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# Application of the combination index (CI)-isobologram equation to study the toxicological interactions of lipid regulators in two aquatic bioluminescent organisms

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ABSTRACT

Pharmaceuticals in the aquatic environment do not appear singly and usually occur as complex mixtures, whose combined effect may exhibit toxicity to the aquatic biota. We report an environmental application of the combination index (CI)-isobologram equation, a method widely used in pharmacology to study drug interactions, to determine the nature of toxicological interactions of three fibrates toward two aquatic bioluminescent organisms, *Vibrio fischeri* and the self-luminescent cyanobacterial recombinant strain *Anabaena* CPB4337. The combination index-isobologram equation method allows computerized quantitation of synergism, additive effect and antagonism. In the *Vibrio* test, the fibrate combinations showed antagonism at low effect levels that turned into an additive effect or synergism at higher effect levels; by contrast, in the *Anabaena* test, the fibrate combinations showed a strong synergism at the lowest effect levels and a very strong antagonism at high effect levels. We also evaluated the nature of the interactions of the three fibrates with a real wastewater sample in the cyanobacterial test. We propose that the combination index-isobologram equation method can serve as a useful tool in ecotoxicological assessment.


## 1. Introduction

Fibrates and statins (HMG-CoA reductase inhibitors) are the main lipid-lowering drugs prescribed either alone or in combination therapy in order to decrease plasma cholesterol levels and reduce the incidence of coronary heart disease. Although partially displaced by statins, the total number of fibrate prescriptions is in constant increase in the United States (Holoshitz et al., 2008). Fibric acids are the active forms of fibrates and belong to the nuclear receptor superfamily of ligand-activated transcription factors. Gemfibrozil and fenofibrate are the fibrates currently marketed in the US, whereas bezafibrate is also available in Europe and other developed countries (Lambropoulou et al., 2008). Fenofibric acid, 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid, is the active metabolite of fenofibrate, the inactive prodrug marketed and dispensed. Gemfibrozil, 5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid and bezafibrate, *p*-[4-[chlorobenzoylamino-

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ethyl]-phenoxy]-b-methylpropionic acid, are also fibric acid derivatives with similar pharmacokinetic behaviour (Miller and Spence, 1998).

The occurrence of lipid regulators in the discharge of treated urban and municipal wastewater has been relatively well documented. Bezafibrate has been detected in effluents of two British STP with averages up to 230 ng/L (Kasprzyk-Hordern et al., 2009). Metcalfe et al. (2003) found around 1 μg/L of gemfibrozil in effluents of Canadian STP, whereas fenofibrate has been reported in concentrations up to 0.5 μg/L in the influent of several Brazilian STP (Stumpf et al., 1999). Andreozzi et al. (2003) found lipid regulators in the effluent of several European STP at concentrations up to 4.76 μg/L (gemfibrozil), 1.07 μg/L (bezafibrate) and 0.16 μg/L (fenofibrate). Rosal et al. (2008), reported the occurrence of bezafibrate and gemfibrozil at levels of 139 and 608 ng/L respectively in the effluent of a Spanish STP. In the same plant Rodríguez et al. (2008) found 165 ng/L of fenofibric acid, 61 ng/L of bezafibrate and 143 ng/L of gemfibrozil.

It is also significant that removal efficiencies observed in current STP are not always high. Fent et al. (2006) reported maximum removal rates of 50–75% for fenofibric acid and gemfibrozil and somewhat higher for bezafibrate, although for the later, efficiencies below 15% have also been reported. Stumpf et al. (1999) reported a 45% removal of fenofibric acid by an activated sludge conventional treatment. Kasprzyk-Hordern et al. (2009) encountered an average degradation of bezafibrate not higher than 67%. On the other hand, Castiglioni et al. (2006) reported that the removal efficiency of bezafibrate during an activated sludge treatment greatly varied from 15% in winter to 87% in summer.

At measured environmental concentrations as those reported above (mostly in the ng/L and μg/L range), many studies have shown that the risk of acute toxicity is unlikely (Fent et al., 2006; Han et al., 2006; Borgmann et al., 2007); however, there is a lack of data on chronic toxicity effects. Moreover, pharmaceuticals in the aquatic environments occur as complex mixtures from different classes, not as single contaminants (Gros et al., 2007); thus, although the concentration of individual pharmaceuticals is low, their mixture could prove ecotoxicologically significant (Brain et al., 2004). Current methods of risk assessment usually focus on the assessment of single chemicals, which may underestimate the risk associated with toxic action of mixtures; probably for this reason, in the last years there is an increasing number of studies dealing with complex mixtures of pharmaceuticals (Cleuvers, 2003, 2004; Crane et al., 2006; Han et al., 2006; Borgmann et al., 2007; Christensen et al., 2007; Pomati et al., 2008; Quinn et al., 2009). However, assessment of combined toxicities is not an easy issue. Basically, two different models are in use for the prediction of mixture toxicity, i.e., concentration addition, when pharmaceuticals have a similar mode of toxic action, and response addition or independent action, when pharmaceuticals have different modes of toxic action (Cleuvers, 2003; Teuschler, 2007). However, toxicological interactions, synergisms or antagonisms, between the pharmaceuticals and their effects can occur independently of mode of action; moreover, in most cases, the pharmacological mechanisms of action is known but the toxic mode of action may remain unknown (Cleuvers, 2003; Chou, 2006). In an effort to overcome this limitation, we report an environmental application of a method widely used in pharmacology to interpret drug interactions; this method, termed as the median-effect/combination index (CI)-isobologram equation (Chou, 2006) allows quantitative determinations of chemical interactions where CI $<1$, $=1$ and $>1$ indicate synergism, additive effect and antagonism, respectively. One important property of the method is that previous knowledge of the mechanisms of action of each chemical is not required. Besides, the method takes into account both the potency and the shapes of the dose-effect curve of each chemical. The method has been computerized allowing an automated simulation of synergism and antagonism at different concentrations and at different effect levels of the chemicals in a mixture.

The aim of our study was to assess the nature of the toxicological interactions of three fibrates, gemfibrozil, bezafibrate and fenofibric acid, by the method of combination index (CI)-isobologram equation. The three pharmaceuticals were used singly or in two- and three-drug combinations. As toxicity endpoint we have chosen the bioluminescent response of two prokaryotes, the naturally luminescent *Vibrio fischeri* and the recombinant bioluminescent cyanobacterium *Anabaena* sp. PCC 7120 CPB4337 (hereinafter, *Anabaena* CPB4337), both bioluminescent organisms have proved very useful in evaluating the toxicity of individual fibrates in a previous study (Rosal et al., 2009). For *Anabaena* CPB4337, we also evaluated the nature of the interactions of the three fibrates with a wastewater sample from a local STP, which already proved very toxic to the cyanobacterium (Rosal et al., 2009).

## 2. Materials and methods

### 2.1. Materials

Gemfibrozil (+99%) and bezafibrate (+98%) were purchased from Sigma–Aldrich. Fenofibric acid was produced from fenofibrate (Sigma–Aldrich, +99% purity) by hydrolysis. A suspension of fenofibrate in isopropanol (30 wt.%, 400 mL) was refluxed during 4 h with an aqueous sodium hydroxide solution (2.0 M, 200 mL). After cooling to less than 70 °C, a solution of hydrochloric acid (1.0 M, 325 mL) was slowly added while keeping the temperature over 60 °C. The product crystallized after cooling and keeping at room temperature during 4 or more h. The product was filtered and rinsed with water and dried overnight at 60 °C under nitrogen. The purity of the product was over 97% checked by HPLC. Solubility of acidic drugs in water is strongly pH dependent with few data considering this variable. Comerton et al. (2007) reported a solubility of 10.9 mg/L of gemfibrozil in water, but we could solve over 125 mg/L in 2 mM MOPS (3-[N-morpholino] propanesulfonic acid) at pH 6 and higher quantities for the pH at which *V. fischeri* bioassays were performed. In all cases, we avoided the use of solvents and the upper limit for the concentrations of the studied compounds was their solubility in pure water or wastewater at the pH of the bioassay.

Wastewater samples were collected from the secondary clarifier of a STP located in Alcalá de Henares (Madrid) that

receives domestic wastewater with a minor contribution of industrial effluents from facilities located near the city. This STP used a conventional activated sludge treatment and has been designed for a total capacity of 375,000 equivalent inhabitants with a maximum flow rate of 3000 $m^3$/h. In a recent previous study (Rosal et al., 2009), we found that this wastewater was very toxic to *Anabaena* cells with a wastewater dilution as low as 0.11 causing 50% luminescence inhibition (wastewater $EC_{50}$).

