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Inhibitory concentrations of 2,4D and its possible intermediates in sulfate reducing biofilms

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ABSTRACT

Different concentrations of the herbicide 2,4-dichlorophenoxyacetic acid (2,4D) and its possible intermediates such as 2,4-dichlorophenol (2,4DCP), 4-chlorophenol (4CP), 2-chlorophenol (2CP) and phenol, were assayed to evaluate the inhibitory effect on sulfate and ethanol utilization in a sulfate reducing biofilm. Increasing concentrations of the chlorophenolic compounds showed an adverse effect on sulfate reduction rate and ethanol conversion to acetate, being the intermediate 2,4DCP most toxic than the herbicide. The monochlorophenol 4CP (600 ppm) caused the complete cessation of sulfate reduction and ethanol conversion. The ratio of the electron acceptor to the electron donor utilized as well as the sulfate utilization volumetric rates, diminished when chlorophenols and phenol concentrations were increased, pointing out to the inhibition of the respiratory process and electrons transfer. The difference found in the IC_{50} values obtained was due to the chemical structure complexity of the phenolic compounds, the number of chlorine atoms as much as the chlorine atom position in the phenol ring. The IC_{50} values (ppm) indicated that the acute inhibition on the biofilm was caused by 2,4DCP (17.4) followed by 2,4D (29.0), 2CP (99.8), 4CP (108.0) and phenol (143.8).

1. Introduction

Mono- and polysubstituted chlorophenols are recalcitrant chemicals widely distributed in terrestrial and aquatic environments due to their extensive use as disinfectants, fungicides, antiseptics, herbicides, pesticides, wood preservatives and as bleaching process by-products in paper mills [1,2].

Small amounts of all types of chlorophenols have been found in marine and fresh waters [3], including coastal Mediterranean and North marine waters [4,5]. Because of their persistence, chlorophenols have been classified as dangerous organic substances that affect human health and the environment [6]. These pollutants accumulate in sediments that induce changes in the biota and modify the rate of natural depuration processes, interfering with the trophic chain [3,7].

Some chlorophenols are used as unspecific biocides, among them, the pesticide 2,4-dichlorophenoxyacetic acid (2,4D) is present in some 1500 commercial products as an active ingredient; its main intermediate, 2,4-dichlorophenol (2,4DCP) may represent 30% in these formulations. Formulations containing these products have been extensively used for weeds control in agricultural and non-crop areas in Mexico.

Chlorophenols have been found in environments where sulfate reducing bacteria (SRB) are present and cause toxicity and inhibition on sulfate respiration. Under anaerobic and reduced conditions, SRB carry out the oxidation of simple organic carbon compounds to CO_2 or to intermediates as acetate, using sulfate as terminal electron acceptor that is reduced to sulfide through a non-assimilative metabolic process. Many SRB are able to metabolize hydroxybenzoate isomers or benzoic acid through the benzoyl coenzyme A pathway, but are not known to degrade phenols or chlorophenols until exposed to these compounds for long periods of time [8]. Moreover, Uberoi and Bhattacharya [9] determined the relative toxicity concentrations of several chlorophenols for propionate degrading SRB.

SRB able to degrade chlorophenols initiate the process through reductive dehalogenation that implies electrons transfer from a donating substance to the aromatic ring to replace the chlorine

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Table 1
Sulfate reduction volumetric rates, q_{max}, effectiveness ratio, $R_{acc/don}$ obtained for each phenolic compound at all concentration levels assayed in ppm.

2,4DCP			2,4D			2CP			4CP		Phenol		
ppm	q_{max}	$R_{acc/don}$	ppm	q_{max}	$R_{acc/don}$	ppm	q_{max}	$R_{acc/don}$	q_{max}	$R_{acc/don}$	ppm	q_{max}	$R_{acc/don}$
0	0.66	0.75	0	0.52	0.58	0	0.52	0.58	0.66	0.75	0	0.66	0.75
10	0.44	0.72	5	0.38	0.59	25	0.32	0.63	0.55	0.71	150	0.32	0.45
25	0.22	0.62	10	0.3	0.54	75	0.28	0.63	0.48	0.65	300	0.27	0.38
50	0.21	0.52	25	0.28	0.5	150	0.20	0.51	0.17	0.54	600	0.24	0.47
75	0.21	0.54	50	0.18	0.48	300	0.17	0.43	0.12	0.52	1200	0.24	0.46
125	0.16	0.47	75	0.12	0.46	600	0.09	0.17	0	0	2400	0.11	0.49

