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Ecotoxicity assessment of lipid regulators in water and biologically treated wastewater using three aquatic organisms

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Abstract
Background, aim, and scope The high consumption of blood lipid regulators is leading to frequent reports of the occurrence of fibrates in natural streams and wastewater effluents. This paper describes a study undertaken to evaluate the acute toxicity of bezafibrate, clofibric acid, gemfibrozil, and fenofibric acid, a metabolite of fenofibrate whose ecotoxicity has not been previously reported.
Materials and methods The bioassays used were based on *Vibrio fischeri*, *Daphnia magna*, and *Anabaena* CPB4337 tests. *Anabaena* CPB4337 is a novel bioassay based on *Anabaena* sp. PCC 7120 strain CPB4337 bearing in the chromosome a Tn5 derivative with *luxCDABE* from the luminescent terrestrial bacterium *Photorhabdus luminescens*.
Results The higher toxicity corresponded to fenofibric acid, with EC_{50} as low as 1.72 mg/l for *V. fischeri*. Gemfibrozil was also toxic for *Anabaena* sp. with EC_{50} of 4.42 mg/l. The study reports the results from toxicity tests using fortified real wastewater samples taken from the effluent of a wastewater treatment plant. The wastewater itself was found to be very toxic to *Anabaena* CPB4337 (84% of bioluminescence inhibition) whereas it did not have any negative effect on *D. magna* or *V. fischeri*. On the contrary, *V. fischeri* luminescence exhibited a stimulatory effect in wastewater.
Discussion Except for fenofibric acid, the *Anabaena* bioassay was more sensitive than the *D. magna* and *V. fischeri* bioassays to bezafibrate, clofibric acid, and gemfibrozil. For the three toxicity tests, fortification resulted in lower measured toxicity for the four compounds, probably indicating a reduced bioavailability due to the interaction with other chemicals in the wastewater or with particulate matter. The observed decrease in toxicity associated to the use of a wastewater matrix was higher for the more hydrophobic compounds reaching one order of magnitude for bezafibrate and gemfibrozil.
Conclusions The *Anabaena* CPB4337 bioassay revealed a certain risk associated with the three less toxic compounds tested. Based on *V. fischeri* and *D. magna* bioassays, bezafibrate and gemfibrozil would have been considered non-toxic and harmful, respectively. The use of EC_{50} data measured in wastewater increases the risk estimation.
Recommendations and perspectives Cyanobacteria, as primary producers with a key role in the carbon and nitrogen cycles, are a substantial component of the microbial food webs. Any detrimental effect on this group may have a negative impact in nutrient availability to organisms of higher trophic levels and should be considered in ecotoxicity assessment tests.

Keywords *Anabaena* sp. · *Daphnia magna* · Ecotoxicity · Lipid regulators · *Vibrio fischeri* · Wastewater

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1 Background, aim, and scope

The occurrence and fate of pharmaceutically active compounds in the aquatic environment have become a major cause for concern due to their effects on humans and aquatic *ecosystems* (Daughton and Ternes 1999; Kümmerer 2001; Gagné et al. 2006). Pharmaceuticals are continuously released into the environment and lead to a widespread distribution (Jørgensen and Halling-Sørensen 2000), with the effluents of sewage treatment plants (STP) being the prevailing path that these compounds follow to enter surface water streams (Gagné et al. 2006). The risks associated with the discharge of pharmaceuticals into the environment are due not only to their acute toxicity but also their genotoxicity, development of pathogen resistance, and endocrine disruption (Halling-Sørensen et al. 1998). The constant presence of these biologically active xenobiotics exposes aquatic organisms to accumulative and multigenerational exposure with a risk of changes that may remain undetected while causing irreversible damage (Daughton and Ternes 1999). Two effects contribute to enhance this risk: first, many pharmaceuticals are not completely destroyed in conventional STP (Carballa et al. 2004); second, the metabolic and environmental degradation of these compounds produce a huge variety of metabolites and degradation products that increase the complexity of wastewater mixtures. The existence of matrix effects and non-additive interactions suggests the necessity of considering combination effects even for less toxic substances (Cleuvers 2003).