### 2.2. Toxicity tests

Bioassays with the photo-luminescent bacteria *Vibrio fischeri* were carried out according to ISO 11348-3 standard protocol (ISO, 2007). This bioassay measures, during the prescribed incubation period, the decrease in bioluminescence induced in the cell metabolism due to the presence of a toxic substance. The bacterial assay used the commercially available Biofix Lumi test (Macherey-Nagel, Germany). The bacterial reagent is supplied freeze-dried (*Vibrio fischeri* NRRL-B 11177) and was reconstituted and incubated at 3 °C for 5 min before use. The desired pH was set by using NaOH or HCl. The analysis media was 0.34 M NaCl (2% w/v) and tests were performed at 15 °C and the measurements of light were made using a luminometer (Optocomp I). The effect of toxicants or toxicant mixtures (i.e., fibrates or fibrate combinations) was measured as percent inhibition with respect to the light emitted under test conditions in the absence of any toxic influence. Toxicity values were routinely obtained after 30 min exposure. Phenol and $ZnSO_4 \bullet 7\ H_2O$ have been used as toxicity standards and all tests have been replicated to ensure reproducibility.

The bioassays using the recombinant bioluminescent cyanobacterium *Anabaena* CPB4337 were based on the inhibition of constitutive luminescence caused by the presence of any toxic substance (Rodea-Palomares et al., 2009; Rosal et al., 2009). *Anabaena* CPB4337 was routinely grown at 28 °C in the light, ca. 65 μmol photons $m^2\ s^{-1}$ on a rotary shaker in 50 mL AA/8 (Allen and Arnon, 1955) supplemented with nitrate (5 mM) in 125 ml Erlenmeyer flasks and 10 μg/mL of neomycin sulphate (Nm). Luminescence inhibition-based toxicity assays were performed as follows: 160 μL from five to seven serial dilutions of each tested toxicant or toxicant mixture (i.e.; fibrates or fibrate combinations) plus a control ($ddH_2O$ buffered with MOPS at pH 5.8) were disposed in an opaque white 96-well microtiter plates. 40 μL cells, grown as described, were washed twice and resuspended in $ddH_2O$ buffered with MOPS at pH 5.8 and were added to the microtiter plate wells to reach a final cell density at $OD_{750\ nm}$ of 0.5. The luminescence of each sample was recorded every 5 min for up to 1 h in the Centro LB 960 luminometer. Three independent experiments with duplicate samples were carried out for all *Anabaena* toxicity assays. $CuSO_4$ has been used as toxicity standard and all tests have been replicated to ensure reproducibility.

### 2.3. Experimental design of fibrate combinations

Solutions of gemfibrozil (Gm), bezafibrate (Bz) and fenofibric acid (Fn) prepared as described above were used singly and in two (Bz + Gm; Fn + Gm; Fn + Bz) and three (Fn + Gm + Bz) combinations. *Anabaena* and *Vibrio fischeri* cells were treated with serial dilutions of each fibrate individually and with a fixed constant ratio (1:1), based on the individual $EC_{50}$ values, in their binary and ternary combinations. Five dilutions (serial dilution factor = 2) of each fibrate and combination plus a control were tested in three independent experiments with replicate samples.

For evaluating the nature of the interaction of fibrates with wastewater, binary combinations of each fibrate plus wastewater (Fn + WW; Gm + WW; Bz + WW) and a quaternary combination of the three fibrates plus wastewater (Fn + Gm + Bz + WW) were also prepared and tested for *Anabaena* CPB4337. *Anabaena* cells were treated with serial dilutions of each fibrate and wastewater individually and with a fixed constant ratio (1:1), based on the individual $EC_{50}$ values, in their binary and quaternary combinations. Five dilutions (serial dilution factor = 2) of each fibrate and wastewater and their combinations plus a control were tested in three independent experiments with replicate samples. The experimental design is shown in Table 1.

All individual fibrate, wastewater and their combination assays were carried out at the same time as recommended by Chou (2006) to maximize computational analysis of data.

### 2.4. Median-effect and combination index (CI)-isobologram equations for determining combined fibrate interactions

The results were analyzed using the median-effect/combination index (CI)-isobologram equation by Chou (2006) and Chou and Talalay (1984) which is based on the median-effect principle (mass-action law) (Chou, 1976) that demonstrates that there is an univocal relationship between dose and effect independently of the number of substrates or products and of the mechanism of action or inhibition. This method involved plotting the dose-effect curves for each compound and their combinations in multiple diluted concentrations by using the median-effect equation:

$$\frac{fa}{fu} = \left(\frac{D}{Dm}\right)^m \tag{1}$$

Where $D$ is the dose, $Dm$ is the dose for 50% effect (e.g., 50% inhibition of bioluminescence or $EC_{50}$), $fa$ is the fraction affected by dose $D$ (e.g., 0.75 if cell bioluminescence is inhibited by 75%), $fu$ is the unaffected fraction (therefore, $fa = 1 - fu$), and $m$ is the coefficient of the sigmoidicity of the dose-effect curve: $m = 1$, $m > 1$, and $m < 1$ indicate hyperbolic, sigmoidal, and negative sigmoidal dose-effect curve, respectively. Therefore, the method takes into account both the potency ($Dm$) and shape ($m$) parameters. If Eq. (1) is rearranged, then:

$$D = Dm[fa/(1 - fa)]^{1/m} \tag{2}$$

The $Dm$ and $m$ values for each fibrate are easily determined by the median-effect plot: $x = \log(D)$ versus $y = \log(fa/fu)$ which is based on the logarithmic form of Eq. (1). In the median-effect plot, $m$ is the slope and $\log(Dm)$ is the $x$-intercept. The conformity of the data to the median-effect principle can be ready manifested by the linear correlation coefficient ($r$) of the data to the logarithmic form of Eq. (1) (Chou, 2006).

**Table 1 – Experimental design for determining toxicological interactions of fenofibric acid [Fn ($D$)$_1$], gemfibrozil [Gm ($D$)$_2$], bezafibrate [Bz ($D$)$_3$] and their binary and ternary combinations for *Vibrio fischeri* and *Anabaena* CPB4337 bioluminescence tests.**

