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Nicolas Mater, Florence Geret, Luis Castillo, Virginie Faucet-Marquis, Claire Albasi, et al.. In vitro tests aiding ecological risk assessment of ciprofloxacin, tamoxifen and cyclophosphamide in range of concentrations released in hospital wastewater and surface water. *Environment International*, 2014, 63, pp.191-200. 10.1016/j.envint.2013.11.011 . hal-01893117

**HAL Id: hal-01893117**

**<https://hal.science/hal-01893117v1>**

Submitted on 11 Oct 2018

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### To cite this version:

Mater, Nicolas and Geret, Florence and Castillo, Luis and Faucet-Marquis, Virginie and Albasi, Claire and Pfohl-Leszkowicz, Annie In vitro tests aiding ecological risk assessment of ciprofloxacin, tamoxifen and cyclophosphamide in range of concentrations released in hospital wastewater and surface water. (2014) *Environment International*, 63. 191-200. ISSN 0160-4120

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# *In vitro* tests aiding ecological risk assessment of ciprofloxacin, tamoxifen and cyclophosphamide in range of concentrations released in hospital wastewater and surface water

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## A B S T R A C T

Ciprofloxacin (CIP), tamoxifen (TAM) and cyclophosphamide (CP) which are often used in anticancer treatment are released in hospital effluent and into the environment. Although the concentrations are low (from ng/L to µg/L), no data exist concerning their ecotoxicological impact. In this study two biomarkers of early effect were performed on hepatic cells (HepG2): cell viability and genotoxicity (DNA breaks) using cell proliferative assay and comet assay, respectively. These data were compared with two standardized ecotoxicological tests: algaltoxkit F™ and microtox®. Cells were exposed to an increasing amount of an individual drug or in a mixture for 24, 48 or 72 h. The time-exposure of bacteria and algae ranged between 5 and 30 min and 72 h, respectively. A non-monotonic dose–response on cell viability was observed when HepG2 cells were exposed to TAM alone or in the presence of CIP. The same scheme was observed with microtox® when the bacteria were exposed to the mixtures. On the other side, an individual drug does not induce any DNA breaks on hepatic cells, whereas a mixture leads to a dose dependent increase of DNA breaks. Similarly a positive response was observed with algaltoxkit F™ only with mixtures. Synergistic effects observed when drugs are in a mixture highlight the importance of investigating the ecotoxicological effects of contaminants at low concentrations and in mixtures.

### Keywords:

Ciprofloxacin  
Tamoxifen  
Cyclophosphamide  
Wastewater  
Hormesis  
Bioassays

## 1. Introduction

All over the world, the number of cancers is in constant progression leading to a dramatic increase of treatments. These treatments are chemotherapy and radiotherapy which can be used combined or not. Chemotherapy is realized by means of anticancer drugs having toxic properties for cells, and of which most are classified in three categories by the International Agency for Research on Cancer (IARC): carcinogenic impact for humans (class 1); likely carcinogenic (class 2); and potentially carcinogenic (class 2B). Cytostatic drugs act on cell growth inhibition or directly kill cells but in an unselective way on both tumoral and healthy cells. Therefore, many antineoplastic agents have cytotoxic, mutagenic, carcinogenic, embryotoxic and/or teratogenic effects. During the treatments, these drugs are eliminated in large proportion under parent compounds or metabolite forms *via* the urine and feces of patients. Hospital effluents which are generally released directly to the sewage

network without pretreatment represent an incontestable release source of many bioactive substances used for medical and research purposes such as pharmaceuticals, radioisotopes or solvents (Emmanuel et al., 2005; Verlicchi et al., 2012). Although many of these compounds are removed by adsorption (Carabineiro et al., 2011) or biodegradation in wastewater treatment plants (WWTP), some molecules are directly released into the environment (Besse et al., 2012; Gartiser et al., 1996; Halling-Sørensen et al., 1998; Kiemmerer et al., 1997) where they represent a potential toxic hazard for ecosystems and human health *via* drinking water (Johnson et al., 2008; Rowney et al., 2009; Zegura et al., 2009). Cytostatic agents are mainly found in hospital wastewater, and their use increases by 10% each year. WWTPs can eliminate some cytostatic agents, but the removal rate is not equal between molecules (e.g. 25% for cyclophosphamide and 75% for methotrexate) (Catastini et al., 2010). Concentrations of contaminants in potable water are usually in the range from subnanogram/L to µg/L, which is generally below the detection limit of biological assays (Besse et al., 2012). Nevertheless many of them or their metabolites raise considerable toxicological concerns particularly when present as components of complex mixtures (Hernando et al., 2006; Zegura et al., 2009). For example, some endocrine active drugs possess an extremely high biological potency down to the µg/day; therefore it is very likely that they also cause effects at very

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low concentrations in the environment. Thus, the contraceptive 17 $\beta$ -ethinylestradiol adversely affects the reproduction of zebrafish (*Danio rerio*) at concentrations as low as 1 ng/L (Wenzel et al., 2001). Data reported by Coe et al. (2008) suggest that exposure to some estrogens at low ng/L concentrations detected in recipient water bodies can even deteriorate the reproductive behavior by alternating mating habits.