Fibrates are a class of drugs derived from fibric acid widely used to reduce plasma triglycerides and raise the level of high-density lipoprotein cholesterol. Their active forms, fibric acids, exert their biological effects by binding peroxisome proliferator-activated receptor α, a member of the nuclear receptor superfamily of ligand-activated transcription factors. It has been stated that most fibrates are excreted unmodified (Isidori et al. 2007). Clofibric acid is a biological metabolite of the active substance clofibrate, ethyl 2-(4-chlorophenoxy)-2-methylpropanoate, a persistent drug used as lipid regulator that has been detected in surface waters even years after it fell out of use (Buser et al. 1998). The presence of clofibric acid in STP has been repeatedly reported after its detection in the effluent of a German treatment plant (Ternes 1998; Andreozzi et al. 2003; Tauxe-Wuersch et al. 2005). Heberer and Stan (1997) reported the presence of clofibric acid in drinking water from the Berlin area at concentrations up to 270 ng/l, probably due to artificial groundwater enrichment. Weigel et al. (2002) detected over 1 ng/l of clofibric acid in samples taken in the North Sea, whereas Boyd et al. (2003) reported 103 ng/l of clofibric acid at the inlet of a drinking treatment plant fed by Detroit River water. Bezafibrate, *p*-[4-[chlorobenzoylamino-ethyl]-phenoxy]-b-methylpropionic acid, is a drug extensively used as a lipid regulator whose consumption in developed countries has greatly increased during the last years (Lambropoulou et al. 2008). Due to its large use and its persistence, bezafibrate has been detected in surface and drinking waters as well as in effluents of STP in the range of nanogram per liter (Metcalfe et al. 2003a, b; Fent et al. 2006). Gemfibrozil, 5-(2,5- dimethylphenoxy)-2,2-dimethylpentanoic acid, is also a growingly used lipid-regulating agent generically classified as a fibric acid derivative. Metcalfe et al. (2003a, b) found levels around 1 μg/l in effluents of Canadian sewage treatment plants. Sanderson et al. (2003) detected similar values (0.75–1.50 μg/l) for the highest concentrations of gemfibrozil in surface waters in North America and Europe. Fenofibric acid, 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid, is the active metabolite of fenofibrate and has been reported in concentrations of up to 0.5 μg/l in the influent of several Brazilian STP (Stumpf et al. 1999). The same authors reported a low (45%) efficiency of removal of fenofibric acid by activated sludge conventional treatments and also reported an average of 40 ng/l in several natural streams in the state of Rio de Janeiro. In Europe, Andreozzi et al. (2003) reported the occurrence of lipid regulators in the effluent of several STP in Italy, France, and Greece at concentrations of up to 0.68 μg/l (clofibric acid), 4.76 μg/l (gemfibrozil), 1.07 μg/l (bezafibrate), and 0.16 μg/l (fenofibrate). In a former work (Rosal et al. 2008), bezafibrate and gemfibrozil were detected in amounts of 139 and 608 ng/l, respectively, in the same STP from which the samples used in this work were taken. In previous analyses for the same STP, Rodríguez et al. (2008) reported 165 ng/l of fenofibric acid, 61 ng/l of bezafibrate, and 143 ng/l of gemfibrozil. In the same sampling program, clofibric acid was detected with an average concentration of 24 ng/l.

The luminescence inhibition bioassay with marine *Vibrio fischeri* photobacteria has proven to be a useful tool in estimating the acute toxicity of many chemicals. Other standard ecotoxicity methods using algae, bacteria, invertebrates, and higher organisms many of which have been defined by Organization for Economic Cooperation and Development (OECD) guidelines for testing chemicals (OECD 2008) are also routinely used. This work used a novel toxicity test based on the strain denominated *Anabaena* sp. PCC 7120 strain CPB4337 that bears in the chromosome a Tn5 derivative with *luxCDABE* from the luminescent terrestrial bacterium *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*) (Szittner and Meighen 1990). This strain shows a high constitutive self-luminescence with no need to add exogenous aldehyde; cell viability is not significantly affected by the Tn5 insertion and the endogenous generation of aldehyde (Fernandez-

Pinas and Wolk 1994). Luminescence was shown to be high in a wide range of temperatures and pH values (Fernandez-Pinas and Wolk 1994; Fernandez-Pinas et al. 2000). Cyanobacteria were the first organisms to carry out the oxygenic photosynthesis and are thought to be the ancestors of the chloroplasts; thus, they are usually models to study photosynthetic processes whose results can be extrapolated to higher plants. Cyanobacteria are found virtually in all regions of the world. Originated during the Precambrian Era, they have been found to be the most abundant photosynthetic organisms on the planet today; they are at the base of the aquatic food webs, making air, light, and water into food for other forms of life. Due to its ecological relevance as a prokaryotic primary producer, this novel cyanobacterial bioreporter may be particularly useful to test toxicity in aquatic environments.