| Pure fibrate experiments | | | | | | | Fibrates plus wastewater experiments | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Vibrio fischeri* | | | *Anabaena* CPB4337 | | | *Anabaena* CPB4337 | | | | |
| Dilutions | Single toxicant | | | Single toxicant | | | | Single toxicant | | | |
| | Fn | Gm | Bz | Fn | Gm | Bz | | Fn | Gm | Bz | WW** |
| | ($D$)$_1$ | ($D$)$_2$ | ($D$)$_3$ | ($D$)$_1$ | ($D$)$_2$ | ($D$)$_3$ | | ($D$)$_1$ | ($D$)$_2$ | ($D$)$_3$ | ($D$)$_4$ |
| ¼ ($EC_{50}$) | 0.4 | 8.75 | 37.5 | 2.5 | 2.5 | 12.5 | ¼ ($EC_{50}$) | 2.5 | 2.5 | 12.5 | 0.025 |
| ½ ($EC_{50}$) | 0.8 | 17.5 | 75 | 5 | 5 | 25 | ½ ($EC_{50}$) | 5 | 5 | 25 | 0.05 |
| 1 ($EC_{50}$) | 1.6 | 35 | 150 | 10 | 10 | 50 | 1 ($EC_{50}$) | 10 | 10 | 50 | 0.1 |
| 2 ($EC_{50}$) | 3.2 | 70 | 300 | 20 | 20 | 100 | 2 ($EC_{50}$) | 20 | 20 | 100 | 0.2 |
| 4 ($EC_{50}$) | 6.4 | 140 | 600 | 40 | 40 | 200 | 4 ($EC_{50}$) | 40 | 40 | 200 | 0.4 |
| | Two toxicant combo | | | Two toxicant combo | | | | Two toxicant combo | | | |
| | ($D$)$_1$ + ($D$)$_2$ (1.6:35) | | | ($D$)$_1$ + ($D$)$_2$ (1:1) | | | | ($D$)$_1$ + ($D$)$_4$ (1:0.01) | | | |
| ¼ ($EC_{50}$) | 0.4 | 8.75 | | 2.5 | 2.5 | | ¼ ($EC_{50}$) | 2.5 | | | 0.025 |
| ½ ($EC_{50}$) | 0.8 | 17.5 | | 5 | 5 | | ½ ($EC_{50}$) | 5 | | | 0.05 |
| 1 ($EC_{50}$) | 1.6 | 35 | | 10 | 10 | | 1 ($EC_{50}$) | 10 | | | 0.1 |
| 2 ($EC_{50}$) | 3.2 | 70 | | 20 | 20 | | 2 ($EC_{50}$) | 20 | | | 0.2 |
| 4 ($EC_{50}$) | 6.4 | 140 | | 25* | 25* | | 4 ($EC_{50}$) | 40 | | | 0.4 |
| | ($D$)$_1$ + ($D$)$_3$ (1.6:150) | | | ($D$)$_1$ + ($D$)$_3$ (1:5) | | | | ($D$)$_2$ + ($D$)$_4$ (1:0.01) | | | |
| ¼ ($EC_{50}$) | 0.4 | | 37.5 | 2.5 | | 12.5 | ¼ ($EC_{50}$) | | 2.5 | | 0.025 |
| ½ ($EC_{50}$) | 0.8 | | 75 | 5 | | 25 | ½ ($EC_{50}$) | | 5 | | 0.05 |
| 1 ($EC_{50}$) | 1.6 | | 150 | 10 | | 50 | 1 ($EC_{50}$) | | 10 | | 0.1 |
| 2 ($EC_{50}$) | 3.2 | | 300 | 20 | | 100 | 2 ($EC_{50}$) | | 20 | | 0.2 |
| 4 ($EC_{50}$) | 6.4 | | 600 | 30* | | 150* | 4 ($EC_{50}$) | | 40 | | 0.4 |
| | ($D$)$_2$ + ($D$)$_3$ (35:150) | | | ($D$)$_2$ + ($D$)$_3$ (1:5) | | | | ($D$)$_3$ + ($D$)$_4$ (1:0.002) | | | |
| ¼ ($EC_{50}$) | | 8.75 | 37.5 | | 2.5 | 12.5 | ¼ ($EC_{50}$) | | | 12.5 | 0.025 |
| ½ ($EC_{50}$) | | 17.5 | 75 | | 5 | 25 | ½ ($EC_{50}$) | | | 25 | 0.05 |
| 1 ($EC_{50}$) | | 35 | 150 | | 10 | 50 | 1 ($EC_{50}$) | | | 50 | 0.1 |
| 2 ($EC_{50}$) | | 70 | 300 | | 20 | 100 | 2 ($EC_{50}$) | | | 100 | 0.2 |
| 4 ($EC_{50}$) | | 140 | 600 | | 40 | 200 | 4 ($EC_{50}$) | | | 200 | 0.4 |
| | Three toxicant combo | | | Three toxicant combo | | | | Four toxicant combo | | | |
| | ($D$)$_1$ + ($D$)$_2$ + ($D$)$_3$ (1.6:35:150) | | | ($D$)$_1$ + ($D$)$_2$ + ($D$)$_3$ (1:1:5) | | | | ($D$)$_1$ + ($D$)$_2$ + ($D$)$_3$ + ($D$)$_4$ (1:1:5:0.01) | | | |
| ¼ ($EC_{50}$) | 0.4 | 8.75 | 37.5 | 2.5 | 2.5 | 12.5 | 1/8 ($EC_{50}$) | 1.25 | 1.25 | 6.25 | 0.0125 |
| ½ ($EC_{50}$) | 0.8 | 17.5 | 75 | 5 | 5 | 25 | ¼ ($EC_{50}$) | 2.5 | 2.5 | 12.5 | 0.025 |
| 1 ($EC_{50}$) | 1.6 | 35 | 150 | 10 | 10 | 50 | ½ ($EC_{50}$) | 5 | 5 | 25 | 0.05 |
| 2 ($EC_{50}$) | 3.2 | 70 | 300 | 20 | 20 | 100 | 1 ($EC_{50}$) | 10 | 10 | 50 | 0.1 |
| 4 ($EC_{50}$) | 6.4 | 140 | 600 | 40 | 40 | 200 | 2 ($EC_{50}$) | 20 | 20 | 100 | 0.2 |

For the *Anabaena* test, the design for the experiment with the wastewater [WW ($D_4$)] sample is also included. The experimental design is based on $EC_{50}$ ratios as proposed by Chou and Talalay (1984). $EC_{50}$ is the effective concentration of a toxicant which caused a 50% bioluminescence inhibition. The combination ratio was approximately equal to the $EC_{50}$ ratio of the combination components (i.e., close to their equipotency ratio). *Upper maximal possible dose due to the solubility limit of fibrates in pure water. **$EC_{50}$ for wastewater is the dilution which caused 50% luminescence inhibition. ($D$)$_1$, ($D$)$_2$ and ($D$)$_3$ in mg/L, ($D$)$_4$ is the dilution of wastewater in dd$H_2O$.

These parameters were then used to calculate doses of the fibrates and their combinations required to produce various effect levels according to Eq. (1); for each effect level, combination index (CI) values were then calculated according to the general combination index equation for $n$ chemical combination at $x$% inhibition (Chou, 2006):

$$^{n}(\mathrm{CI})_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n}\left\{[D]_j / \sum_1^n [D]\right\}}{(D_m)_j \left\{(f_{ax})_j / \left[1-(f_{ax})_j\right]\right\}^{1/mj}} \quad (3)$$

where $^{n}(\mathrm{CI})_x$ is the combination index for $n$ chemicals (e.g., fibrates) at $x$% inhibition (e.g., bioluminescence inhibition); $(D_x)_{1-n}$ is the sum of the dose of $n$ chemicals that exerts $x$% inhibition in combination, $\{[D_j]/\sum_1^n [D]\}$ is the proportionality of the dose of each of $n$ chemicals that exerts $x$% inhibition in combination; and $(D_m)_j\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the dose of each drug alone that exerts $x$% inhibition. From Eq. (3), CI <1, =1 and >1 indicates synergism, additive effect and antagonism, respectively.

### 2.5. Analysis of results

Computer program CompuSyn (Chou and Martin, 2005, Compusyn Inc, USA) was used for calculation of dose-effect curve parameters, CI values, *fa*-CI plot (plot representing CI versus *fa*, the fraction affected by a particular dose; see Eq. (1)) and polygonograms (a polygonal graphic representation depicting synergism, additive effect and antagonism for three or more drug combinations). Linear regression analyses were computed using MINITAB Release 14 for Windows (Minitab Inc; USA).

## 3. Results

### 3.1. Toxicological interactions of fibrate combinations in Vibrio fischeri and Anabaena CPB4337 bioluminescence tests

Applying the combination index-isobologram method, we evaluated the nature of gemfibrozil (Gm), fenofibric acid (Fn) and bezafibrate (Bz) interactions both in *Vibrio fischeri* and *Anabaena* CPB4337 bioluminescence tests. Table 2 shows the dose-effect curve parameters (*Dm*, *m* and *r*) of the three fibrates singly and their binary and ternary combinations, as well as mean combination index (CI) values of fibrate combinations. *Dm* was the dose required to produce the median-effect (analogous to the $EC_{50}$); *Dm* values for Fn were the lowest both, in *Vibrio* and *Anabaena* tests, *Dm* values for Gm were in the same range for both *Vibrio* and *Anabaena* while Bz