substituent by hydrogen. In a second step, the remaining phenol is carboxylated in the para-position and dehydroxylated to yield benzoate which oxidation is through sulfidogenesis, the whole process is known as dehalorespiration and occurs as a detoxification mechanism. Nevertheless, the presence of sulfate and other sulfur oxyanions (thiosulfate or sulfite), may cause partial or total inhibition of the aromatic ring dehalogenation, due to a competence between the sulfur oxyanions and the aromatic compound, which acts as an alternate electron acceptor [10]. In this way dehalogenation may be stimulated by the addition of inducers (electron donors) such as lactate, 2–4 carbons organic acids, ethanol, pyruvate, formate and hydrogen, particularly [11–13].

There are few reports about chlorophenols degradation by SRB. The degradation of 2-, 3- and 4-chlorophenol and 2,4-dichlorophenol coupled to sulfate reduction in estuarine and river sediments has been observed [14,15]. Chang et al. [16] demonstrated the degradation of 2-chlorophenol in a membrane reactor in sulfate reducing conditions. Some specialized SRB able to degrade monochlorinated compounds coupled to sulfate respiration have been isolated, as *Desulfomonile tiedjei*, strain 2CB-1 and *Desulfovibrio dechloroacetivorans* SF3 among others [13,17], while, some bacteria from the genus Desulfitobacterium like *Desulfitobacterium dehalogenans*, are able to degrade mono, tri and pentachlorophenols using sulfite as terminal electron acceptor [17].

Inhibiting concentrations of 2,4D has been reported for several algae species and toxic tests were assayed in *Vibrio fisheri* [6]. No inhibitory concentrations for 2,4D have been reported for aquatic bacteria like SRB. The aim of this investigation was to evaluate the inhibition of the sulfate respiratory process in the presence of this herbicide and the most probable intermediates suggested by Gibson and Suflita [11]. The IC_{50} values for 2,4-dichlorophenol (2,4DCP), 4-chlorophenol (4CP), 2-chlorophenol (2CP) and phenol were determined as a function of the decrease in sulfate rate consumption, q_{max}, in sulfate reducing biofilms using ethanol as the electron donor. This work is part of a research on the physiological effect of chlorophenols on several anaerobic respiratory processes and the effect of herbicides and its intermediates in aquatic environments.

2. Materials and methods

2.1. Biofilm cultivation

The biofilm was cultivated in a down flow fluidized bed reactor (DFFBR) 2.5 L volume. The support particles consisted of grinded low-density polyethylene of 400 kg/m^3 and a mean diameter of 0.6 mm, the bed was inoculated with 0.025 L of a SRB enriched consortia. The reactor was fed with mineral medium consisting of (g/L): $NaHCO_3$ (2.0), $NaH_2PO_4{\cdot}2H_2O$ (0.795), K_2HPO_4 (0.6), NH_4Cl (0.28); $MgSO_4{\cdot}7H_2O$ (0.111), $CaCl_2{\cdot}7H_2O$ (0.01), yeast extract (0.02), supplemented with a trace elements solution (1 mL/L) consisting of (mg/L): $FeCl_2{\cdot}4H_2O$ (2000), $MnCl_2$ (500), resazurin (500), EDTA (500), Na_2SeO_3 (100), H_3BO_3 (50), $ZnCl_2$ (50), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (50), $AlCl_3$ (50), $NiCl_2{\cdot}6H_2O$ (50), $CoCl_2{\cdot}2H_2O$ (50), $CuCl_2{\cdot}2H_2O$ (50), amended with 1.1 g ethanol/L and 1.1 g sulfate/L to obtain a COD to sulfate ratio of 2, pH was adjusted to 6.6. For biomass immobilization the reactor was batch operated at 30 °C for 16 days until the biomass concentration obtained was of 0.81 ± 0.26 kg immobilized volatile solids (IVS)/m^3 dry support. Afterwards, the reactor was operated continuously at a HRT of 1 day and fed with the formulation described above. After 1 month in stationary phase, sulfate reduction was the only respiratory process detected and the biomass remained at the same concentration.

