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Use of the microorganism *Bacillus stearothermophilus* as a model to evaluate toxicity of the lipophilic environmental pollutant endosulfan

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Abstract

Microorganisms are very powerful tools for the supply of information about the toxic effects of lipophilic compounds, since an impairment of cell growth usually occurs as a result of perturbations related, in most cases, with the partition of toxicants in membranes. The thermophilic eubacterium *Bacillus stearothermophilus* has been used as a model system to identify α- and β-endosulfan interactions with the membrane possibly related with the insecticide toxicity. Two approaches have been pursued: (a) bacterial growth is followed and the effects of endosulfan isomers determined; (b) biophysical studies with the fluorescent fluidity probe 1,6-diphenyl-1,3,5-hexatriene (DPH) were performed to assess the effects of α- and β-endosulfan on the organization of the membrane lipid bilayer. The effects on growth were quantitatively evaluated by determination of growth parameters, namely the lag phase, the specific growth rate and the cell density reached by cultures in the stationary phase. Growth inhibition by α and β-endosulfan dependent on the concentration is diminished or removed by the addition of 2.5 mM Ca^{2+} to bacterial cultures. Fluorescence DPH polarization consistently showed opposite effects of Ca^{2+} and α- and β-endosulfan on the physical state of bacterial polar lipid dispersions.

Keywords: α-Endosulfan; β-Endosulfan; Fluorescence polarization; *Bacillus stearothermophilus*; Bacterial growth; Lipid membrane

1. Introduction

The environmental pollutant effects of lipophilic compounds on microorganisms have drawn considerable attention in the field of ecotoxicology. The interest in microorganisms has also been stimulated by their application in biotransformations and environmental biotechnology. Additionally, microorganisms have been used in the laboratory for the toxicological characterization of pollutants, e.g. polycyclic aromatic hydrocarbons, industrial solvents and agrochemicals (Sikkema et al., 1995). Inhibition of growth (Calder and Lader, 1976; Luxo et al., 1996; Donato et al., 1997a), effects on respiration and other vital functions (Sikkema et al., 1995; Donato et al., 1997b) have been used to assess the toxicity in vitro of several environmental pollutants. Data on the toxic effects of these xenobiotics, from the molecular to whole organism level, together with a knowledge of the physicochemical properties of the compounds, are useful for the establishment of structure–activity relationships.

The widespread and indiscriminate use of organochlorine insecticides to fight plagues resulted in accumulation in the biosphere and the troposphere and to spreading to areas at long distances away from the place of application, thus contaminating the global ecosystem as persistent environmental pollutants. Endosulfan, a mixture of α- and β-isomers, is a broad-spectrum insecticide, acaricide and miticide used to control a variety of pests that affect fruit, vegetables and ornamental plants (Berrill et al., 1998). This formulation with two isomers with different water solubilities and volatilities (Antonious et al., 1998) has been studied for its toxicity to aquatic organisms possibly affected by direct applications or drift from aerial spraying (Ernst et al., 1991; Berrill et al., 1998). Accumulated data concerning the toxicity hazard of endosulfan led to the recommendation

Abbreviations: DDE, 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene; DDT, 2,2-bis(*p*- chlorophenyl)-1,1,1-trichloroethane; DPH, 1,6-diphenyl-1,3,5-hexatriene

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that it should be replaced by less toxic alternatives wherever possible (Berril et al., 1998).

The knowledge of the interactions of these compounds with biosystems is obviously important for the design and synthesis of more efficient and selective compounds. Owing to the high lipophilicity (Antonious et al., 1998), a strong interaction with biomembranes is predictable. It was shown that the endosulfan isomers have significant effects on membrane organization and permeability (Videira et al., 1999, 2002). Prokaryotic cells, mainly Gram-positive eubacteria, provided with a simple membrane system, are particularly useful as models of drug toxicity at membrane level. Previous studies have shown that bacteria of the genus *Bacillus* are sensitive to the toxicity of the organochlorine insecticide DDT and its metabolite DDE (Donato et al., 1997a,b, 2000). Growth, respiratory and membrane physical data collected with *Bacillus stearothermophilus* was in agreement with studies carried out in other model systems, revealing that the metabolite DDE is less toxic (Matsumuraand Narahashi, 1971; Arhem and Frankenhaeuser, 1974; Khan and Cutkomp, 1982). Similar studies have been carried out with α and β isomers of endosulfan to establish and further develop a bacterial model as a simple methodology for screening tests for chemical toxicity. It was shown that the growth of *B. stearothermophilus*, directly dependent on the physical state of membrane lipids, was affected by the addition of α and β-endosulfan to the growth medium. In parallel, the effects were studied on the physical state of lipid bilayers reconstituted with the bacterial polar lipid fraction, amounting to about 90% of the total lipids (Jurado et al., 1991). β-Endosulfan effects were also identified in the presence of Ca^{2+}, owing to its action as membrane stabilizer (Mosley et al., 1976; Livingstone and Schachter, 1980; Jurado et al., 1991).