**Table 2 – Dose-effect relationship parameters and mean combination index (CI) values (as a function of fractional inhibition of luminescence) of gemfibrozil (Gm), fenofibric acid (Fn), and bezafibrate (Bz) individually and of their binary and ternary combinations on *Vibrio fischeri* and *Anabaena* CPB4337 bioluminescence tests.**

| Drug combo | *Vibrio fischeri* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Dose-effect parameters | | | | CI values | | | | | |
| | *Dm* | | *m* | *r* | $EC_{10}$ | | $EC_{50}$ | | $EC_{90}$ | |
| | mg/L | (μM) | | | | | | | | |
| Fn | 1.45 | (4.01) | 0.78 | 0.989 | – | | – | | – | |
| Gm | 20.58 | (82.11) | 1.53 | 0.966 | – | | – | | – | |
| Bz | 252.07 | (696.46) | 1.15 | 0.975 | – | | – | | – | |
| Gm + Bz | 78.20 | (234.20) | 1.54 | 0.991 | 1.13 ± 0.13 | Add | 0.97 ± 0.04 | Add | 0.86 ± 0.05 | Syn |
| Fn + Bz | 153.79 | (424.93) | 1.09 | 0.981 | 2.98 ± 0.15 | Ant | 1.71 ± 0.03 | Ant | 1.17 ± 0.06 | Ant |
| Fn + Gm | 9.84 | (38.74) | 1.15 | 0.973 | 0.99 ± 0.17 | Add | 0.75 ± 0.05 | Syn | 0.86 ± 0.08 | Syn |
| Fn + Gm + Bz | 55.69 | (166.69) | 1.23 | 0.993 | 1.46 ± 0.06 | Ant | 1.01 ± 0.02 | Add | 0.99 ± 0.03 | Add |
| | *Anabaena* CPB4337 | | | | | | | | | |
| | Dose-effect parameters | | | | CI values | | | | | |
| | *Dm* | | *m* | *r* | $EC_{10}$ | | $EC_{50}$ | | $EC_{90}$ | |
| | mg/L | (μM) | | | | | | | | |
| Fn | 8.53 | (23.62) | 0.96 | 0.971 | – | | – | | – | |
| Gm | 10.69 | (42.67) | 0.81 | 0.959 | – | | – | | – | |
| Bz | 12.56 | (34.70) | 1.08 | 0.990 | – | | – | | – | |
| Gm + Bz | 19.17 | (56.88) | 0.84 | 0.972 | 1.06 ± 0.15 | Add | 1.57 ± 0.06 | Ant | 2.5 ± 0.22 | Ant |
| Fn + Bz | 13.92 | (38.49) | 0.76 | 0.965 | 0.55 ± 0.06 | Syn | 1.19 ± 0.04 | Ant | 2.59 ± 0.14 | Ant |
| Fn + Gm | 12.26 | (41.45) | 0.46 | 0.955 | 0.13 ± 0.02 | Syn | 1.29 ± 0.05 | Ant | 12.9 ± 2.33 | Ant |
| Fn + Gm + Bz | 6.62 | (19.45) | 0.53 | 0.960 | 0.09 ± 0.01 | Syn | 0.57 ± 0.02 | Syn | 3.92 ± 0.19 | Ant |

The parameters *m*, *Dm* and *r* are the antilog of *x*-intercept, the slope and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose-effect curve, the potency ($EC_{50}$), and conformity of the data to the mass-action law, respectively (Chou, 1976; Chou and Talalay, 1984; Chou, 2006). *Dm* and *m* values are used for calculating the CI values (Eq. (3)); CI <1, =1, and >1 indicate synergism (Syn), additive effect (Add), and antagonism (Ant), respectively. $EC_{10}$, $EC_{50}$ and $EC_{90}$, are the doses required to inhibit bioluminescence 10, 50 and 90%, respectively. Computer software CompuSyn was used for automated calculation and simulation.

*Dm* values were an order of magnitude higher in the *Vibrio* test (Rosal et al., 2009); *m* was the Hill coefficient used to determine the shape of the dose-response curve, hyperbolic ($m = 1$), sigmoidal ($m > 1$) or negative sigmoidal ($m < 1$); also shown in the table, linear regression correlation coefficients (*r*-values) of the median-effect plots were >0.95 in all cases, indicating the conformity of the data to the median-effect principle which qualifies for further studies using this method.

The *Dm* and *m* values for single fibrates and for their combination mixtures were used for calculating synergism or antagonism based on the CI Eq. (3) (Chou, 2006). Fig. 1 shows the *fa*-CI plot of fibrate interactions both for *Vibrio* (Fig. 1a) and *Anabaena* tests (Fig. 1b); the *fa*-CI plot depicts the CI value versus *fa* (effect level or fraction of luminescence inhibited by a fibrate singly or in combination with respect to the control) for two (Fn + Bz; Fn + Gm and Bz + Gm) and three fibrate (Fn + Gm + Bz) combinations. The *fa*-CI plot is an effect-oriented plot that shows the evolution of the kind of interaction (synergism, antagonism, additive effect) as a function of the level of the effect (*fa*) of a particular toxicant on the reference organism (*fa*, where $EC_a = fa \times 100$; i.e., $EC_{10} = f10 \times 100$). In the *Vibrio* test (Fig. 1a), the Bz + Gm and Fn + Gm binary combination showed a slight antagonism at very low *fa* values and slight synergism (Fn + Gm) or nearly additive effects (Bz + Gm) at the highest *fa* values, the Fn + Bz combination showed a strong antagonism at low effect levels but the antagonism decreased and approached an additive kind of interaction at the highest *fa* levels; the ternary combination (Fn + Gm + Bz) showed a moderate antagonism at low *fa* values that also turned into a nearly additive effect at *fa* values above 0.4. Correlation analyses were made between CI values of the fibrate ternary combination and CI values of each of the fibrate binary combinations to determine which binary combination interaction was predominant in the ternary mixture (Table 3); the highest correlation coefficient was found for the Fn + Bz combination ($r = 0.91$), suggesting that this combination interaction predominated in the three fibrate mixture. The *fa*-CI plot of the *Anabaena* test (Fig. 1b) showed the opposite pattern of interactions as the three binary and the ternary combinations showed from slight to strong synergism at the lowest *fa* values that turned into a very strong antagonism at *fa* values over 0.5; the ternary combination (Fn + Gm + Bz) closely followed the interaction pattern of the binary Fn + Gm combination, this is confirmed by the highest correlation coefficient found between the CI values of the ternary combination and the CI values of the Fn + Gm combination ($r = 0.996$) which suggests that in the *Anabaena* test, this particular combination seemed to be the predominant in the ternary toxicological interaction. Selected average CI values for both *Vibrio fischeri* and *Anabaena* CPB4337 tests at three representative dose levels ($EC_{10}$, $EC_{50}$ and $EC_{90}$) and the combined effects are summarized in Table 2.

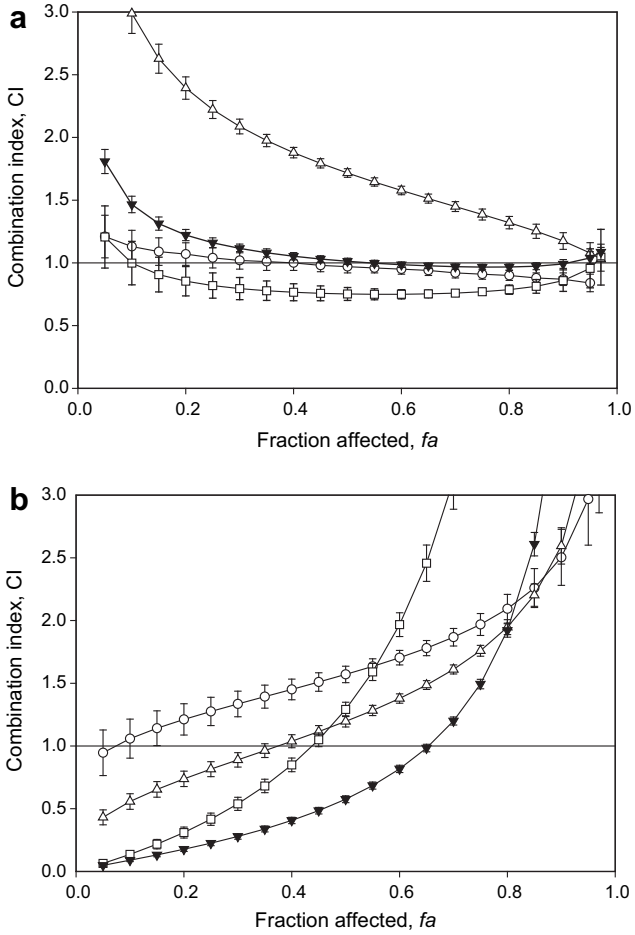


**Fig. 1 – Combination index plot (*fa*-CI plot) for a set of three fibrate combinations: Fn + Bz (–△–), Bz + Gm (–○–), Fn + Gm (–□–) and Fn + Gm + Bz (–▼–) for *Vibrio fischeri* test (a) and *Anabaena* CPB4337 test (b). CI values are plotted as a function of the fractional inhibition of bioluminescence (*fa*) by computer simulation (CompuSyn) from *fa* = 0.10 to 0.95. CI <1, =1 and >1 indicates synergism, additive effect and antagonism, respectively. At least three independent experiments with two replicates were used. The vertical bars indicate 95% confidence intervals for CI values based on sequential deletion analysis (SDA) (Chou and Martin, 2005). Fn = fenofibric acid, Bz = bezafibrate and Gm = gemfibrozil.**