2.2. Inhibition assays

The immobilized support particles were withdrawn from the DFFBR and used for the inhibition assays, performed in duplicate in 60 mL serological bottles at pH 6.6. Each bottle contained 25 mL of the mineral medium described above without substrates and 10 mL of immobilized support. Bottles were sealed with rubber stoppers and aluminum crimps followed by gas phase exchange with N_2:CO_2 (80:20) to provide anaerobic reducing conditions. Ethanol and sulfate were added separately to each bottle at 1.9 g COD/L of ethanol (20.0 mM) and 0.96 g/L of sulfate (10 mM), to keep a COD/SO_4^{2-} ratio around 2. Chlorophenols were added individually spiking the bottles with stock concentrated solutions to obtain increasing concentration levels. The herbicide and its main intermediate (2,4DCP) were evaluated at concentrations ranging from 5 to 125 ppm. Monochlorophenols (2CP and 4CP) were evaluated from 25 to 600 ppm, and phenol from 150 to 2400 ppm (see Table 1). The control assays were fed with ethanol and sulfate and did not contain the toxic compound. All assays were performed at 30 °C without agitation. The respiratory process was assessed during 72 h at 12-h intervals through the measurement of sulfate and ethanol consumption, sulfide and acetate production and phenolic compounds evolution.

2.3. Inhibitory concentration calculations

In order to find out the concentration that caused 50% inhibition of the sulfate respiratory process (IC_{50}), the sulfate consumption volumetric rates, q_{max}, were determined by the Gompertz model for each phenolic compound at each concentration tested. The IC_{50} values were determined graphically. For comparison, some volumetric ethanol consumption rates were also calculated. Additionally, an effectiveness ratio of the sulfate reducing process, $R_{acc/don}$, that relates the amount of electrons from the acceptor to the amount of electrons from the donor consumed or the whole oxidative–reductive process that is taking place, was established (Eq. (1)), to evaluate the inhibitory effect on the respiratory process of the phenolic compound added during the exposure time:

$$R_{acc/don} = \frac{\text{mmol electron acceptor consumed}}{\text{mmol electron donor consumed}} \quad (1)$$

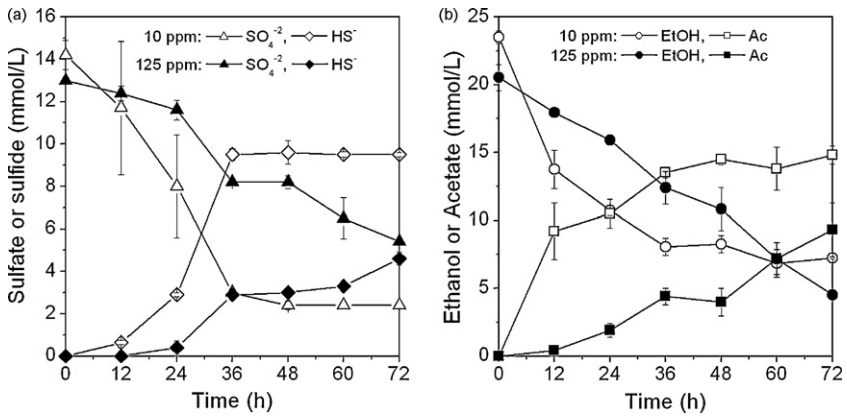

Fig. 1. Concentration profiles of (a) sulfate reduction and (b) ethanol oxidation in the presence of 2,4DCP at 10 and 125 ppm. EtOH: ethanol; Ac: acetate; HS^-: sulfide; SO_4^{2-}: sulfate.

2.4. Analytical techniques

Ethanol and acetate were quantified by gas chromatography/flame ionization detector (Hewlett Packard 5890 series II) equipped with an AT-1000 Alltech capillary column (0.53 mm × 10 m). Chlorophenols and phenol were quantified by HPLC (Waters 2996) equipped with a diode array detector (Waters PDA 600) at 280 nm using an Econosphere C18 reverse phase column (5 μm particle size, 4.6 mm × 250 mm) with a 5 mM acetate–acetonitrile (50:50) mobile phase. Sulfate was quantified with the turbidimetric method with barium chloride [18] and sulfide with a copper spectrophotometric technique. The biofilm attached to the support was detached with consecutive distilled water washes using an ultrasonic bath, volatile suspended solids in the washes were determined according to standard methods [18].