The risk posed by cytostatic molecules and by mixtures is still not well documented and it is not possible to conclude on their long-term effects on non-target organisms. Until today, ecotoxicological effects have been assessed by standardized tests or by non specific *in vitro* assays. Such a test however may not be suitable for anticancer drugs (Zoukova et al., 2010). Tools to characterize the drug removal process efficiency and to assess the impact of these pollutants on aquatic life and human health must be further developed and refined.

The aim of this paper was to compare potential biomarkers sensitive enough for toxic evaluation of low doses of drugs found in hospital effluents, river or drinking water. We selected three compounds (ciprofloxacin (CIP), cyclophosphamide (CP), tamoxifen (TAM), Fig. 1) frequently used in cancer treatment and found at low doses in hospital wastewater, municipal wastewater treatment plants (WWTPs) and surface water (Hartmann et al., 1998; Thomas and Hilton, 2004; Zuccato et al., 2005). Cyclophosphamide (CP) is primarily used as an anticancer chemotherapeutic but is also used as an immunosuppressive agent in several treatments of arthritis, interstitial pneumonia, etc. or during organ transplantation. Tamoxifen (TAM) is a selective estrogen receptor modulator used in breast cancer treatment, which acts as an antagonist competitor in the  $\alpha$ -ER1 estrogen receptor (mammary tissues) and like an agonist competitor in cholesterol metabolism (Coezy et al., 1982; Jordan and Koerner, 1975). TAM belongs to compounds put on the prioritization list, due to the potential of this substance to bioaccumulate in the human body and probably in aquatic organisms (Jean et al., 2012). In fish, dose-dependent effects have been reported on the rate of vitellogenin synthesized in males and on sex-ratio disturbance of the population (also dose-dependent) whose parents were exposed to concentrations of tamoxifen higher than 5  $\mu$ g/L (Liwei et al., 2007). Ciprofloxacin (CIP), which belongs to the fluoroquinolone class, is an antibiotic that inhibits the enzyme activity necessary for replication, transcription, repair and recombination of bacterial DNA (McLellan et al., 1996). This drug is often used against the side effects of treatment protocols, particularly in fever prevention and infections arising after chemotherapy of breast cancer, cancer of the ovaries or the small cell lung cancer (SCLC) (Tjan-Heijnen et al., 2001).

The three drugs can be found in wastewater in the range of 10 ng/L to 10  $\mu$ g/L for TAM and CP, and 10 ng/L to 100  $\mu$ g/L for CIP (Lindberg et al., 2004, 2005; Roberts and Thomas, 2006; Thomas and Hilton, 2004; Zorita et al., 2009; Zuccato et al., 2005). As CIP is widely used for therapeutic treatments it is 10-fold more concentrated in hospital effluents and 2-fold more concentrated in municipal WWTP effluents than TAM and CP. Among the fluoroquinolones, ciprofloxacin (CIP) was the most frequently detected in wastewater and surface waters with concentrations of several hundred ng/L (Karthikeyan and Meyer, 2006; Kolpin et al., 2002; Miao et al., 2004). Higher concentrations up to 150  $\mu$ g/L were even reported in the effluents from hospitals (Martins et al., 2008).

The application of bioassays is an important approach for investigating the causal relationship between exposure to environmental pollutants and the effects on individuals and populations. For example, some microalgae are known to be highly sensitive to the contamination of aquatic environments (Eguchi et al., 2004). Algae play an important role in the equilibrium of aquatic systems where they are involved in nutrient cycling and O<sub>2</sub> production. They therefore form the base of many aquatic food chains; consequently, death of algae will impact aquatic life. They are considered indicators of bioactivity, such as industrial waste, and vary in response to a variety of toxicants (Ma et al., 2006) and reflect chronic aquatic toxicity. On the other hand Microtox® reflects acute aquatic toxicity.