### 3.2. *Toxicological interactions of wastewater and fibrate combinations in the* Anabaena *CPB4337 bioluminescence test*

In a recent previous study (Rosal et al., 2009), we found that a wastewater sample collected from a local STP was very toxic to *Anabaena* cells with a wastewater dilution of 0.11 causing 50% luminescence inhibition (wastewater $EC_{50}$). The observed toxicity was attributed to the combined toxicities of over thirty micropollutants, which included fibrates as well as other pharmaceuticals (Rosal et al., 2008). We sought to investigate the nature of the interaction between the wastewater (WW) and the three fibrates in binary (Fn + WW; Bz + WW and Gm + WW) and quaternary (Fn + Gm + Bz + WW) combinations; for these experiments, the wastewater itself was regarded as a toxicant; the experimental design was analogous to the one for the three fibrate interactions and is also shown in Table 1. The *r*-values of the median-effect plots were >0.95 in all cases, indicating that the data conformed to the median-effect principle (not shown). Fig. 2 shows the *fa*-CI plot for each

**Table 3 – Correlation analyses between CI values of fibrate ternary and fibrate + wastewater quaternary combinations (*y*) and their binary combinations (*x*) for *Vibrio fischeri* and *Anabaena* CPB4337 tests.**

| Test organism | Combinations | | | Regression parameters | | |
|---|---|---|---|---|---|---|
| | | | | $x_o$ | *m* | *r* |
| *V. fischeri* | Fn + Gm + Bz | versus | Gm + Bz | −0.614 | 1.77 | 0.83 |
| | | | Fn + Bz | 0.594 | 0.281 | 0.91 |
| | | | Fn + Gm | −0.067 | 1.40 | 0.81 |
| *Anabaena* CPB4337 | Fn + Gm + Bz | versus | Gm + Bz | −5.876 | 4.39 | 0.91 |
| | | | Fn + Bz | −2.716 | −3.00 | −0.941 |
| | | | Fn + Gm | 0.282 | 0.247 | 0.996 |
| | WW + Fn + Gm + Bz | versus | Gm + Bz | −0.079 | 0.372 | 0.999 |
| | | | Fn + Bz | 0.199 | 0.246 | 0.998 |
| | | | Fn + Gm | 0.464 | 0.017 | 0.897 |
| | | | Fn + WW | −0.253 | 1.31 | 0.999 |
| | | | Gm + WW | 2.131 | −2.41 | −0.89 |
| | | | Bz + WW | 0.003 | 0.865 | 0.999 |

Fn = fenofibric acid, Bz = bezafibrate, Gm = gemfibrozil, WW = wastewater. The parameters of linear regression equations: $x_0$ (value of *y* when *x* = 0); *m* (slope) and *r* (correlation coefficient) with all *p*-values of 0.001. Analyses were computed using MINITAB Release 14 for Windows.

of the binary fibrate-wastewater combination and the quaternary combination; as can be observed, in a broad range of *fa* values, the binary combinations showed a strong synergism; however, at *fa* values above 0.8, the binary Fn + WW and Bz + WW combinations approached an additive effect and at *fa* values above 0.95, these two combinations yielded antagonism; by contrast, the Gm + WW combination became even more synergistic. The quaternary combination interaction showed a strong synergism through a broad range of *fa* values but also turned into slight antagonism at *fa* values above 0.95,

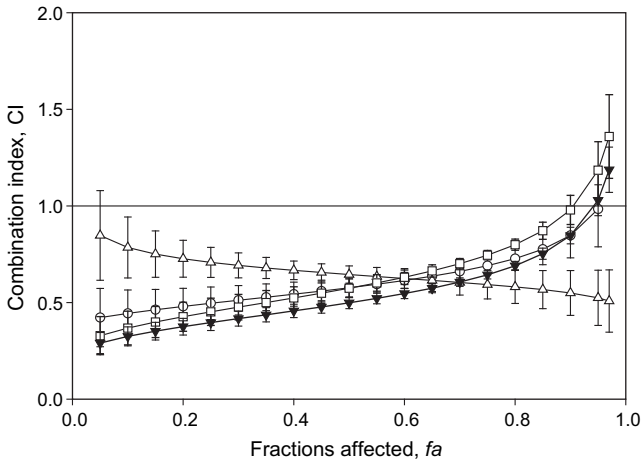


**Fig. 2 – Combination index plot (*fa*-CI plot) for a set of three fibrates and toxic wastewater sample in their binary and quaternary combinations: Gn + WW (–△–), Fn + WW (–○–), Bz + WW (–□–) and Fn + Gm + Bz + WW (–▼–) for the *Anabaena* CPB4337 test. CI values are plotted as a function of the fractional inhibition of bioluminescence (*fa*) by computer simulation (CompuSyn) from *fa* = 0.10 to 0.95. CI <1, =1 and >1 indicates synergism, additive effect and antagonism, respectively. At least three independent experiments with two replicates were used. The vertical bars indicate 95% confidence intervals for CI values based on sequential deletion analysis (SDA) (Chou and Martin, 2005). Fn = fenofibric acid, Bz = bezafibrate, Gm = gemfibrozil, WW = wastewater.**

closely resembling the pattern of the Fn + WW and Bz + WW interactions which is confirmed by the highest *r* value (*r* = 0.999) in the correlation analyses (Table 3), which suggests a predominant effect of Fn and Bz in the quaternary interaction.

The computer software CompuSyn (Chou and Martin, 2005) displays a type of graphic termed polygonogram, which is a semiquantitative method of representing interactions between three or more compounds at a determined *fa* value. This graphic allows a simplified visual presentation of the overall results. Fig. 3 shows the polygonogram for the three fibrates and the wastewater at four *fa* values; synergism is indicated by solid lines and antagonism by broken ones; the thickness of the lines indicates the strength of the interaction. The polygonogram clearly shows the synergistic interaction of wastewater in combination with each of the three fibrates at low *fa* values and the antagonistic interaction that appeared at the highest *fa* value, 0.99, for the Fn + WW and the Bz + WW combinations.

The same wastewater sample collected from a local STP was proved as responsible of stimulation of the bioluminescence activity of *Vibrio fischeri* to 110–120% of that of the control. Moreover, the $EC_{50}$ values for the fibrates in the wastewater were higher than those for fibrates in pure water (Rosal et al., 2009). The same trend was observed comparing the dose-effect curve parameters (*Dm*, *m* and *r*) for the ternary combination (Fn + Gm + Bz) of fibrates in $ddH_2O$ and wastewater. The dose required to produce the median-effect (*Dm*) in *Vibrio fischeri* test when (Fn + Gm + Bz) were solved in wastewater was 131.936 compared to 55.6951 mg/L required when $ddH_2O$ was employed. CI values could not be calculated for *Vibrio fischeri* due to the fact that the wastewater itself was not toxic to this bacterium; synergism or antagonism could not be properly estimated (Chou, 2006).