2. Materials and methods

2.1. Chemicals

The isomers α- and β-endosulfan [C,C′-(1,4,5,6,7,7-hexachloro-8,9,10-trinorborn-5-en-2,3-ylene (dimethyl sulphite)] were obtained from Supelco (Bellefonte, PA, USA). The probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was obtained from Sigma Chemical co.

2.2. Cultures

The strain of *B. stearothermophilus* and the conditions for its maintenance and growth have been described previously (Jurado et al., 1987). α and β-endosulfan from concentrated ethanolic solutions, were added to the growth medium (diluted L-Broth) either supplemented or not with 2.5 mM Ca^{2+}, in order to obtain concentrations from 2.5 to 15 μM. Growth was measured by turbidimetry at 610 nm in a Bausch & Lomb Spectronic 21 spectrophotometer.

2.3. Lipid analysis

Cells grown up to the beginning of the stationary phase were harvested by low-speed centrifugation and washed three times with buffer (10 mM Tris–Cl, pH 7.0). The lipids were extracted by the Bligh and Dyer method (1959) and quantified by measuring the amount of inorganic phosphate (Bartlett, 1959) after hydrolysis of the extracts at 180 °C in 70% $HClO_4$ (Bottcher et al., 1961). The polar lipids were isolated by preparative thin layer chromatography, as previously described (Jurado et al., 1991).

2.4. Liposomes

Aliquots from lipid solutions in $CHCl_3$ (polar lipid extract) containing 2.325 mg of lipid were evaporated to dryness on a rotary evaporator. The dry residues were hydrated under N_2 atmosphere at 55 °C by gentle shaking with 5 ml of 50 mM KCl and 10 mM Tris–maleate (pH 7.0) and multilamellar vesicles were obtained. Then, the suspensions were vortexed for 1 min to disperse aggregates.

2.5. Fluorimetric measurements

The fluidity probe 1,6-diphenyl-1,3,5-hexatriene (DPH) in dimethylformamide was injected (few μl) into liposomes suspensions (600 μM in phospholipid), as previously described (Antunes-Madeira et al., 1994) to give a lipid/probe molar ratio of 200. The mixture was vigorously vortexed for 10 s, and then endosulfan was added from concentrated ethanolic solutions. The mixture was incubated at 55 °C in the dark, for a period of 18–20 h to reach equilibrium. Control samples received equivalent volumes of dimethylformamide and ethanol. Added solvent volumes (few μl) had negligible effects on measurements.

The fluorimetric measurements were performed with a Perkin-Elmer spectrofluorimeter, model MPF-3, with a thermostated cell holder. The excitation was set at 336 nm and the emission at 450 nm (5 nm excitation and 6 nm band pass).

All fluorescence measurements were corrected for the contribution of light scattering by using appropriate blanks without added probes. The degree of fluorescence polarization (P) was calculated according Shinitzky and Barenholz (1978) from the equation:

$$P = \frac{I_{\parallel} - G.I_{\perp}}{I_{\parallel} + G.I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the light emitted with its polarization plane parallel (‖) and perpendicular (⊥) to that of exciting beam. G is the correction factor for instrument polarization, given by the ratio of the vertically to the horizontally polarized emission components when the excitation light is polarized in the horizontal plane.

