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Inhibition of selected bacterial growth by three hydrocarbons: Mathematical evaluation of toxicity using a toxicodynamic equation

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HIGHLIGHTS

- Toxicity of three hydrocarbons was evaluated by bacterial growth assays.
- The toxicodynamic equation modeled perfectly the experimental data.
- Maximum growth and maximum growth rate were the main parameters inhibited.
- Aniline and naphthalene were respectively the least and most toxic hydrocarbons.

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ABSTRACT

The individual toxicity of different hydrocarbons (naphthalene, cyclododecane and aniline) on the growth of selected bacteria (*Pseudomonas* sp., *Phaeobacter* sp. and *Leuconostoc mesenteroides*) was studied by means of a toxicodynamic model combination of two sigmoid equations (logistic and Weibull). All the toxicological effects on growth parameters and kinetic properties were characterized and the global toxicity of such chemicals was evaluated. It was observed that two kinetic parameters (maximum growth and maximum growth rate) were in almost all cases influenced by the hydrocarbons studied. Aniline was less toxic than cyclododecane and naphthalene. The presented approach is a reasonable starting point for understanding and modeling complete and real assessment of chemical toxic effects on bacterial growths. The values of $EC_{50,\tau}$ could be used for a most efficient comparison of the individual toxicity of chemicals.

1. Introduction

Hydrocarbons are organic compounds formed by hydrogen and carbon and divided in different families depending on chemical structure that, in general, present high toxicity, are very persistent, exist widespread in the environment and are accumulated in living organisms. During their extensive production and utilization since XIX century, they have been the cause of large pollution of groundwater, soil, aquifers and marine coasts as well as effects on human health (*e.g.*, polycyclic aromatic hydrocarbons are a significant group of chemical carcinogens). Among hydrocarbons, we have selected three compounds from the different hydrocarbon groups: aniline, cyclododecane and naphthalene due to their high number of industrial and commercial applications and the contamination generated by their manipulation and disposal.

Aniline is widely used as precursor in the production of different materials (rubbers, dyes, herbicides, etc.) and is commonly present in effluents of many industries producing serious environmental problems. Most of the reports about aniline investigate the ability of certain microorganism to degrade it by metabolic pathways; but, it is still unresolved (Konopka et al., 1989; Urata et al., 2004; Tang et al., 2010). Naphthalene is historically employed as a household and pesticide fumigant due to its high toxicity against insects besides presenting antibacterial and antifungi properties. This characteristic has demonstrated to be also harmful for other organisms including humans and therefore substituted in mothballs by other benzene and naphthonitrile derivatives (NTP, 2000; Fadda et al., 2013). Its biodegradability under anaerobic and aerobic conditions has been the subject of numerous works (Haritash and Kaushik, 2009). However, the studies of bacterial growth inhibition has been commonly focussed on naphthalene-degrading bacteria as *Pseudomonas putida* (Pumphrey and Madsen, 2007) or genotoxicity capacity of naphthalene (Mortelmans et al., 1986) but scarcely for obtaining environmental risk assessment

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data using naphthalene-non degrading bacteria. Cyclododecane is very persistent in the environment and not easily biodegradable. Due to lipophilic features, cyclododecane is adsorbed on the surface of soil particles and is readily bioaccumulated in the fatty tissues of living organisms. The toxicity values (LC_{50}, EC_{50}, NOEC) for algae, fish and aquatic invertebrates, obtained by dose–response methods, are well-established (ECHA, 2008) but for bacteria are still unexplored.

In general, the use of biological indicators is the main tool for hazard evaluation of chemicals (Eisentraeger et al., 2008; Murado et al., 2011; Khan et al., 2012). Specifically, the application of bacteria from different origins to assess ecotoxicological effects of toxic compounds and environmental pollutants, has developed an increasing interest in recent years (Sütterlin et al., 2008; Rial et al., 2011; Song and Bielefeldt, 2012; Su et al., 2012). The works concerned with determining the effects of hydrocarbons in bacteria are commonly executed using dose–response analysis at fixed times of bacterial growth without taking into account all the phases of growth. In most cases, the difference among authors in the toxicity of chemicals for similar bioassays are mainly due to the fact that the values of EC_{50} are fully dependent on the growth phase in which they are defined (Mezcua et al., 2002; Vosahlikova et al., 2006; Bartos et al., 2008; Murado and Vázquez, 2010). The joint evaluation of toxic influence on the growth of microorganism by bivariate sigmoid equations is a resource that has showed excellent results of modeling and characterization of kinetic effects with different chemicals and target microorganisms (Kooijman et al., 1983; Rial et al., 2011; Vázquez et al., 2011). It is a very valuable mathematical tool for the real comparison of compounds toxicity.

The objective of the present work is to determine experimentally the inhibitory effects and the actual toxicity of the three hydrocarbons (aniline, cyclododecane and naphthalene) on the growth of three bacteria (*Pseudomonas* sp., *Phaeobacter* sp. and *Leuconostoc mesenteroides*) using a highly predictive toxicodynamic equation.