## 4. Discussion

The three fibrates that we have used in our study are lipid modifying agents that are effective in lowering elevated

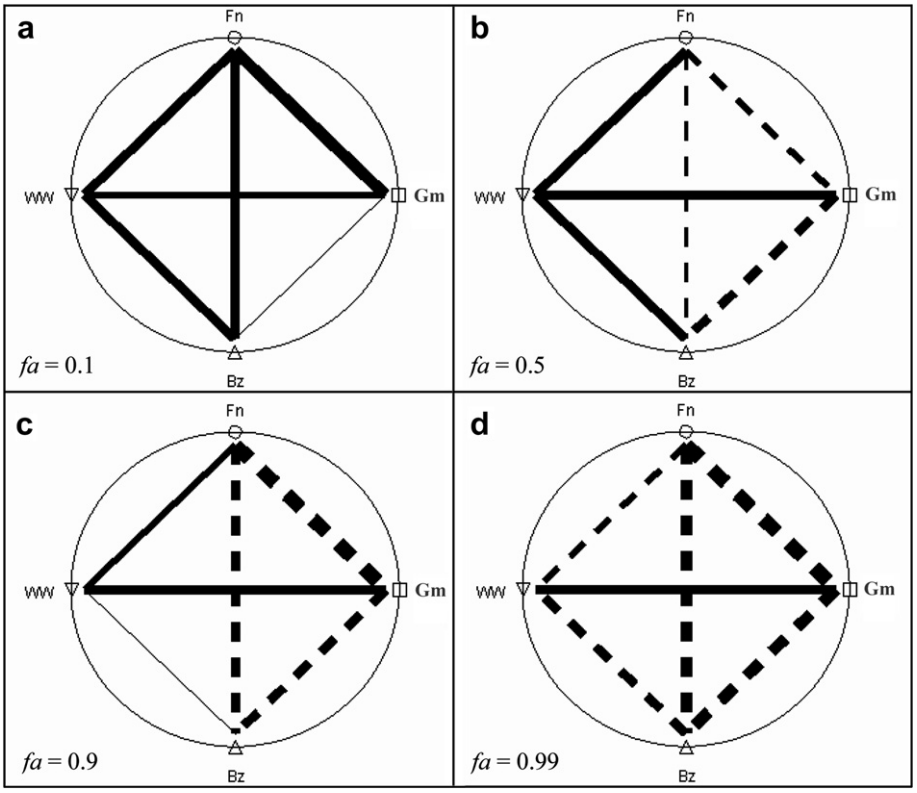


**Fig. 3 – Polygonograms showing the toxicological interactions of three fibrates and a toxic wastewater sample in their binary combinations (Fn + Bz, Bz + Gm, Fn + Gm, Gm + WW, Fn + WW, Bz + WW) as calculated by CompuSyn (Chou and Martin, 2005) for *Anabaena* CPB4337 test at four effect levels: *fa* = 0.1 (a), *fa* = 0.5 (b), *fa* = 0.9 (c) and *fa* = 0.99 (d). Solid lines indicate synergism, broken lines indicate antagonism. The thickness of the line represents the strength of synergism or antagonism. Figure generated by CompuSyn (Chou and Martin, 2005).**

plasma triglycerides and cholesterol in humans (Staels et al., 1998). These pharmaceuticals are highly used, ubiquitous and persistent (Daughton and Ternes, 1999), they are found at ng/L to μg/L levels in many STP effluents, surface waters, estuaries of rivers and even in sea water (for a review, see Hernando et al., 2007). Although non-target organisms; the continuous release of these substances into the environment may cause acute or chronic toxicity to the aquatic biota. Regarding fibrates, in the recent literature there are many reports dealing with individual toxicity of different fibrates in a range of aquatic organisms from primary producers to consumers; a great variability has been found in the sensitivity of the different test organisms toward these pharmaceuticals (Hernando et al., 2007). However, pharmaceuticals such as fibrates do not occur singly in a polluted environment and are usually found as mixtures, therefore, for risk assessment strategies it is important to know the combined effects of pharmaceuticals in non-target organisms (Teuschler, 2007).

There are two concepts widely used for the prediction of mixture toxicity: concentration addition (CA) and independent action (IA) (Backhaus et al., 2003; Vighi et al., 2003; Backhaus et al., 2004; Junghans et al., 2006). CA is used for mixtures whose components act in a similar mode of action while IA is based on the idea of dissimilar action, meaning that the compounds have different mechanisms of action; however, as discussed by Cleuvers (2003) the terms similar/dissimilar action may be misleading. Pharmaceuticals such as fibrates may have the same pharmacological mechanism of action [i.e., interaction with the binding peroxisome proliferator-activated receptor α (PPARα)] in their target organism, humans; however, if fibrates released in the aquatic environments prove toxic to different non-target organisms, the exact mechanism of toxicity (probably different to the pharmacological mode of action) should be investigated in depth before choosing which approach, CA or IA, to use. In fact, only if toxicity is regarded as non-specific at all, the concept of CA may be used although it may also have limitations. Cleuvers (2003) found that two totally different pharmaceuticals, a fibrate and an anti-epileptic drug, followed the concept of CA in the *Daphnia* toxicity test and the concept of IA in an algal test; both pharmaceuticals apparently shared the same non-specific toxic mode of action for both organisms; so it appeared that the concept of CA or IA did not depend on a similar/dissimilar mode of action but on the tested organism. The author also discussed that by definition, when using CA, substances applied below their individual non-effect concentration (NOEC) will contribute to the total effect of the mixture while when using IA, substances applied below their NOEC will not contribute to the total effect of the mixture, meaning that any combination effect will probably be higher if the substances follow the concept of CA and this may be misleading when considering the terms synergism or

antagonism because as also discussed by Chou (2006), synergism or antagonism may occur independently of a similar or dissimilar mode of action. In this context, Fent et al. (2006) tested mixtures of different kinds of pharmaceuticals (including fibrates) that might have estrogenic activity in a yeast reporter system; they applied the CA model and found that it had severe limitations when the dose-response curves of the individual pharmaceuticals were not identical or at low effect concentrations. As pharmaceuticals released in the environment may have such diverse dose-effect relationships, the lack of appropriate prediction suggests limitation of the CA mixtures concept.

To study the nature of the combined fibrate interactions (synergism, additive effect, antagonism) for the *Vibrio fischeri* and *Anabaena* CPB4337 bioluminescence tests, we have followed the combination index (CI)-isobologram equation method of Chou (2006) and Chou and Talalay (1984); a method widely used to study drug interactions in pharmacology. This method may be considered a fractional analysis technique for drug interactions (Berenbaum, 1981; Bovill, 1998) that is independent of the mode of action and considers both the potency ($EC_{50}$, *Dm*) and the shape (*m*) of the dose-effect curve for each drug. The method allows prediction of synergism/antagonism at all effect levels (*fa*) for a combination of *n* drugs; in contrast with the classical graphical isobologram method (Berenbaum, 1981; Bovill, 1998) that cannot be used for more than three compounds and have also graphical limitations to show all effect levels. By using this method, we have been able to determine the nature of interactions for a wide range of effect levels of three fibrates in binary and ternary combinations in two different bioluminescent organisms. However, the nature of these interactions was not uniform along the *fa* levels range in any of the two organisms. In *Vibrio fischeri*, antagonism predominated at low and intermediate *fa* levels but at the highest effect levels, interactions became additive or slightly synergistic. In *Anabaena*, a dual synergistic/antagonistic behaviour was observed with synergism predominating at *fa* levels below 0.4–0.5 and strong antagonism above these *fa* values. It is difficult to give an explanation to this phenomenon because the combination index method only allows quantitative determination of synergism or antagonism and the elucidation of the mechanism by which synergism or antagonism occurs is a separate issue that needs a different kind of approach. However, tentatively, antagonism, which could be considered the predominant interaction in *Vibrio fischeri* and *Anabaena*, might be explained by the structural similarity of fibrates which are related pharmaceuticals that share a common structural motif, a cyclic head and a hydrophobic tail (Rosal et al., 2009); at the *fa* levels where antagonism is found in both organisms, fibrates may compete with one another for the same target/receptor sites. The slight synergism found at very high levels in *Vibrio fischeri* could perhaps be explained by the fact that at very high concentrations, fibrates may somehow combine to increase toxicity by an unspecific way of action that is probably not related to their pharmacological mechanism. Perhaps, the most puzzling interaction is the observed high synergism at very low *fa* levels in *Anabaena*; the mechanism of such synergistic interaction is not readily apparent. One could speculate that these fibrates at very low concentrations could involve what Jia et al. (2009) in their extensive review of mechanisms of drug combinations call ''facilitating actions'' that means that secondary actions of one drug enhances the activity or level of another drug in the mixture or alternatively ''complementary actions'' when drugs act at the same target at different sites, at overlapping sites or at different targets of the same pathway. However, in the literature there are very few reports on possible targets of fibrates on the prokaryotic cell; English et al. (1994) reported that peroxisome proliferators such as fibrates have been shown to induce cytochrome $P450_{BM\text{-}3}$ which catalyzes the hydroxylation of fatty acids, in *Bacillus megaterium*. Garbe (2004) reported that fibrates induced methyltransferase Rv0560c with a function in the biosynthesis of isoprenoid compounds in *Mycobacterium tuberculosis*; Garbe (2004) suggested that both effects may act on the plasma membrane, modulating its properties. In mitochondria, which have significant features that resemble those of prokaryotes, fibrates have been found to inhibit respiratory complex I (NDH-1 complex) and to interfere with mitochondrial fatty acid oxidation (Scatena et al., 2007). Whether fibrates may exert similar effects in *Vibrio fischeri* and *Anabaena* to those observed in *Bacillus* or mitochondria needs further research. In this context, we have found that, as the *fa*-CI plots show, fibrate interactions do not follow the same pattern in both bacteria, this may be due to the different origin and position in the food web of *Vibrio fischeri*, a heterotrophic marine prokaryote and *Anabaena* CPB4337, a recombinant strain of an obligate phototrophic freshwater prokaryote; in fact, *Anabaena* presents intracellular photosynthetic membranes called thylakoids where several functionally distinct NDH-1 complexes have been found with roles both in respiration and photosynthesis (Battchikova and Aro, 2007). If fibrates are also affecting NDH-1 complexes in *Anabaena*, their effects might be very different to those in *Vibrio fischeri*; so, although we have measured the same toxicity endpoint in both bacteria, i.e., luminescence inhibition, the combined effects of fibrates seem to depend on the test organism.