The objective of this study was to evaluate the toxicity of several fibric acid derivatives commonly used to treat hypercholesterolemia and universally found in urban and domestic wastewaters even after biological conventional treatments. The ecotoxicity tests used combined the well-established tests based on OECD and Deutsches Institut für Normung standards for *Daphnia magna* and bioluminescent bacteria *V. fischeri* with the novel bioassay that used a self-luminescent cyanobacterium denominated *Anabaena* sp. PCC 7120 strain CPB4337 (hereinafter, *Anabaena* CPB4337). Toxicity bioassays have also been performed in a real wastewater from the effluent of a secondary clarifier in an STP. Special attention has been paid to fenofibric acid, a compound whose presence has been recently reported in wastewaters but whose toxicity has not been experimentally assessed before.

2 Material and methods

2.1 Materials

Wastewater samples were collected from the secondary clarifier of an STP located in Alcalá de Henares (Madrid) that receives domestic wastewater with a minor contribution of industrial effluents from facilities located near the city, notably chemical and pharmaceutical small plants. The main wastewater characterization parameters are shown in Table 1. This STP used conventional activated sludge treatment and has been designed for a total capacity of 375,000 equivalent inhabitants with a maximum flow rate of 3,000 m^3/h. More detailed information has been given elsewhere and revealed that the individual pollutants detected in higher amounts are those commonly encountered in biologically treated wastewater, with a high contribution of stimulants, anti-inflammatories, antibiotics, β-blockers, and lipid regulators (Rosal et al. 2008).

Table 1 Main wastewater parameters

	Anions and cations (mg/L)		
pH	7.73	NO_3^-	36.03
Turbidity (NTU)	2.68	PO_4^{3-}	3.18
Conductivity (μS/cm)	702	SO_4^{2-}	81.66
TOC (mg/l)[a]	8.1	Cl^-	89.17
COD (mg/l)	62	Na^+	83.12
PO_4-P (mg/l)	1.1	K^+	15.10
NH_4^+-N (mg/l)	1.5	Mg^{2+}	19.45
NO_3-N (mg/l)	7.0	Ca^{2+}	38.02

[a] Filtered at 0.45 μm

Gemfibrozil (+99%), clofibric acid (97%), and bezafibrate (+98%) were purchased from Sigma Aldrich. Fenofibric acid was produced from fenofibrate (Sigma Aldrich, +99% purity) by way of 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 room temperature during the course of four more hours. The product was filtered and rinsed with water and dried overnight at 60°C under nitrogen. The purity of the product was over 97% and thereafter checked by high-performance liquid chromatography (HPLC) as described. Table 2 shows the main physicochemical properties of the four compounds studied. Solubility, being an important property, has not been included as the 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 3-(*N*-morpholino)propanesulfonic acid (MOPS) at pH 6 and higher quantities for the pH at which *V. fischeri* and *D. magna* 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.

2.2 Standard toxicity tests

Bioassays with the photo-luminescent bacteria *V. fischeri* were carried out according to ISO 11348-3 standard protocol (International Organization for Standardization 2007). During the prescribed incubation period, this bioassay measures 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 (*V. fischeri* NRRL-B 11177) and was reconstituted and incubated at 3° C for 5 min before use. The analysis media was 0.34 M NaCl (2% *w*/*v*) adjusted to the desired pH with NaOH or HCl. The measurements were performed at 15°C using an Optocomp I luminometer. The effect of toxics was measured as percent inhibition with respect to the light emitted under test conditions in the absence of any toxic influence. Toxicity values are routinely obtained after 15- or 30-min exposure and are usually expressed as the median effective concentration values (EC_{50}) at which a 50% loss of luminescence is obtained. Phenol and $ZnSO_4$•$7H_2O$ have been used as toxicity standards and all tests have been replicated to ensure reproducibility and in order to obtain acceptable confidence intervals. In analyses performed using wastewater as a matrix, the reference was always the same wastewater after adjusting osmotic pressure.