The use of a battery of bioassays in field monitoring has been increasing over the past 15 years (Orias and Perrodin, 2013). Genotoxicity biomarkers are now considered to be an integral part of this approach because exposure to genotoxic agents may exert damage beyond that of individuals and may be detected through several generations (Frenzilli et al., 2009). The Comet assay has already been included in the strategy guidelines for the testing of chemical mutagenicity (EFSA, 2012). The Comet assay was chosen as an indicator test for its sensitivity to detect small amounts of DNA damage, for being flexible and for requiring only small numbers of cells (Tice et al., 2000). Especially the alkaline Comet assay with sperm – a relatively new technique – may become important, as it is very sensitive in assessing genotoxic damage (Baumgartner et al., 2007). The *in vitro* evaluation of DNA damage on cells will help establish feasible, adequate and expedient biomarkers of exposure.

For this reason to be close to the real contamination levels found in waste water, human hepatoma cell line HepG2 has been exposed to the drugs in a range of concentrations from 1 ng/L to 100  $\mu$ g/L. HepG2 cells have been chosen because they are extensively used for toxicity and genotoxicity evaluation of pure compounds and retained many of the properties of primary liver cells, including the metabolic activation (Lu and Huang, 1994). Owing to their endogenous expression of a variety of xenobiotic metabolizing enzymes, this cell lines are useful for detecting the combined effects of chemicals in a mixture (Mersch-Sundermann et al., 2004; Zegura et al., 2009). Furthermore, it has been widely described that zearalenone (a phytoestrogen) and its metabolites competitively bind to estrogen receptors in different cell models including HepG2 cells (Breithofer et al., 1998). Both viability and genotoxicity revealed by comet assay were evaluated on cells and a comparison was made with two standard tests: microtox® and algaltoxkit F™.

## 2. Materials and methods

### 2.1. Chemicals

Ciprofloxacin (CIP), tamoxifen citrate (TAM), cyclophosphamide (CP) and agarose were obtained from Sigma-Aldrich (France). Cell culture reagents including fetal calf serum and material were obtained from Fisher Scientific (France). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium inner salt

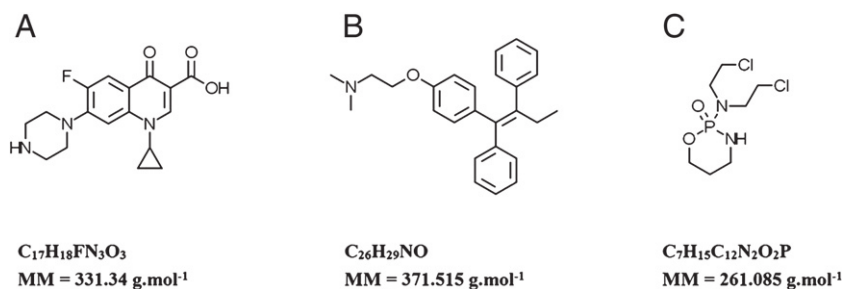


Fig. 1. Chemical structure of ciprofloxacin [CIP] (A), tamoxifen [TAM] (B) and cyclophosphamide [CP] (C).

(MTS) was obtained from Promega (France). All solutions were prepared using ultrapure water.

## 2.2. Cell culture

The HepG2 cell line (American Type Culture Collection, HB-8065) was cultured in 75-cm<sup>2</sup> polystyrene flasks with minimum essential medium supplemented with 10% heat-inactive fetal bovine serum, 10% L-glutamine (200 mM), 1% penicillin (100 U/mL), 1% streptomycin (100 mg/mL), and 1 mM sodium pyruvate under an atmosphere of 5% CO<sub>2</sub> at 37 °C. Viable cell count was assessed by Trypan Blue exclusion before toxic evaluation.

## 2.3. Cell, algae and bacteria treatments

Ciprofloxacin (CIP), tamoxifen citrate (TAM) and cyclophosphamide (CP) were dissolved in HCl, methanol and water respectively for stock solution and stored at -20 °C. Prior to the treatments, the compounds were diluted in the culture medium in order to give a final solvent concentration below 0.1%. None of these solvents at this dose affected cell viability, bacteria viability, and algae growth.

Hepatic cells (HepG2), algae and bacteria were exposed to increasing concentrations of the respective drugs (CIP, TAM, CP), or to a binary mixture of CIP + TAM or to a mixture of the 3 drugs (CTC) in the range of concentrations found in water as described in Table 1. Conditions 1 and 2 represent low doses close to environmental concentrations (surface water), condition 3 close to WWTP concentrations with a 2-fold factor of CIP, and conditions 4 and 5 represent hospital wastewater concentrations with a 10-fold factor of CIP. In condition 0 cells received only medium. This latter condition corresponds to control.