3. Results

3.1. Effect of α- and β-endosulfan on the growth of *B. stearothermophilus*

The strain of *B. stearothermophilus* was grown at 65 °C (in the optimal temperature range) in a complex medium (diluted L-Broth) with an endogenous concentration of 0.115 μM Ca^{2+} (Jurado et al., 1987). To this basal medium, α- and β-endosulfan from concentrated ethanolic solutions were added to obtain concentrations ranging from 2.5 to 15 μM; control cultures were grown in a medium without insecticides but with 0.0375% (v/v) ethanol (i.e. the maximum amount of solvent used). Growth in media with increasing concentrations of β-endosulfan (Fig. 1A) was characterized by progressively lower bacterial yields (Table 1), although no significant effects were detected on the specific growth rate. Increasing concentrations of α-endosulfan (Fig. 1B) also inhibit growth, inducing the beginning of the stationary phase at progressively lower cell densities, although final cell densities reached by cultures with α-endosulfan were closer to the control culture, as compared with cultures grown with β-endosulfan (Table 1). The perturbation of growth in the dependence of α-endosulfan concentration also affected the lag phase (Table 1). The length of the lag period depends on several factors, e.g. the age of the inoculum. The variability of this growth parameter in different growth experiments is therefore considerable. However, since the same inoculum was used, the comparison of lag times in different growth conditions may provide information about cell growth behaviour.

The inhibition of bacterial growth by β-endosulfan depends on the presence or the absence of Ca^{2+}. The addition of 2.5 mM of Ca^{2+} to the basal medium had no appreciable effect on bacterial growth at 65 °C (data not shown; Jurado et al., 1987), but counteracted the β-endosulfan effect on cell density (Fig. 2). The extension of growth in the Ca^{2+}-supplemented medium containing 10 μM β-endosulfan was similar to that of the control culture, the lag time being significantly reduced (Table 1).

3.2. Effect of α and β-endosulfan on the physical state of membrane polar lipids of *B. stearothermophilus*

The effects of α- and β-endosulfan on the physical properties of membrane polar lipids of *B. stearothermophilus* were studied by DPH fluorescence polarization. DPH is a fluidity probe localized within the hydrophobic core of the membrane (Shinitzky and Barenholz, 1978) and has been widely used to monitor membrane organization. This technique, which is very sensitive and reproducible, provides a physical parameter (*P*) that can be readily interpreted in terms of fluidity in lipid membrane systems (Shinitzky and Barenholz, 1978). *P* values are mainly dependent on the restriction of the fluorophore probe rotation influenced

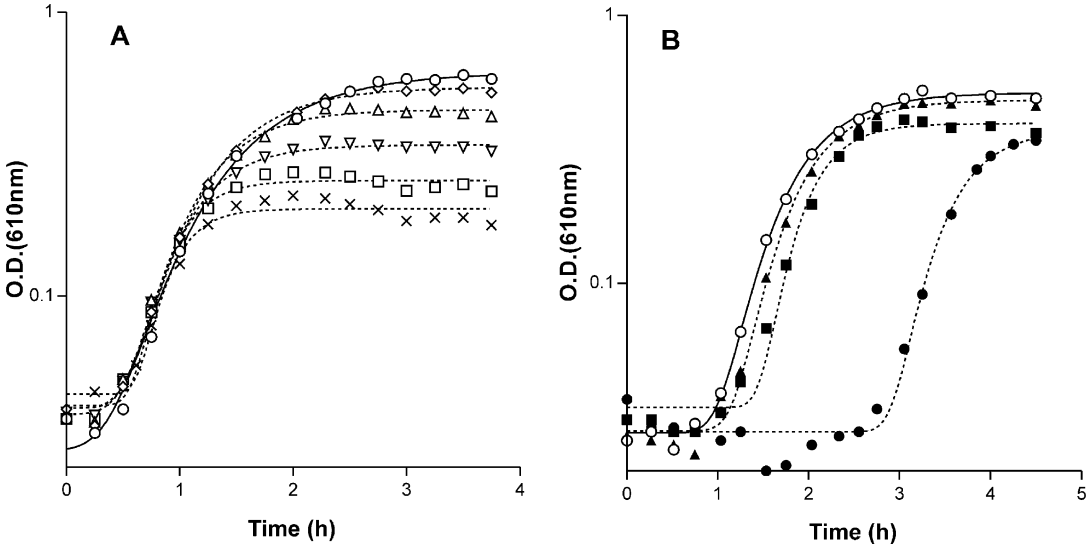

Fig. 1. Effects of β-endosulfan (A) and α-endosulfan (B) on the growth of cultures of *B. stearothermophilus*, at 65 °C. (A) Cells were grown in a basal medium (dilute L-Broth) without (○) and with 2.5 μM (◇), 5.0 μM (△), 7.5 μM (▽), 10 μM (□) and 15 μM (x) β-endosulfan. (B) Cells were grown in the basal medium without (○) and with 5.0 μM (▲), 10 μM (■) and 15 μM (●) α-endosulfan. The results are typical of two independent experiments.

