thus toxicity, with treatment time.

Regression analysis using Eq. (7) and the two sets of data shown in Fig. 2 resulted in inhibition coefficients \( (K_\text{i}) \) equal to \( 23.64 \pm 3.61 \) and \( 5.69 \pm 0.08 \, \text{ppm} \), respectively, depending on whether the cultures were exposed to treated or untreated 2,4-DCP solutions. The statistically significant increase in inhibition coefficients between the untreated and the treated solutions is strong evidence that the treatment resulted in a reduction of the toxicity effect experienced by the \( P. \ putida \) cultures.

A comparison between the experimental data and the results of the regression analysis (Fig. 2, where the solid lines are plots of Eq. (7) in which the above-mentioned \( K_\text{i} \) values were used) shows that the curves adequately described the experimental data, further supporting the assumption that noncompetitive inhibition is responsible for the observed effects on the specific growth rate of \( P. \ putida \).

The decrease in 2,4-DCP toxicity following the treatment was related to a decrease in 2,4-DCP concentration, as demonstrated by HPLC analysis. This decrease was obtained only with treatments utilizing all three components simultaneously (iron(III)-porphyrin, HA, and \( \text{H}_2\text{O}_2 \)), and it was a function of treatment time (Table 3). At low 2,4-DCP concentrations (<5 ppm) and long treatment times complete removal of 2,4-DCP was achieved, while significant concentration reduction was observed at higher initial 2,4-DCP concentrations. The initial concentration of 10 ppm, for example, was reduced by 89% after a 120-h treatment and that of 25 ppm by 81% (Table 3). Treatment with individual components did not substantially affect the final 2,4-DCP concentrations (Table 3), supporting the conclusion that adsorption phenomena are not responsible for the reduction of 2,4-DCP.

The nominal 2,4-DCP concentrations in Table 2 are not the actual concentrations in the treated solutions (and experienced by the microorganisms in the toxicity experiments). This implies that the use of the initial (nominal) 2,4-DCP concentrations obtained prior to the oxidative treatment (shown in Table 2) in the interpretation of the biological growth rate data (in Fig. 2) has limitations. Therefore, the microbial growth rates \( \mu \) were replotted in Fig. 3 as a function of the actual 2,4-DCP concentrations obtained after the solutions underwent the oxidative catalytic treatment described here (as measured by HPLC) and listed in Table 3 instead of the nominal concentrations listed in Table 2. In Fig. 3 all points now collapse on the solid line that was obtained previously for the untreated case using the inhibition coefficient \( K_\text{i} = 5.69 \pm 0.08 \, \text{ppm} \). Thus, the relationship between growth rate and 2,4-DCP concentration is adequately represented by the one-parameter inhibition curve given by Eq. (7). Conversely, this equation can be used to predict the residual 2,4-DCP concentrations in samples using growth rate determination.

4. Discussion

The standardized inoculation method used here produced a reliable determination of the specific growth rate \( \mu \) for \( P. \ putida \) in all treatments, thus providing the standardized conditions necessary for comparative analyses of the growth rates impacted by external factors (Abbondanzini et al., 2003). Growth rates are a function of all metabolic activities during the different life cycles of the bacterium. Thus, the determination of growth inhibition in
response to contamination might be considered more ecologically relevant than commonly used approaches utilizing specific metabolic activities such as, for example, bioluminescence of the bacterium *Vibrio fisheri* (Gellert, 2000). The latter assay is well established in many laboratories around the world since it is extremely sensitive, rapid, and inexpensive (Steinberg et al., 1995; Backhaus et al., 1997; Froehner et al., 2000). Growth inhibition studies are supposed to be less sensitive, although the toxic effects of 3,5-DCP were reported to be detectable at concentrations below 1 ppm in both growth inhibition and bioluminescence studies (Gellert, 2000). Results obtained with *V. fisheri* showed growth inhibition values comparable to those of other bacteria, such as *P. putida* (Reinke et al., 1995). Our experimental setup with *P. putida* as a test organism and 2,4-DCP concentrations between 0.5 and 25 ppm should be useful for the determination of 2,4-DCP toxicity by growth inhibition.