Acute immobilization tests with *D. magna* were conducted following the standard protocol described in the European Guideline (Commission of the European Communities 1992). The *D. magna* bioassay used a commercially available text kit (Daphtoxkit F™ magna, Creasel, Belgium). The dormant eggs were incubated in standard culture medium imitating natural freshwater at 20±1°C under continuous illumination of 6,000 lx in order to induce hatching. Between hatching and test steps, the daphnids were fed with the microalgae *Spirulina* to avoid mortality during tests. The pH of samples was adjusted to be in the tolerance interval of the test organisms (Seco et al. 2003). Test plates with *D. magna* neonates were incubated for 24–48 h in the dark at 20°C. Acute toxicity was assessed by observing the effects of the test compounds on the mobility of *D. magna*. The neonates are considered immobilized if they lie on the bottom of the test plate and do not resume swimming within a period of 15 s. Acute toxicity is expressed in this test as the median effective concentration (EC_{50}) leading to the immobilization of 50% of the daphnids after 48 h.

2.3 Cyanobacterial test methods

2.3.1 Strain and culture conditions

Anabaena CPB4337 was routinely grown at 28°C in the light, approximately 65 µmol photons per square meter per second 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 sulfate (Nm).

2.3.2 Determination of acute toxicity

The toxicity bioassays using *Anabaena* CPB4337 are based on the inhibition of constitutive luminescence caused by the presence of a toxic substance. Acute (1 and 24 h of exposure) luminescence inhibition-based toxicity assays were performed as follows: 160 µl from five to seven serial dilutions of each tested toxicant plus a control (ddH_2O buffered with MOPS at pH 5.8) were disposed in an opaque white 96-well microtiter plates. 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. For 1-h toxicity testing, the luminescence of each sample was recorded every 5 min for up to 1 h in the Centro LB 960 luminometer. For 24-h toxicity testing, the 96-well microtiter plates were kept at room temperature (28°C) at low light (approximately 30 µmol photons per square meter per second) during 24 h and luminescence was recorded for 5 min. Three independent experiments with quadruplicate samples were carried out for all *Anabaena* toxicity assays.

2.3.3 Reference toxicant and inter-assay variability calibration

Copper sulfate ($CuSO_4$) was selected as reference toxicant for calibration in all assays. The calibration allows one to calculate the mean $EC_{50\text{-}1\,h}$ and $EC_{50\text{-}24\,h}$ of copper in order to refuse or accept the experiment if those EC_{50} values fall in or out of the 95% of confidence limits previously fixed for this reference toxicant (USEPA 1994, 2002). To achieve

Table 2 Physicochemical properties of bezafibrate, clofibric acid, fenofibrate, and gemfibrozil

	Bezafibrate	Clofibric acid	Fenofibrate	Gemfibrozil
CAS no.	41859-67-0	882-09-7	49562-28-9	25812-30-0
Molecular formula				
Molecular weight (g/mol)	361.819	214.645	360.831	250.333
pK_a	3.6 (1)	3.18 (2)	2.86 (3) [a]	4.8 (4)
log K_{ow}	4.25 (5)	2.88 (6)	5.19 (7)	4.77 (5)
Percent removal in STP (8)	27–50	15–34	6–45 [a]	16–46

(*1*) Huber et al. 2003, (*2*) Packer et al. 2003, (*3*) Lewis and Lakeb 1998, (*4*) Lin et al. 2006, (*5*) Han et al. 2006, (*6*) Scheytt et al. 2005, (*7*) Hernando et al. 2007, (*8*) Stumpf et al. 1999

[a] Fenofibric acid

this, five copper dilutions were tested by quadruplicate in control wells of each assay.