Table 1
Lag times and cell densities (A) in the beginning of the stationary phase and 2 h after (final cell densities) of cultures of *B. stearothermophilus* grown in media with different concentrations of α or β-endosulfan, supplemented or not with 2.5 mM Ca^{2+} (cell densities are expressed as % of control cultures)

Additives to the growth medium	Lag time (h)	Cell density in the beginning of stationary phase (% of control)[a]	Final cell density (% of control)[b]
none	0.663	100	100
2.5 μM β-endosulfan	0.683	94.4	91.4
5.0 μM β-endosulfan	0.652	80.8	76.9
7.5 μM β-endosulfan	0.605	69.9	58.1
10 μM β-endosulfan	0.622	60.4	43.4
15 μM β-endosulfan	0.647	53.8	34.5
10 μM β-endosulfan + 2.5 mM Ca^{2+}	0.354	111.5	106.8
none	1.217	100	100
5.0 μM α-endosulfan	1.352	82.0	94.2
10μM α-endosulfan	1.567	72.8	78.0
15 μM α-endosulfan	3.054	63.3	n.d.

n.d., not determined.
[a] Cell densities were determined at the point of intersection between the tangent to the beginning of stationary phase and the straight line representing the exponential phase. as indicated in Fig. 2 for one of the growth curves.
[b] Cell densities were determined using the best-fitted curve to the experimental data.

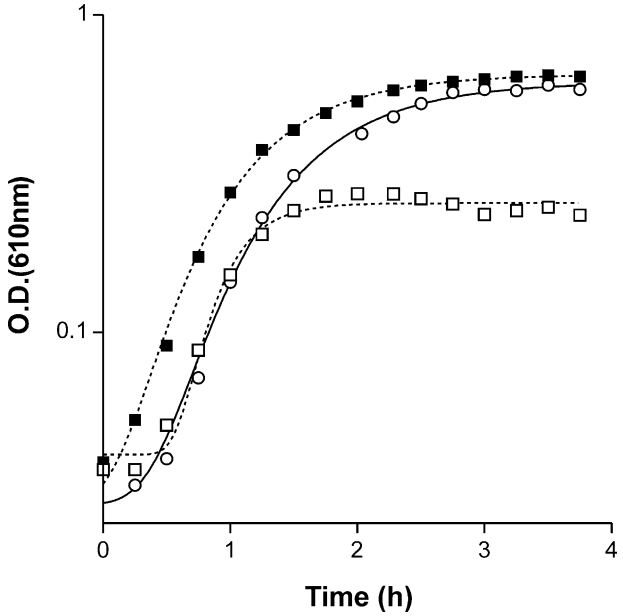

Fig. 2. Effect of β-endosulfan and Ca^{2+} on growth of *B. stearothermophilus*, at 65 °C. Cells were grown in the basal medium without (○) or with 10 μM β-endosulfan (□) and in a Ca^{2+}-supplemented medium with 10 μM β-endosulfan (■).

by the degree of molecular packing of the lipids. Thus, the term fluidity is used here in an operational sense and defined as being directly proportional to the reciprocal of fluorescence polarization.

Fig. 3 shows the thermograms of liposomes prepared with the polar lipid fraction of the membrane of *B. stearothermophilus* grown at 65 °C, in the basal medium. Because of its heterogeneous composition, the liposomes exhibit a broad transition phase with an amplitude of about 20 °C, as detected by DPH fluorescence polarization (Fig. 3) in accordance with other physical techniques (Jurado et al., 1991).

The addition of β- or α-endosulfan to liposomes (concentrations of 50 and 100 μM) induced a decrease in the polarization values in the gel phase and along the range of the thermotropic transition phase. This concentration-dependent effect reflects a decrease in molecular packing promoted by the perturbation of the lipid bilayer order induced by the compounds. However, disordering effects of β-endosulfan are absent in the fluid phase (temperatures above the transition) whereas the perturbation induced by α-endosulfan was noticed along all the temperature range, i.e. from 10 to 50 °C.