3. Results and discussion

Ethanol was used as substrate to form the biofilm in the DFFBR and was added to the inhibition assays as the electron donor for the sulfate respiratory process at the following stoichiometric ratio:

$$C_2H_5OH + 0.5SO_4^{2-} \rightarrow 0.5HS^- + CH_3COO^- + 0.5H^+ + H_2O$$

During biofilm cultivation at stationary phase conditions, the sulfate reducing volumetric rate, q_{max}, attained by the biofilm in the reactor varied from 0.52 to 0.66 mmol SO_4^{2-}/L h, the effectiveness ratio of the electron donating/accepting process, $R_{acc/don}$, varied from 0.58 to 0.75 and the sulfate reduction efficiency was of 67 ± 3.2%, indicating a stable sulfate respiratory process in the biofilm formed.

Related to the inhibition assays, Table 1 shows the sulfate reduction volumetric rates, q_{max}, in mmol SO_4^{2-}/L h and the effectiveness ratios of the sulfate respiratory process ($R_{acc/don}$) at different concentrations of the five phenolic compounds evaluated, during the inhibition assays. According to the IC_{50} values the intermediate 2,4DCP was the inhibitoriest to the sulfate respiratory process (IC_{50} of 17.4 mg/L), whereas phenol was the less inhibitory with an IC_{50} of 143.8 mg/L. In all the assays added with phenolic compounds, sulfate reduction was inhibited along with ethanol consumption, as the toxic compound concentration increased, diminishing the sulfate utilization volumetric rates.

Concerning the $R_{acc/don}$ lower values obtained than the values without the addition of chlorophenols indicate that the biofilm diminished its whole capability for electrons transfer from the donating substance (ethanol) to the final acceptor (sulfate), while being exposed to the phenolic compounds, which probably affected the respiratory process at the membrane level as has been suggested by Sikkema et al. [19].

The concentration of all chlorophenols and phenol tested remained constant throughout the experiments. None of the herbicide intermediates or the herbicide itself were dehalogenated during the exposure time, because phenol or benzoate accumulation was not detected, regardless of an inducer was added and reducing conditions were established, as sulfide production demonstrated. This suggests that no competence between electron acceptors for dehalogenation was established, as Ensley and Suflita [10] pointed out due to the inhibitory effect of the chlorophenolic compounds.

3.1. Inhibitory effect of dichlorophenolic compounds

The consumption profile followed by sulfate and ethanol when the biofilm was exposed to increasing concentrations of 2,4DCP, the chemical that caused the highest inhibition is shown in Fig. 1. As the chlorophenol concentration increased from 10 to 125 ppm, the sulfate reduction and ethanol oxidation volumetric rates decreased from 0.44 to 0.16 mmol SO_4^{2-}/L h and from 0.3 to 0.22 mmol ethanol/L h, respectively. The intermediate 2,4DCP was not dehalogenated and its concentration remained constant during the assay (data not shown); the IC_{50} value found was 17.4 ppm, the lowest concentration among all intermediates and the herbicide itself indicating its acute inhibitory effect.

The herbicide 2,4D presented similar consumption and production profiles of sulfur and carbon compounds (data not shown) to the profiles presented in Fig. 1. In the case of the herbicide, not even the phenoxy bond was hydrolyzed that is the common initial step in the degradation pathway of 2,4D [12], and the intermediate 2,4DCP that would be the main hydrolysis product, was not detected.

The q_{max} value decreased 77% at a 2,4D concentration of 75 ppm, in comparison to the control (no 2,4D added) while $R_{acc/don}$ diminished only 20%. Similarly, for 2,4DCP the ratio diminished 28% at the same concentration (Table 1), indicating the less inhibitory effect of the herbicide and 2,4DCP on the utilization of the electron donor by the biofilm (see Fig. 1b) than on the sulfate reduction process (see Fig. 1a). Finally, the IC_{50} of 2,4D was found at 29.0 ppm.