2.4 Data analysis

The quantitative response to toxic exposure in *V. fischeri* and *D. magna* tests was estimated by fitting the experimental luminescence inhibition or immobilization data to a logistic function:

$$E[Y|x] = \frac{\alpha}{1 + \left(\frac{x}{EC_{50}}\right)^{\beta}} \quad (1)$$

Where E[$Y \mid x$] represents the average response at dosage x, and α and β are the upper asymptote of response and parameters related to the rate of change at the inflection point of the curve, respectively. The elaboration of experimental data was performed by non-linear regression analysis using conventional statistical inference tools through which EC_{50} results were expressed with their corresponding intervals at a given p level of confidence (Schabenberger et al. 1999).

Toxicity response of the cyanobacterium was estimated also as EC_{50} values, the median effective concentration of a toxicant that causes 50% of bioluminescence inhibition with respect to a non-treated control. EC_{50} and its related statistical parameters, standard deviation, coefficient of variation, and confidence intervals (CI) were estimated using the linear interpolation method (Norberg-King 1993; USEPA 1994, 2002).

2.5 Fortification of wastewater

In order to assess the matrix effect associated with real wastewater samples, bezafibrate, clofibric acid, fenofibric acid, and gemfibrozil were dissolved in wastewater at the pH required by each bioassay. The concentration range was adapted as required to obtain acceptable standard deviation in the computing of EC_{50} values.

2.6 Stability analyses

The stability of target compounds under bioassay conditions was assessed according to OECD guidance (OECD 2008). In this work, analyses have been performed at the start and at the end of tests lasting 24 h (*Anabaena* CPB4337) and 48 h (*D. magna*). The test has been carried out for the lower and higher concentrations as well as for a concentration near EC_{50} in pure water for each compound. Analyses were conducted at room temperature using an HPLC–diode array liquid chromatograph (Varian) equipped with automated injection of 50-µl sample volumes. The column used was a C18 of 250×4.6 mm, 5 µm (Phenomenex). Isocratic elution of gemfibrozil, clofibric, and fenofibric acids was performed using a mixture of acrylonitrile and deionized water (with 4 ml/l of phosphoric acid and 50 ml/l of methanol) at 1 ml/min and 60:40 (fenofibric acid and gemfibrozil) or 70:30 (clofibric acid). Bezafibrate was analyzed using isocratic elution of methanol and deionized water (70:30) with 0.09 M of acetic acid at 1 ml/min. Detection was performed at 280 nm for gemfibrozil and fenofibric acid, at 230 nm for clofibric, and at 228 nm for bezafibrate.

3 Results and discussion

The toxicity can be assessed from EC_{50} values according to the categories established in the technical guidance on risk assessment of substances in the European Union (Commission of the European Communities 1996). In it, wastewater samples or standard compounds are considered "harmful to aquatic organisms" if EC_{50} falls in the 10–100-mg/l range, "toxic" if $1 < EC_{50} < 10$ mg/l and "very toxic" if $EC_{50} <$ 1 mg/l. The results of toxicity tests for fibrates in pure water are reported in Table 3 together with their 95% confidence intervals. The data showed that fenofibric acid could be considered toxic based on *V. fischeri* and *D. magna* tests. Gemfibrozil and bezafibrate exhibited $EC_{50} < 10$ mg/l using

Table 3 Toxicity of lipid regulators in pure water as EC_{50} values (mg/l) with confidence limits (95% probability)

Compounds	*V. fischeri*[a]	*V. fischeri*[b]	*Anabaena* sp. 4337[c]	*Anabaena* sp. 4337[d]	*D. magna*
Bezafibrate	178.73 (162.06–197.12)	172.73 (155.52–191.85)	37.28 (32.60–41.79)	7.62 (7.01–8.41)	240.40 (230.12–250.68)
Clofibric acid	290.04 (269.24–310.84)	240.65 (202.57–278.73)	48.08 (45.82–55.92)	30.80 (26.50–42.39)	83.52 (70.41–96.63)
Fenofibric acid	1.86 (1.64–2.08)	1.72 (1.48–1.96)	10.82 (8.46–13.35)	10.85 (6.16–13.16)	4.90 (3.74–6.06)
Gemfibrozil	35.34 (33.22–37.66)	29.07 (26.77–31.37)	8.44 (7.81–9.24)	4.42 (4.06–4.57)	22.85 (17.01–28.69)

