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Study on the toxicity of binary equitoxic mixtures of metals using the luminescent bacteria *Vibrio fischeri* as a biological target

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Abstract

Results from two mathematical approaches to predict the toxicity of all the possible binary equitoxic mixtures of Co, Cd, Cu, Zn and Pb were compared to the observed toxicity of these mixtures to *Vibrio fischeri* bacteria. Combined effect of the metals was found to be antagonistic for Co–Cd, Cd–Zn, Cd–Pb and Cu–Pb, synergistic for Co–Cu and Zn–Pb and merely additive in other cases, revealing a complex pattern of possible interactions. Besides, Cd appears much less toxic to the bacterial model than to animal cells. The synergistic effect of the Co–Cu combination and the strong lowering of Pb toxicity in the presence of Cd deserve much attention when establishing environmental safety regulations.

Keywords: Microtox®; EC50; Metal interaction models; Equitoxic mixtures; Antagonism; Synergism

1. Introduction

Environmental pollution most often results from the combination of several factors. In the case of water pollution this combination can include changes in pH, temperature, oxygen concentration and concomitant increase in concentration of one or more chemicals. In cases of contamination from metal mining, smelting and manufacturing processes, the adverse effect will be due to mixtures of pollutants. Different mathematical algorithms have been proposed to predict the toxicity of a mixture of several contaminants. Investigating the toxicity on guppies of phenol and different chlorophenols in equitoxic mixtures, Koneman (1981) predicted the resulting toxicity by considering that joint effects were either merely similar or totally independent. Merely similar effect means that each chemical can be replaced by an equitoxic concentration of another chemical without changing the response, implying that they act via similar mechanisms. Such case was called concentration-addition by Anderson and Weber (1975). Independent effect means that chemicals are not interchangeable as they have different mechanism of toxic action. When studying the toxicity of different mixtures of phenol and chlorinated phenols with the Microtox® test, which uses the luminescent bacteria *Vibrio fischeri* as test organism, Ribó and Rogers (1990) proposed a mathematical model which assumes that the mechanism

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of toxic action of all compounds tested is similar. In general, they found a good correlation between the experimental and the predicted data. However, the compounds tested in their study had similar chemical structures and, thus, the mechanism of toxic actions could be supposed to be similar. The Ribó and Rogers's mathematical model was used by Fernandez-Alba et al. (2001) when studying the toxicity of pesticides mixtures on *Daphnia magna* with the BioTox™ and MitoScan™ tests. In this case, however, the predicted toxicity failed to match the experimental data. Thomulka and Lange (1996, 1997) found evidence of independent action of tin, dibutyl- and tributyl-tin bi- nary mixtures by looking at the shape of isobolic diagrams when using the additive index method, identical to the Ribó and Rogers's one, to predict mixtures toxicity on the marine bacteria *Vibrio harveyi*. Some studies concerning metals-containing mixtures are found in the bibliography. Studying the toxicity of sublethal mixtures of metals and organic chemicals on fathead minnow (*Pimephales promelas*), Parrot and Sprague (1993) were able to predict the joint interaction of toxicants from isoboles graphs. Ince et al. (1999) assessed the type of interaction of metals mixtures at different concentration levels of Zn(II), Cd(II), Co(II) and Cr(VI) using either the Microtox® test or the duckweed (*Lemna minor*) model, by a statistical approach based on the null hypothesis of "additive toxicity" at a 95% confidence level. Mowat and Bundy (2002) developed a different mathematical algorithm to calculate the combined toxicity of different metal mixtures using the Microtox® test.

Basically, the use of different mathematical models reported in the bibliography leads to three possible types of interactions between two toxicants: the effects can be described as antagonistic, synergistic or simply additive. The extent of deviation from a simple additive effect generally depends on (1) the measured parameter, (2) the chemical nature of toxicants and (3) the relative contribution of each toxicant to the toxicity of the mixture.

The aim of the present work is to assess the adverse effect of either individual or equitoxic binary combinations of metals, using the Microtox® test. Equitoxic mixtures are solutions in which each metal of the mixture is present at the concentration which would exert the same toxic effect (in this study, a 50% light inhibition) if given separately. Hence, a lowering or an increase of the resulting measured toxicity of the mixture as compared to the expected one would reveal either an antagonistic or a synergistic effect, respectively. Not significant (at 95% confidence level) deviation of the observed actual toxicity of the mixture from the expected one would indicate that the effect is merely additive.

In the present investigation, EC50 values of single metals and binary mixtures of Co(II), Cd(II), Zn(II), Cu(II) and Pb(II) were evaluated using two different predictive mathematical models, namely, the one proposed by Ribó and Rogers (1990) and the one proposed by Ince et al. (1999), respectively.

2. Materials and methods

2.1. Test reagents and chemicals

The freeze-dried luminescent bacteria, *V. fischeri* (NRRL B-11177), and the reconstitution solution were supplied by Azur Environmental (Carlsbad, CA, USA). Metal standard solutions for flame atomic absorption (FAA) calibration of Cd(II), Zn(II), Cu(II) and Pb(II) ($1000\,mg\,l^{-1}$ in 0.5 M HNO_3) were purchased from Panreac (Barcelona, Spain). Cobalt chloride $CoCl_2 \cdot 6H_2O$, NaOH, HCl and NaCl purchased from Merck (Darmstadt, Germany) were analytical grade. All solutions were prepared with ultra-pure water from a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Apparatus

The tests were performed using the Microtox® Model 500 Toxicity Analyzer from Microbics Corporation (Carlsbad, CA, USA). The analyzer was equipped with a 30-well temperature-controlled incubator chamber at 15 °C. A small compartment held at 5 °C was used to store the bacteria before dilution. The light output was recorded from a digital display. The pH was determined using a Crison Digilab 517 pH-meter (Crison, Barcelona, Spain).

2.3. Samples preparation

Metal solutions were prepared by diluting the FAA standard metal solutions or dissolving the corresponding salt in ultra-pure water. Solutions were then adjusted to pH 6.0 by addition of either 0.1 N HCl or NaOH solutions. Before toxicity tests, the pH was checked and adjusted if necessary. Diluent solution (2% NaCl), used in Microtox® basic test protocol, was also adjusted to pH 6.0 to ensure pH stability during the assays as previously explained (Fulladosa et al., 2004).

2.4. Effective concentration (EC50) determination

EC50 values, defined as the concentration which provokes a 50% light reduction on *V. fischeri*, were obtained by following the Microtox® basic test protocol (Villaescusa et al., 1997). Practically, the EC50 values were calculated by regression analysis of the linear relationship between the logarithm of the metal concentration against the logarithm of the lost/remaining light intensity ratio.