4. Discussion

This work is framed in a large project aiming to the development of efficient in vitro toxicity assessment methodologies as an alternative to animal use, attempting to validate the use of microorganisms to evaluate the toxicity of lipophilic compounds. In biochemical research, microorganisms have been used as efficient tools to study the action mechanisms of several chemical compounds, permitting to relate effects at the molecular level with physiological events. A strain of *B. stearothermophilus* has been used as a suitable model to study the membrane-mediated effects of drugs, e.g. citostatics (Luxo et al., 1996, 1998, 2000), antiarrythmics (Rosa et al., 2000a,b) and pesticides (Donato et al., 1997 a,b, 2000). The following studies have been implemented: (a) studies in vivo of drugs effects on cell growth, viability and respiratory activity of protoplasts; (b) studies in vitro with isolated membranes and bacterial lipid dispersions, evaluating effects on the physical properties of

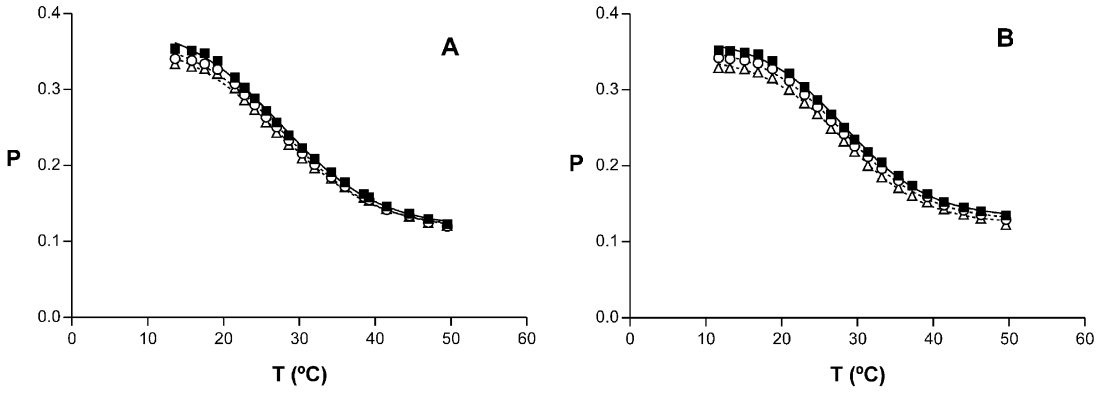

Fig. 3. Thermograms of fluorescence polarization (P) of DPH in liposomes prepared with the polar lipids of cells grown in the basal medium, at 65 °C. (A) Liposomes were incubated without (■) or with 50 (○) and 100 μM (△) β-endosulfan. (B) Liposomes were incubated without (■) or with 50 (○) and 100 μM (△) α-endosulfan. The thermotropic profiles are typical assays of three independent experiments. Polarization values derived from different readings of fluorescence intensities, for the same assay, provided means with error bars (S.D.) that, for most points, are encompassed by the size of the symbols.

the lipid bilayer and on membrane-associated functions, namely enzyme activities and transport systems. The strain of *B. stearothermophilus* was chosen because its growth is sensitive to the physical state of membrane lipids (Jurado et al., 1991). Thus, previous studies suggested a possible relationship between Ca^{2+}–lipid interactions affecting membrane physical properties and the improvement of growth induced by divalent cations at supraoptimal temperatures (Jurado et al., 1991). Therefore, this strain is potentially useful for the elucidation of possible relationships between drug-induced changes in membrane organization and effects on growth, promising a significant biotechnological interest for the screening of chemical toxicity of membrane-active compounds. In addition to the general advantages of bacterial cells (i.e. rapidly and economically obtained cell cultures with high cell densities; fast isolated homogeneous membrane preparations; the possibility of membrane chemical composition to be manipulated by modifying the growth conditions; enzyme dynamics, membrane permeability and membrane physical changes easily monitored in intact cells and isolated membranes), *B. stearothermophilus* grows at high temperatures, optimally at 65 °C, preventing contamination, and it is provided with efficient molecular mechanisms of adaptation to adverse growth conditions, e.g. changing its membrane lipid composition.