[a] Fifteen minutes of exposure

[b] Thirty minutes of exposure

[c] One hour of exposure

[d] Twenty-four hours of exposure

the new ecotoxicity test based on *Anabaena* CPB4337 and both could also be considered toxic to the cyanobacterium, whereas, according to *V. fischeri* and *D. magna* tests, they would have been classified only as harmful and non-toxic, respectively. Exposure of *Anabaena* to bezafibrate, clofibric acid, and gemfibrozil for 24 h resulted in a significant increase of toxicity (Student's t test, $P<0.05$). It is interesting to point out that the four fibrates can be considered at least harmful to *Anabaena* CPB4337 even in the case of substances that do not evidence toxicity such as bezafibrate or the less toxic clofibric acid when using classic tests. The stability assessment tests showed that the concentrations of all tested compounds were essentially constant during the analyses, always inside the boundary established by OECD (2008).

Table 4 shows toxicity data reported by other authors that used ecotoxicity tests based on *V. fischeri* with 30 min of exposure and *D. magna* at 48 h. The estimations of Sanderson et al. (2003) that used a model based on structure activity relationship (SAR) have been included for comparison. Our results show that gemfibrozil proved more toxic to *V. fischeri* and *D. magna* than most data reported in the literature. The cyanobacterial bioassay that we have developed showed the greatest sensitivity towards bezafibrate, clofibric acid, and gemfibrozil, thus indicating the suitability of this new test to check toxicity of these types of pollutants. Table 4 also evidences an apparent variability of data both within the same and between different tests that is usually found in ecotoxicity data reported from different sources. For example, bezafibrate toxicity to *D. magna* (EC_{50}, 48 h) varies between 30.3 and over 200 mg/l (168.3 mg/l in this work). This low reproducibility due to the complexity of biological tests has been attributed to different actual exposure concentrations, changing sensitivities of the organisms, and diverse laboratory performances (Fent et al. 2006).

The toxicity of fenofibric acid has been assessed for the first time in this work. Previously, only an estimation based on SAR models whose precision was determined to be low not only for fenofibric acid but also for other fibrates, as indicated in Table 4 (Sanderson et al. 2003), was reported. Among the four fibrates, the toxicity of fenofibric acid was highest for *V. fischeri* and lowest for *Anabaena* CPB4337, a result opposite to that obtained with bezafibrate, clofibric acid, and gemfibrozil, for which the new test exhibited a higher sensitivity. These results stress the need for gathering information from different organisms before assessing the environmental risks associated with drugs spread into the environment. A restriction to conventional well-established tests could yield a risk underestimation.

Table 5 shows the toxicity of wastewater to the three test organisms used in this work. The wastewater, whose main

Table 4 Literature toxicity data for bezafibrate, clofibric acid, and gemfibrozil

Compound	Bioassay	EC_{50} (mg/l) and 95% CI	Reference
Bezafibrate	*V. fischeri* (30 min)	>100	Isidori et al. 2007
	D. magna (24 h)	100.08 (80.02–120.54)	Isidori et al. 2007
	D. magna (48 h)	>200	Hernando et al. 2004
	D. magna (48 h)	30.3	Han et al. 2006
	D. magna (48 h)	25.00[a]	Sanderson et al. 2003
Clofibric acid	*V. fischeri* (30 min)	100	Henschel et al. 1997
	V. fischeri (30 min)	91.8	Ferrari et al. 2003
	D. magna (48 h)	>200	Ferrari et al. 2003
	D. magna (48 h)	106	Henschel et al. 1997
	D. magna (48 h)	150	Hernando et al. 2004
	D. magna (48 h)	72	Cleuvers 2003
	D. magna (48 h)	141.2	Han et al. 2006
	D. magna (48 h)	293.00[a]	Sanderson et al. 2003
Fenofibric acid	*D. magna* (48 h)	38.00[a]	Sanderson et al. 2003
Gemfibrozil	*V. fischeri* (15 min)	180 (174–186)	Zurita et al. 2007
	V. fischeri (30 min)	85.74 (77.22–91.74)	Isidori et al. 2007
	D. magna (24 h)	74.30 (66.15–83.45)	Isidori et al. 2007
	D. magna (24 h)	228 (214–242)	Zurita et al. 2007
	D. magna (48 h)	100	Hernando et al. 2004
	D. magna (48 h)	170 (156–184)	Zurita et al. 2007
	D. magna (48 h)	10.4	Han et al. 2006
	D. magna (48 h)	6.00[a]	Sanderson et al. 2003