2. Materials and methods

2.1. Microbiological and analytical methods

Phaeobacter sp. was kindly provided by Dr. Lone Gram (DTU Aqua, Denmark), *L. mesenteroides* was supplied by Dr. B. Ray (University of Wyoming, Laramie, USA) and *Pseudomonas* sp. (CECT 4355) was purchased to Colección Española de Cultivos Tipo (CECT, Universidad de Valencia, Spain). Stock cultures of bacteria were kept at −80 °C in commercial marine (*Phaeobacter* sp. and *Pseudomonas* sp.) and MRS medium (Mann Rogosa Sharpe broth) for *L. mesenteroides* with 25% glycerol (Cabo et al., 2001; Vázquez et al., 2004). Marine broth was from Difco (Becton, Dickinson and Company, MD, USA) and MRS medium from Pronadisa (Hispanlab S.A., Spain), both prepared under commercial specifications. Inocula and cultures were prepared according to previously methods reported by Rial et al. (2011). Bacterial cultivations were performed with orbital shaking at 200 rpm and 22 °C (*Phaeobacter* sp.), 27 °C (*Pseudomonas* sp.) and 30 °C (*L. mesenteroides*).

After predetermined incubation times, samples from each culture condition were centrifuged at 4000*g* for 15 min. Sediments (bacterial cells) were washed and resuspended in distilled water to the appropriate dilution for measuring the bacterial growth by optical density at 700 nm. In the case of *L. mesenteroides* cultivations, the corresponding supernatants were used for the determination of glucose, lactic acid, acetic acid and ethanol by HPLC analysis (refractive-index detector), using an ION-300 column (Interaction Chromatography, USA) with 6 mM sulphuric acid as a mobile phase (flow = 0.4 mL min^{-1}), at 65 °C.

2.2. Hydrocarbons preparation

Cyclododecane and naphthalene were diluted in acetone and 20 μL of each dilution was added to each 30 mL culture tube with 10 mL of corresponding culture medium to obtain the concentrations selected. Previously, the effect of that acetone concentration on bacterial growth was studied and inhibition of growth was not detected (data not shown). Ranges tested (mg L^{-1}) were 0–400 (naphthalene), 0–100 (cyclododecane) and 0–25 g L^{-1} (aniline). Concentrations assayed of naphthalene and cyclododecane were higher than their solubility limits in water: 30 mg L^{-1} and 10 mg L^{-1} for naphthalene and cyclododecane, respectively. Two factors involve the formation of hydrocarbon dispersions above the solubility limit: orbital shaking and the presence of surfactants (1 g L^{-1} of Tween® 80 in MRS medium). Therefore, the toxicity assessment implies higher concentrations than expected on a real environment. All chemicals were purchased to Sigma–Aldrich Inc. (St. Louis, USA).

2.3. Modeling of bacterial growth inhibition

The mathematical description of the hydrocarbons toxicity was done by means of a bivariate equation based on the combination of Weibull function as hydrocarbon-concentration model modifying the most important parameters of the reparametrized logistic equation used for bacterial growth description (Rial et al., 2011; Vázquez et al., 2011):

$$X = \frac{X_{m*}}{1 + \exp[2 + \frac{4\upsilon_{m*}}{X_{m*}}(\lambda_* - t)]}; \quad \text{where :} \tag{1}$$

$$X_{m*} = X_m\{1 - K_x[1 - \exp(-\ln 2(C/m_x)^{a_x})]\}$$

$$\upsilon_{m*} = \upsilon_m\{1 - K_\upsilon[1 - \exp(-\ln 2(C/m_\upsilon)^{a_\upsilon})]\}$$

$$\lambda_* = \lambda\{1 + K_\lambda[1 - \exp(-\ln 2(C/m_\lambda)^{a_\lambda})]\}$$

where, υ_m is the maximum growth rate, X_m is the maximum growth, λ is the lag phase and C is the hydrocarbon concentration. The meanings of other symbolic notations as well as the corresponding units are summarized in Table 1. A global parameter ($EC_{50,\tau}$) was also calculated for the overall description of hydrocarbon effects on bacterial growth studies according the procedure described in Appendix A (Rial et al., 2011).

2.4. Numerical methods and statistical analysis

The fitting procedures and parametric estimates from the experimental results were performed by minimizing the sum of quadratic differences between the observed and model-predicted values using the nonlinear least-squares (quasi-Newton) method provided by the '*Solver*' macro from Microsoft Excel spreadsheet. The confidence intervals of the best-fit values for the parametric estimates (Student's t test, $\alpha = 0.05$), consistency of the mathematical models (Fisher's F test; $p < 0.05$) and covariance and correlation matrices were calculated using the '*SolverAid*' macro available from Levie's Excellaneous website http://www.bowdoin.edu/~rdelevie/excellaneous/.

3. Results and discussion

The microorganisms were mainly selected due to its different cell wall structure (Gram-positive and Gram-negative) and habitat (marine and terrestrial). *L. mesenteroides* are heterofermentative