A significant perturbation of growth was induced by α and β isomers of endosulfan (Fig. 1). However, a more detailed analysis of the growth parameters, namely the bacterial yields (final cell densities reached by cultures in the stationary phase), shows differential effects of α- and β-endosulfan. Apparently, the perturbations caused by α-endosulfan accumulation in the membrane are more successfully overcome by the bacterium than those of β-endosulfan. Thus, using the same inoculum, growth of cells in a medium containing 15 μM β-endosulfan showed a shorter lag time but a lower final cell density when compared with the growth of cells in a medium with the same concentration of α-endosulfan. Bacterium adaptation to the presence of α- and β-endosulfan, by alteration of its membrane lipid composition, is a matter currently under study.

In accordance with previous studies with DDT and DDE (Donato et al., 1997a, 2000), we observed an antagonistic effect of 2.5 mM Ca^{2+} on the growth inhibition induced by β-endosulfan, reflected in a higher bacterial yield and a shorter lag phase. Since calcium ions have direct effects on the physical state of lipids (Jurado et al., 1991), we attempted to correlate insecticide growth effects with a physical perturbation of bilayer lipids. Therefore, the effect of α- and ß-endosulfan on the thermotropic transition of bilayers composed of the bacterial polar lipids, including several phospholipid classes (phosphatidylethanolamine, phosphatidylglycerol, cardiolipin and a phosphoglycolipid; Jurado et al., 1991), has been studied. As illustrated in Fig. 3, the main perturbation of α- and β-endosulfan is the disordering of the bacterial lipids, which is reflected by a decrease of DPH fluorescence polarization, in accordance with studies carried out with synthetic lipids (Videira et al., 1999, 2002). This physical perturbation of membrane lipids may well be related to the Ca^{2+}/β-endosulfan antagonistic effects on bacterial growth, since divalent cations can effectively counteract the structural disordering induced by other membrane-active drugs, e.g. DDT (Donato et al., 1997a) and tamoxifen (Luxo et al., 2000). On the other hand, previous studies have shown that xenobiotic-induced growth inhibition is potentiated by changing the growth temperature from the optimal to the supraoptimal temperature range (Luxo et al., 1996) and that the Ca^{2+} protective effect in xenobiotic-stressed cultures is impaired or attenuated at supraoptimal temperatures (Luxo et al., 2000). These observations are in agreement with the hypothesis of a strong dependence between

growth impairment and membrane effects, since the xenobiotics tested and high temperatures have additive effects whereas Ca^{2+} and high temperatures have opposite effects on the lipid bilayer organization. A relationship between xenobiotic-induced perturbation of membrane bilayer and growth inhibition may also be predicted on the basis of lipid composition changes induced by xenobiotics, which is a common strategy for the adaptation to stress in bacteria, namely in *B. stearothermophilus*. It has been shown that this bacterium changes its membrane lipid composition to counteract the lipid bilayer destabilizing effects of different lipophilic compounds (Luxo et al., 1998; Donato et al., 2000; Rosa et al., 2000b). Similar studies will be carried out with α- and β-endosulfan.

Different effects of α- and β-endosulfan were detected in the fluid phase of bacterial polar lipids (temperatures above T_m). To understand the significance of these different effects, the relative localization of the endosulfan isomers along the bilayer thickness has to be determined. This requires the use of other fluidity probes, e.g. the propionic acid derivative of DPH, which places in the outer region of the membrane because of its charged propionic group that anchors it to the bilayer surface (Trotter and Storch, 1989). On the other hand, additional significant data would be obtained if membrane order parameters could be directly measured in native membranes or intact cells. Fluorescence studies could not be carried out with cell membranes, since native fluorophores are present and fluorescence quenching of probes is very significant. Calorimetric experiments are scheduled and can be carried out with the total lipid extracts and, possibly, with membranes and even cells.

In conclusion, the use of a bacterial model enabled alterations of the membrane physical properties promoted by xenobiotics to be related to cell growth impairment, used as an index of citotoxicity.

Furthermore, data show that endosulfan toxic effects depend to a very large extent on the incubation conditions, e.g. presence of divalent cations, which suggests that environmental conditions are important in the development of bioassays to evaluate membrane interactions of contaminants in microorganisms. This is particularly significant in the use of microorganisms for technological purposes, where the environmental conditions should be improved in order to improve the resistance of cells to the toxic actions of xenobiotics.

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