[a] Predicted by a structure activity relationship model

Table 5 Toxicity values obtained for an STP effluent

toxicity test	EC_{50}[a]	SD	CV (%)	CI_L 95%	CI_U 95%
V. fischeri	ls				
Anabaena sp. 4337[b]	0.11	0.01	9.09	0.09	0.16
Anabaena sp. 4337[c]	0.66	0.08	12.12	0.47	0.42
D. magna	nt				

Ls luminescence stimulation (effect described in the text), *nt* non-toxic with immobilization <5%

[a] STP effluent dilution that causes 50% inhibition of luminescence in *V. fischeri* and *Anabaena* 4337 tests and 50% immobilization of daphnids in *D. magna* test. CI_L 95% and CI_U 95% are lower and upper 95% confidence limits, respectively

[b] One hour of exposure

[b] Twenty-four hours of exposure

characteristics are indicated in Table 1, was non-toxic to *D. magna*, with immobilization of <5% in three replicates. *V. fischeri* tests exhibited a luminescence increase of near 50% during the first 5 min to decay thereafter that resulted in negative toxicity values when compared to the blank. Although rarely reported in *V. fischeri* bioassays, this effect has been repeatedly observed in our laboratory for wastewaters of a different origin. Early works have shown that subinhibitory concentrations of a toxic substance may yield stimulatory effects on an organism (Southman and Ehrlich 1943). This behavior, called hormetic, has been attributed to the salinity correction of the sample in the *V. fischeri* bioassay that may alter the pollutant bioavailability, thereby developing a false-negative toxicity response (Abbondanzi et al. 2003). Recently, Deryabin and Aleshina (2008) found a stimulatory effect of some salts such as chlorides and sulfates on the luminescence response of *Photobacterium phosphoreum* and recombinant *Escherichia coli* with cloned *lux*CDABE genes of *Photobacterium leiognathi* 54D10. Opposite the result found with *D. magna* and *V. fischeri*, the wastewater was very toxic to *Anabaena* CPB4337, causing a luminescence inhibition of 84.24% (76.14–91.42). As shown in the table, a wastewater dilution as low as 0.11 caused 50% inhibition of luminescence in the acute toxicity test. The observed toxicity could be due to the combined toxicities of the chemicals, pharmaceuticals, herbicides, or metals present in this wastewater. As a reference, an analysis of over 30 micropollutants in wastewater from the same STP has been given elsewhere (Rosal et al. 2008). The fact that the wastewater itself was already toxic to *Anabaena* but non-toxic to *V. fischeri* or *D. magna* highlights the need to develop and use new toxicity tests based on organisms of different trophic levels or origins in order to achieve a more complete assessment of the ecotoxicological risk of pollutants.

In order to assess the risk of fibrates in a real matrix, ecotoxicity tests have been applied to wastewater fortified with different concentrations of the compounds studied in this work. The effect of fibrates in wastewater, measured as EC_{50} and corresponding to wastewater fortified with different amounts of the target compounds, is shown in Table 6. When using the *V. fischeri* and *D. magna* tests, the EC_{50} values found in wastewater were considerably higher than those obtained in pure water by a factor in the range of 2–13×. The lower measured toxicity that resulted from fortification of real wastewater is probably reflecting a reduced bioavailability due to interactions with other chemicals or with particulate matter. The observed decrease

Table 6 Toxicity effects of lipid regulators in fortified STP effluent expressed as EC_{50} values (mg/l) with confidence limits (95% probability)

Compounds	*V. fischeri*[a]	*V. fischeri*[b]	*Anabaena* sp. 4337[c]	*Anabaena* sp. 4337[d]	*D. magna*
Bezafibrate	>250	>250	>100	>100	>500[e]
Clofibric acid	>500	>500	>400	62.70 (56.30–66.03)	366.69 (337.39–395.99)
Fenofibric acid	4.31 (3.97–4.65)	4.11 (3.75–4.47)	>20	13.80 (13.38–14.50)	16.88 (14.13–19.63)
Gemfibrozil	134.15 (123.15–145.15)	153.29 (146.91–212.59)	4.81 (2.90–5.70)	59.16 (57.00–66.00)	121.12 (113.89–128.36)