Toxicity or inhibition data involving pesticides and microorganisms is scarce because most of the studies focus on microbial degradation of pesticides. DeLorenzo et al. [7] reported that for Vibrio fisheri the EC_{50} value found for 2,4D was of 100.7 mg/L while for the intermediate 2,4DCP was 5.0 mg/L. The herbicide 2,4D showed a similar toxicity for microalgae species since EC_{50} value close to that for *V. fisheri*. This information as well as the results reported here, point out the higher inhibitory or toxic effect caused by the intermediate (2,4DCP) rather than by the original compound (2,4D).

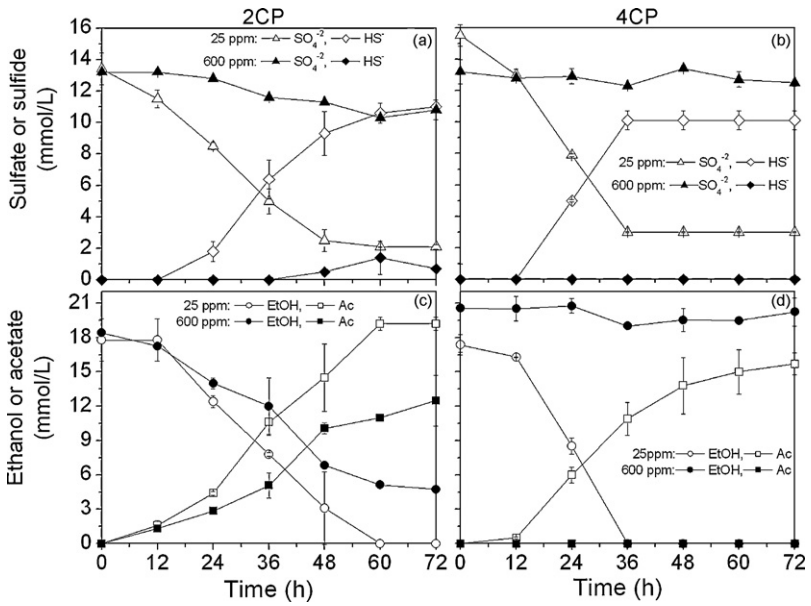

Fig. 2. Concentration profiles of (a and b) sulfate reduction and (c and d) ethanol oxidation in the presence of 2CP (left panels) and 4CP (right panels), at 25 and 600 ppm. EtOH: ethanol; Ac: acetate; HS^-: sulfide; SO_4^{2-}: sulfate.

3.2. Inhibitory effect of monochlorophenolic compounds

The profiles of sulfate and ethanol consumption as well as the sulfide and acetate production at 25 and 600 ppm of 2CP and 4CP are shown in Fig. 2. Both monochlorinated phenols exhibited a similar detrimental effect on sulfate reduction at high concentrations (Fig. 2a and b), being the inhibition more acute on the entire respiratory process (sulfate reduction and ethanol oxidation) when 4CP was assayed. At 150 ppm of 4CP, the sulfate reduction volumetric rate was the same to that found at 300 ppm for 2CP (Table 1). At these concentration levels, the decay in sulfate reduction was around 74% for 4CP and 67% for 2CP.

At 600 ppm, 2CP inhibited 82% of the sulfate reduction volumetric rate (q_{max}) and the $R_{acc/don}$ diminished 70% because sulfate consumption was almost completely inhibited (Fig. 2c). In contrast, at 600 ppm of 4CP, sulfate reduction and ethanol consumption were totally inhibited (Fig. 2b and d), indicating the influence of the chlorine in the para-position on the electron donating process. These results are similar to previous reports for methanogenic consortia in which the para-isomer was more toxic [20].

The IC_{50} values for both chlorophenolic compounds were very similar; being of 99.8 ppm in the case of 2CP and 108.0 ppm for 4CP. However, as it was mentioned, 4CP inhibited completely the consumption of the electron donor besides sulfate reduction according to the values of q_{max} and $R_{acc/don}$ (Table 1).