Ince et al. (1999) assessed toxic interactions of heavy metals in binary mixtures on *Vibrio fischeri* and the freshwater aquatic plant *Lemna minor* and found that most binary metal mixtures exhibited only antagonistic interactions in the plant opposed to fewer antagonistic and some synergistic interactions in the heterotrophic bacterium. These authors also found that in the bacterium, the nature of the interaction (synergism or antagonism) also changed with the effect level of the binary metal combinations, although the authors did not provide a mechanistic explanation for this variability. Cheng and Lu (2002) made a comparison of joint interactions of organic toxicants in binary mixtures in *Escherichia coli* and *Vibrio fischeri* and found that toxicants with the same mechanisms of toxicity displayed mostly additive or antagonistic interactions in *E. coli* and *Vibrio fischeri*; however a synergistic interaction was found between glutardialdehyde and butyraldehyde in *Vibrio*. Synergistic effects in both bacteria were mostly associated with toxicants with different mechanisms of toxicity, although antagonism clearly predominated. They also found that for a total of 44 organic binary mixtures, only six mixtures resulted in identical type of interaction in both bacteria. From our results and those of other authors' (Ince et al., 1999; Cheng and Lu, 2002; Cleuvers, 2003) one may

conclude that previous knowledge of the mechanism of toxic action of a compound is not useful enough to predict which kind of interactions it will display when combined with other toxicants with the same or different toxic mechanism; also, as we have shown, the nature of the interaction may depend on the effect level of the mixture. In addition, different types of organisms will show completely different responses to mixtures of potential toxicants.

We previously found that a local wastewater was very toxic for the *Anabaena* CPB4337 test but non-toxic at all for the *Vibrio fischeri* or *Daphnia magna* tests. This wastewater is a mixture of over thirty micropollutants, mostly pharmaceuticals of different therapeutics groups that, besides the fibrates used in this study, included antibiotics, analgesics/anti-inflammatories, β-blockers, antidepressants, anti-epileptics/psychiatrics, ulcer healing compounds, diuretics and bronchodilators; personal care products and some priority organic pollutants are also present (Rosal et al., 2008). The method of Chou allows to combine one drug mixture with another drug mixture and determine their interactions; therefore, we studied the nature of the interaction of fibrates and wastewater in the *Anabaena* bioluminescence test; interestingly, we found that in a wide range of effect levels, the interaction of wastewater and the three fibrate combination was synergistic; particularly, at very low *fa* values which means that fibrates are at low concentrations and the wastewater is diluted several-fold, the method predicted a strong synergism; this may be due, as discussed above, to the observed synergistic interactions of fibrates with one another as well as interactions with some of the detected micropollutants when present at very low concentrations. This observed synergism may be environmentally relevant since most pharmaceuticals such as fibrates do not usually show acute toxicity on non-target organisms when tested at real environmental concentrations (Hernando et al., 2007) but in a mixture, if they act synergistically, they could prove toxic for a test organism even at low concentrations; these results agree with those found by Hernando et al. (2004) who reported synergistic toxic effects for *Daphnia magna* test when wastewater was spiked with environmental concentrations of several pharmaceuticals including fibrates. By contrast, our results show that at high *fa* values ($fa > 0.8$), the combined interaction of the quaternary fibrates + wastewater combination, the binary Fn + WW and Bz + WW combinations approached an additive effect and eventually became antagonistic; in our previous study, the wastewater itself decreased *Anabaena* bioluminescence by 84% with a lower confidence limit of 76% and an upper confidence limit of 91%; when the wastewater was spiked with increasing concentrations of each fibrate we found that, with the exception of gemfibrozil, the $EC_{50}$ values for the fibrates in the wastewater were higher than those for fibrates in pure water; this was attributed either to reduced bioavailability or to antagonistic effects of fibrates with other chemicals present in the wastewater; although we did not use the method of Chou, we obtained similar results to the ones we report in this study; that is, at high effect levels (>84% luminescence inhibition) the interaction of fibrates with wastewater, except the Gm + WW combination, showed antagonism.

Based on our results, we propose that the combination index (CI)-isobologram equation, a method widely used in pharmacology both for *in vitro* and *in vivo* bioassays, may also be applied in environmental toxicology as a general method to define interactions of potential toxicants in mixtures in any test organism and/or toxicological endpoint of interest and could be especially useful for risk assessment strategies that take into account the toxicological interactions of substances in a mixture.

## 5. Conclusions

We report an environmental application of the combination index (CI)-isobologram equation to study the nature of the interactions of fibrate combinations in two bioluminescent aquatic organisms. The method allowed calculating synergism or antagonism of binary and ternary fibrate combinations at all effect levels simultaneously; we could also test the method with a real wastewater sample in binary and quaternary combination with the fibrates, finding that at very low effect levels, the fibrates acted synergistically with the wastewater in the *Anabaena* test. The proposed method may be used with other test organisms and/or toxicological endpoints and could be particularly useful for risk assessment approaches to toxicity of complex mixtures.

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## REFERENCES

Allen, M.B., Arnon, D.I., 1955. Studies on nitrogen-fixing blue green algae. I Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. Plant Physiol 30 (4), 366–372.

Andreozzi, R., Raffaele, M., Nicklas, P., 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. Chemosphere 50 (10), 1319–1330.

Backhaus, T., Altenburger, R., Arrhenius, Å., Blanck, H., Faust, M., Finizio, A., Gramatica, P., Grote, M., Junghans, M., Meyer, W., Pavan, M., Porsbring, T., Scholze, M., Todeschini, R., Vighi, M., Walter, H., Horst Grimme, L., 2003. The BEAM-project: prediction and assessment of mixture toxicities in the aquatic environment. Continental Shelf Res. 23 (17–19), 1757–1769.

Backhaus, T., Arrhenius, A., Blanck, H., 2004. Toxicity of a mixture of dissimilarly acting substances to natural algal communities: predictive power and limitations of independent action and concentration addition. Environ. Sci. Technol 38 (23), 6363–6370.

Battchikova, N., Aro, E.-M., 2007. Cyanobacterial NDH-1 complexes: multiplicity in function and subunit composition. Physiol. Plant 131 (1), 22–32.

Berenbaum, M.C., 1981. Criteria for analyzing interactions between biologically active agents. Adv. Cancer Res. 35, 269–335.

Borgmann, U., Bennie, D.T., Ball, A.L., Palabrica, V., 2007. Effect of a mixture of seven pharmaceuticals on *Hyalella azteca* over multiple generations. Chemosphere 66 (7), 1278–1283.