[a] Fifteen minutes of exposure

[b] Thirty minutes of exposure

[c] One hour of exposure

[d] Twenty-four hours of exposure

[e] Immobilization <10% at 500 mg/l

in toxicity associated with the use of a wastewater matrix was higher for the more hydrophobic compounds, reaching one order of magnitude for bezafibrate and gemfibrozil, and with a more pronounced effect on *Anabaena* bioassay. In this regard, it should be noted that the wastewater itself was already very toxic to *Anabaena*. One would expect that the addition of fibrates to the wastewater, also toxic to *Anabaena*, would completely inhibit luminescence. However, except for fenofibric acid, for which there was no significant difference, the EC_{50} values were also higher than those reported in pure water. These results favor the already mentioned hypothesis of antagonistic effects of fibrates with some of the chemicals that are in the wastewater (Rosal et al. 2008). A wide diversity of pharmaceuticals, personal care products, or pesticides that are not completely eliminated in conventional wastewater treatments may reach freshwater sources, leading to a long-term exposure of aquatic organisms in a mixture of these compounds; a systematic analysis of the mixture toxicities of these chemicals should be carried out in order to better understand their biological effects.

As indicated before, the reported concentrations of individual pollutants typically exceed the 10 ng/l cutoff value established in the European Medicines Evaluation Agency (EMEA) tiered procedure that corresponds to 100 ng/l in STP effluents which was once considered the conventional dilution factor, as indicated before (European Medicines Evaluation Agency 2006). Predicted no-effect concentrations (PNEC) can be estimated from their corresponding EC_{50} values obtained in acute toxicity tests by application of an assessment factor as indicated in the EMEA procedure. The guideline prescribes an assessment factor of 1,000 when using the information of short-term EC_{50} from each of three trophic levels of the base set (fish, *Daphnia*, and algae). The results show a clear evidence of adverse effects in water as result of measured environmental concentration of fenofibric acid for which *V. fischeri* was particularly sensitive. For the other tested compounds, the cyanobacterium *Anabaena* CPB4337 led to the lowest estimation of PNEC. If ecotoxicity is measured in wastewater, the observed increase in EC_{50} values roughly corresponds with the dilution factor leading to similar risk estimations for the four compounds. The results show the risk of extrapolation of PNEC based on EC_{50} values obtained from the conventional set of toxicity tests on freshwater, as the introduction of a new species or a change in the matrix may considerably alter the risk level for a certain substance. An additional cause for concern is the potential bioaccumulation of fibrates, as indicated by log $K_{ow}>3$ with the lowest value (clofibric acid) very close to the boundary and with the rest clearly above, as shown in Table 2.

4 Conclusions

A novel bioassay that used a self-luminescent cyanobacterium denominated *Anabaena* sp. PCC 7120 strain CPB4337 has been compared to standard ecotoxicity tests based on *V. fischeri* and *D. magna* for the ecotoxicity assessment of four lipid regulators. The toxicity of fenofibric acid, a compound whose ecotoxicity had not been previously assessed, was particularly high with EC_{50} values as low as 1.72 mg/l for *V. fischeri*. The new test of *Anabaena* CPB4337 showed a greater sensitivity for bezafibrate, clofibric acid, and gemfibrozil. The introduction of *Anabaena* bioassay would allow one to reclassify bezafibrate and clofibric acid from "non-toxic" ($EC_{50}>$ 100 mg/l) to "harmful to aquatic organisms" (10 mg/l< $EC_{50}<100$ mg/l). The assessment of toxicity using a matrix of real wastewater leads to higher EC_{50} values that correspond with a lower evidence for toxicity. The more hydrophobic the compound, the higher was the difference, thus suggesting that the effect is probably linked to a reduced bioavailability of the less polar compounds. The results encourage the development of new and more sensitive toxicity tests for the detection of unwanted toxic effects that might become overlooked using conventional bioassays.

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