Since incubation of 2,4-DCP in the presence of HA alone did not produce growth rates any different from those with 2,4-DCP alone (Table 2), adsorption to HA or even biota which has been observed to a certain extent in other studies (Murin and Soeyik, 1979; Ning et al., 1996; Peuravuori et al., 2002) can be ruled out as a cause for the reduction of 2,4-DCP in our treatments. Other mechanisms for the removal of 2,4-DCP must be considered. Removal through enhanced degradation of 2,4-DCP in the presence of synthetic iron(III)-porphyrin might be one mechanism, similar to that observed for the biomimetic oxidation of lignin compounds by synthetic iron and manganese porphyrins in the presence of H$_2$O$_2$ (Crestini et al., 1999). HA might also help to enhance the degradation of xenobiotic compounds in the environment (Fava and Piccolo, 2002), although this effect would be more pronounced in the presence of a catalyst and an oxygen donor (Fukushima and Tatsumi, 2001). Both mechanisms, however, were not likely to play a major role in our system since all three components, i.e., iron(III)-porphyrin, HA, and H$_2$O$_2$, are required to reduce 2,4-DCP availability and toxicity. More likely mechanisms are degradations based on radical-based oxidative reactions in the presence of a catalyst, humic acids, and an oxygen donor. Such systems have been shown to remove pentachlorophenol from solutions, accompanied by dechlorination (Fukushima et al., 2003). Dechlorination was not assessed in our study. Therefore, degradation of 2,4-DCP can neither be confirmed nor rejected.

Small heterogeneous molecules present in humic matter, as in weakly associated superstructures, can be covalently bound into true oligomers or polymers by an oxidative coupling reaction catalyzed by enzymes such as peroxidase (Piccolo et al., 2000; Cozzolino and Piccolo, 2002) or by a biomimetic catalyst (Piccolo et al., 2005). Structural analysis of 2,4-D removal has previously demonstrated covalent binding of 2,4-D to humic substances (Piccolo et al., 2001). A similar mechanism, i.e., the oxidative coupling of 2,4-DCP to humic substances catalyzed by the synthetic iron(III)-porphyrin, could therefore be the basis for removal of 2,4-DCP from solution and the reduction in toxicity to *P. putida* observed in our study. This speculation

### Table 3

HPLC analysis of residual 2,4-DCP concentrations (ppm) in solutions treated with individual compounds or a complete mixture of iron(III)-porphyrin, HA, and H$_2$O$_2$, for different times (0, 24, 96, and 120 h)

<table>
<thead>
<tr>
<th>Addition of 2,4-DCP (ppm)</th>
<th>HA*</th>
<th>Mixture of iron-porphyrin, HA, and H$_2$O$_2$, incubated for 120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0h</td>
</tr>
<tr>
<td>1</td>
<td>0.84 (0.01)</td>
<td>0.57 (0.04)</td>
</tr>
<tr>
<td>5</td>
<td>4.61 (0.05)</td>
<td>2.33 (0.02)</td>
</tr>
<tr>
<td>10</td>
<td>8.95 (0.04)</td>
<td>4.62 (0.01)</td>
</tr>
<tr>
<td>15</td>
<td>14.08 (0.05)</td>
<td>6.93 (0.01)</td>
</tr>
<tr>
<td>25</td>
<td>23.08 (0.07)</td>
<td>8.80 (0.02)</td>
</tr>
</tbody>
</table>

*Values are those after 120 h of incubation and are representative for those of all other incubations without or with individual compounds only, at different incubation times. Data represent means±standard error (SE) (n = 3).
is supported by studies of others in which the toxicity of by-products of pentachlorophenol oxidation in other biomimetic systems was significantly reduced in the presence of humic acids (Fukushima et al., 2003). The reduction in toxicity determined by using bioluminescence measurements of the bacterium *V. fisheri* was related to a covalent binding of the chlorinated byproducts to humic acids.

In conclusion, the results of this work have implications in environmental soil management since they show that naturally occurring humic superstructures and organic pollutants can be catalytically and covalently inactivated in situ. In addition, the approach described here appears to be promising to decrease the concentration of 2,4-DCP in the effluents of many industrial facilities and hence reduce its bioavailability and toxicity upon release in the environment.

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**References**