Bovill, J.G., 1998. Analysis of drug interactions. Baillière's Clin. Anaesthesiol 12 (2), 135–168.
Brain, R.A., Johnson, D.J., Richards, S.M., Hanson, M.L., Sanderson, H., Lam, M.W., Young, C., Mabury, S.A., Sibley, P.K., Solomon, K.R., 2004. Microcosm evaluation of the effects of an eight pharmaceutical mixture to the aquatic macrophytes *Lemna gibba* and *Myriophyllum sibiricum*. Aquat. Toxicol 70 (1), 23–40.
Castiglioni, S., Bagnati, R., Fanelli, R., Pomati, F., Calamari, D., Zuccato, E., 2006. Removal of pharmaceuticals in sewage treatment plants in Italy. Environ. Sci. Technol 40 (1), 357–363.
Chen, C.-Y., Lu, C.-L., 2002. An analysis of the combined effects of organic toxicants. Sci. Total Environ 289 (1–3), 123–132.
Chou, T.C., 1976. Derivation and properties of Michaelis–Menten type and Hill type equations for reference ligands. J. Theor. Biol. 59 (2), 253–276.
Chou, T.C., 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. 58 (3), 621–681.
Chou, T.C., Martin, N., 2005. CompuSyn for Drug Combinations: PC Software and User's Guide: A Computer Program for Quantification of Synergism and Antagonism in Drug Combinations and the Determination of $IC_{50}$ and $ED_{50}$ and $LD_{50}$ Values. ComboSyn, Inc., Paramus, NJ.
Chou, T.C., Talalay, P., 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul 22, 27–55.
Christensen, A.M., Faaborg-Andersen, S., Ingerslev, F., Baun, A., 2007. Mixture and single-substance toxicity of selective serotonin reuptake inhibitors toward algae and crustaceans. Environ. Toxicol. Chem. 26 (1), 85–91.
Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142 (3), 185–194.
Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotoxicol. Environ. Saf 59 (3), 309–315.
Comerton, A.M., Andrews, R.C., Bagley, D.M., Yang, P., 2007. Membrane adsorption of endocrine disrupting compounds and pharmaceutically active compounds. J. Memb. Sci. 303 (1–2), 267–277.
Crane, M., Watts, C., Boucard, T., 2006. Chronic aquatic environmental risks from exposure to human pharmaceuticals. Sci. Total Environ 367 (1), 23–41.
Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ. Health Perspect 107 (6), 907–938.
English, N., Hughes, V., Wolf, C.R., 1994. Common pathways of cytochrome P450 gene regulation by peroxisome proliferators and barbiturates in *Bacillus megaterium* ATCC14581. J. Biol. Chem. 269 (43), 26836–26841.
Fent, K., Escher, C., Caminada, D., 2006. Estrogenic activity of pharmaceuticals and pharmaceutical mixtures in a yeast reporter gene system. Reprod. Toxicol 22 (2), 175–185.
Garbe, T.R., 2004. Co-induction of methyltransferase Rv0560c by naphthoquinones and fibric acids suggests attenuation of isoprenoid quinone action in *Mycobacterium tuberculosis*. Can. J. Microbiol. 50 (10), 771–778.
Gros, M., Petrovic, M., Barcelo, D., 2007. Wastewater treatment plants as a pathway for aquatic contamination by pharmaceuticals in the Ebro river basin (northeast Spain). Environ. Toxicol. Chem. 26 (8), 1553–1562.
Han, G.H., Hur, H.G., Kim, S.D., 2006. Ecotoxicological risk of pharmaceuticals from wastewater treatment plants in Korea: occurrence and toxicity to *Daphnia magna*. Environ. Toxicol. Chem. 25 (1), 265–271.
Hernando, M.D., Petrovic, M., Fernández-Alba, A.R., Barceló, D., 2004. Analysis by liquid chromatography-electrospray ionization tandem mass spectrometry and acute toxicity evaluation for beta-blockers and lipid-regulating agents in wastewater samples. J. Chromatogr. A 1046 (1–2), 133–140.
Hernando, M.D., Agüera, A., Fernández-Alba, A.R., 2007. LC-MS analysis and environmental risk of lipid regulators. Anal. Bioanal. Chem. 387 (4), 1269–1285.
Holoshitz, N., Alsheikh-Ali, A.A., Karas, R.H., 2008. Relative safety of gemfibrozil and fenofibrate in the absence of concomitant cerivastatin use. Am. J. Cardiol 101 (1), 95–97.
Ince, N.H., Dirilgen, N., Apikyan, I.G., Tezcanli, G., Üstün, B., 1999. Assessment of toxic interactions of heavy metals in binary mixtures: a statistical approach. Arch. Environ. Contam. Toxicol 36, 365–372.
International Organization for Standardization, 2007. Water quality - Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test), ISO 11348-3 Revised version, Geneva, Switzerland.
Jia, J., Zhu, F., Ma, X., Cao, Z.W., Li, Y.X., Chen, Y.Z., 2009. Mechanisms of drug combinations: interaction and network perspectives. Nat. Rev. Drug Discov 8 (2), 111–128.
Junghans, M., Backhaus, T., Faust, M., Scholze, M., Grimme, L.H., 2006. Application and validation of approaches for the predictive hazard assessment of realistic pesticide mixtures. Aquat. Toxicol 76 (2), 93–110.
Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2009. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. Water Res. 43 (2), 363–380.
Lambropoulou, D.A., Hernando, M.D., Konstantinou, I.K., Thurman, E.M., Ferrer, I., Albanis, T.A., Fernandez-Alba, A.R., 2008. Identification of photocatalytic degradation products of bezafibrate in $TiO_2$ aqueous suspensions by liquid and gas chromatography. J. Chromatogr. A 1183 (1–2), 38–48.
Metcalfe, C.D., Miao, X.S., Koenig, B.G., Struger, J., 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environ. Toxicol. Chem. 22 (12), 2881–2889.
Miller, D.B., Spence, J.D., 1998. Clinical pharmacokinetics of fibric acid derivatives (fibrates). Clin. Pharmacokinet 34 (2), 155–162.
Pomati, F., Orlandi, C., Clerici, M., Luciani, F., Zuccato, E., 2008. Effects and interactions in an environmentally relevant mixture of pharmaceuticals. Toxicol. Sci. 102 (1), 129–137.
Quinn, B., Gagne, F., Blaise, C., 2009. Evaluation of the acute, chronic and teratogenic effects of a mixture of eleven pharmaceuticals on the cnidarian, *Hydra attenuata*. Sci. Total Environ 407 (3), 1072–1079.
Rodea-Palomares, I., González-García, C., Leganés, F., Fernández-Piñas, F., 2009. Effect of pH, EDTA, and anions on heavy metal toxicity toward a bioluminescent cyanobacterial bioreporter. Arch. Environ. Contam. Toxicol. doi:10.1007/s00244-008-9280-9.
Rodríguez, A., Rosal, R., Perdigón, J.A., Mezcua, M., Agüera, A., Hernando, M.D., León, P., Fernández-Alba, A.R., García-Calvo, E., 2008. In: Barceló, D., Petrovic, M. (Eds.), Emerging Contaminants from Industrial and Municipal Waste. Springer-Verlag, Berlin, pp. 127–175.
Rosal, R., Rodríguez, A., Perdigón-Melón, J.A., Mezcua, M., Hernando, M.D., León, P., García-Calvo, E., Agüera, A., Fernández-Alba, A.R., 2008. Removal of pharmaceuticals and kinetics of mineralization by $O_3/H_2O_2$ in a biotreated municipal wastewater. Water Res. 42 (14), 3719–3728.
Rosal, R., Rodea-Palomares, I., Boltes, K., Fernández-Piñas, F., Leganés, F., Gonzalo, S., Petre, A., 2009. Ecotoxicity assessment of lipid regulators in water and biologically treated wastewater using three aquatic organism. Environ. Sci. Pollut. Res. doi:10.1007/s11356-009-0137-1.

Scatena, R., Bottoni, P., Botta, G., Martorana, G.E., Giardina, B., 2007. The role of mitochondria in pharmacotoxicology: a reevaluation of an old, newly emerging topic. Am. J. Physiol. Cell Physiol 293 (1), C12–C21.

Staels, B., Dallongeville, J., Auwerx, J., Schoonjans, K., Leitersdorf, E., Fruchart, J.C., 1998. Mechanism of action of fibrates on lipid and lipoprotein metabolism. Circulation 98 (19), 2088–2093.

Stumpf, M., Ternes, T.A., Wilken, R.D., Rodrigues, S.V., Baumann, W., 1999. Polar drug residues in sewage and natural waters in the state of Rio de Janeiro. Brazil. Sci. Total Environ 225 (1–2), 135–141.

Teuschler, L.K., 2007. Deciding which chemical mixtures risk assessment methods work best for what mixtures. Toxicol. Appl. Pharmacol 223 (2), 139–147.

Vighi, M., Altenburger, R., Arrhenius, A., Backhaus, T., Bodeker, W., Blanck, H., Consolaro, F., Faust, M., Finizio, A., Froehner, K., Gramatica, P., Grimme, L.H., Gronvall, F., Hamer, V., Scholze, M., Walter, H., 2003. Water quality objectives for mixtures of toxic chemicals: problems and perspectives. Ecotoxicol. Environ. Saf 54 (2), 139–150.