3.3. IC_{50} values and molecule complexity

Phenol, the simplest molecule assayed, was the less toxic compound tested because at a concentration of 2400 ppm the sulfate reduction volumetric rate decreased 83%, comparing the q_{max} value when phenol was not added (Table 1). The $R_{acc/don}$ ratio diminished only 34% indicating that at this large concentration the respiratory process was not completely inhibited. The IC_{50} was 143.8 ppm and phenol neither was oxidized nor mineralized by the biofilm during the assays.

From Fig. 3, it can be observed that the increasing number of chlorine atoms on the benzene ring and its position, exerted an acute inhibitory effect on the sulfate reduction process. The most complex molecules, 2,4D and 2,4DCP, presented the lowest IC_{50} values, 17.4 and 29.0 ppm, being 6 and 3 times smaller, respectively than the IC_{50} values found for monochlorinated compounds, which were around 100 ppm. In addition it was found that similar sulfate reducing rate values were obtained adding a concentration four times higher (300 ppm) of 2CP or 4CP, than when 75 ppm of 2,4D and 2,4DCP were added (Table 1). The IC_{50} values indicated that the highest inhibition towards the sulfate reducing biofilm was observed for 2,4DCP followed by 2,4D > 2CP > 4CP and phenol.

These results can be compared to those obtained by Uberoi and Bhattacharya [9] on propionate utilization by a sulfate reducing consortium, where 2,4DCP turned out to be more toxic than 2CP and 4CP, although 4CP was more toxic than 2CP. In this case, the para-isomer was more toxic than the ortho-isomer, while a disubstituted phenol in the ortho- and the para-positions resulted more toxic than de monosubstituted compounds.

In general, it was noticed that the inhibitory effect was acute on the sulfate reduction rate (q_{max}) that is the metabolic process carried out by the respiratory chain located in the cell membrane, which may have been destabilized by the effect of the toxic com-

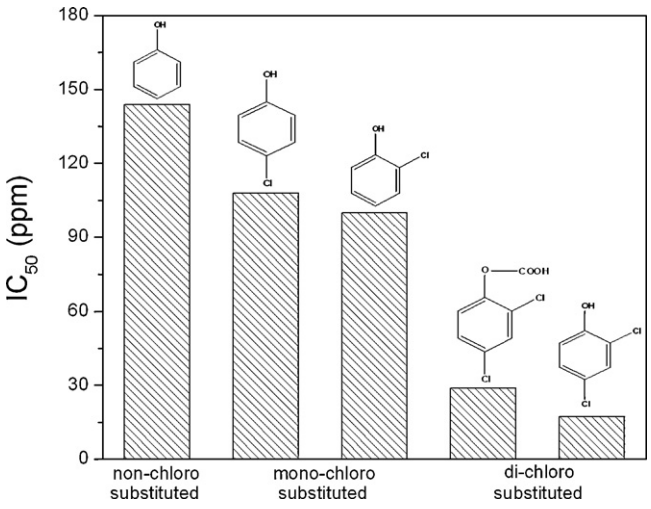

Fig. 3. The IC_{50} values obtained for chlorophenolic compounds and phenol.

pounds. The ratio $R_{acc/don}$ shows the inhibitory effect on the electron transfer process.

The toxic effect and the lack of biodegradability of all phenolic compounds may be explained by the fact that the enzymes responsible for reductive dechlorination as well as phenol degradation are induced enzymes, as Holliger et al. [15] had pointed out. Microbial cultures that have been exposed to these compounds, may exhibit subsequently the capacity to remove the halogen and mineralize phenol than cultures that have not been ever exposed. In another study, dechlorination and phenol mineralization of 2CP, 3CP and 4CP was observed after 5 years of exposure of a sulfate reducing consortia from estuarine sediments [8].

4. Conclusions

Chlorophenols affected the sulfate respiratory activity of the biofilm, inhibiting sulfate reduction and ethanol oxidation. The phenolic compounds were not degraded by the non-acclimated culture immobilized with ethanol, not even at very small concentrations (5 ppm of 2,4D), although an alternative electrons source was added. Phenol low inhibitory effect on sulfate respiration also indicated that chlorine is the source of toxicity to sulfate reduction and that the number of chlorine atoms and structural complexity of the chlorophenolic compounds increased the inhibition levels.

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