## 2.4. MTS cell proliferation assay

The assay was performed in accordance with the method by Barltrop et al. (1991). In brief, cell metabolic activity was determined by monitoring the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) using a commercial assay (CellTiter 96 Aqueous, Promega). Subcultures for experiments were prepared the day preceding in 96-multiwell plates at a density of  $5.5 \times 10^3$  cells/well and 18 h after plating, the medium was discarded and fresh medium containing drugs individually or in mixtures as described in Table 1 at different concentrations was added. Cells were exposed to drugs during 24 to 72 h then 20 µL of the MTS reagent was added to each well and incubated for 2 h at 37 °C, 5% CO<sub>2</sub>. The reduction of MTS was monitored by recording the change in absorbance (A) at 490 nm using the automated Dynex plate reader. The amount of reduced MTS was calculated as a percentage of the absorbance determined by comparison with the standard condition.

$$\text{Percent surviving cells} = \left[ \frac{A_{\text{test sample}}}{A_{\text{control sample}}} \right] \times 100.$$

**Table 1**  
Nominal concentrations of drugs used for treatments.

	Unit	Code conditions					
		0	1	2	3	4	5
Drugs (individually)							
CIP	µg/L	0	0.01	0.1	1	10	100
TAM	µg/L	0	0.01	0.1	0.5	1	10
CP	µg/L	0	0.01	0.1	0.5	1	10
Drugs (in mixture)							
CIP	µg/L	0	0.01	0.1	1	10	100
TAM	µg/L	0	0.01	0.1	0.5	1	10
CIP	µg/L	0	0.01	0.1	1	10	100
TAM	µg/L	0	0.01	0.1	0.5	1	10
CP	µg/L	0	0.01	0.1	0.5	1	10

## 2.5. Alkaline single cell gel electrophoresis (SCGE) assay

### 2.5.1. Treatment

Subcultures for experiments were prepared the day preceding the test. HepG2 cells were plated onto 12-multiwell plates at a density of  $1 \times 10^5$  cells/well and allowed to attach overnight. The medium was then discarded and replaced by 2 mL of test medium and treated cells and control cells were incubated for 24 h to 72 h.

### 2.5.2. Slide preparation

The comet assay was performed according to Singh et al. (1988) with minor modifications. All the procedures were conducted in the dark and on ice to minimize spurious sources that might cause DNA damage. Conventional microscope slides were dipped in 1.6% agarose and allowed to dry on a flat surface at room temperature before the experiment. The first layer with a 0.8% normal melting point (NMP) in PBS (phosphate saline buffer containing NaCl 137 mM, KCl 2.7 mM, Na<sub>2</sub>HPO<sub>4</sub> 4.3 mM, KH<sub>2</sub>PO<sub>4</sub> 1.47 mM, pH 7.4) kept at 37 °C was added onto frosted slides and gently covered with a cover-slide. The cell pellet obtained by centrifugation for 5 min at 4 °C, 4000 rpm, was resuspended in 150 µL to a final cell-agarose solution containing approximately  $5 \times 10^4$  cells in 0.5% low melting point (LMP) agarose in PBS and 75 µL was added as the second layer onto duplicate slides. The third layer (75 µL 0.5% LMP agarose) was added and the slides were placed in fresh lysis solution (2.5 M NaCl, 100 mM EDTA-Na<sub>2</sub>, 10 mM Tris, pH 10; 1% Triton X-100, 10% DMSO, pH 10) at 4 °C for 1 h. The slides were transferred on a horizontal electrophoresis tray and submerged in denaturation buffer (1 mM EDTA, 300 mM NaOH, pH 13) for 20 min. Electrophoresis was carried out using the same solution for 20 min at 26 V and 350 mA (0.8 V/cm) then the slides were gently washed three times for 5 min in a cold neutralizing buffer (400 mM Tris, pH 7.4). Slides were dried in methanol and stored in a low humidity environment until analysis.

### 2.5.3. Slide analysis

Few minutes before analysis, 50 µL of ethidium bromide (40 µg/mL) was dropped onto the agarose and covered with a coverslip. Fifty cells per slide were randomly scored at 250× magnification with an Olympus BX50 fluorescence microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. DNA damage was quantified by tail DNA and median values were calculated by a Komet 5.5 image analysis system (ANDOR Technology, Belfast, UK).

Positive control was run using ethyl-methanesulfonate (EMS).

## 2.6. Algaltoxitkit F<sup>TM</sup> assay: *Selenastrum capricornutum*

The growth inhibition of green algae *S. capricornutum* bioassays from MicroBioTests Inc. (Gent, Belgium) was performed following the standard operational procedures of algaltoxitkit F<sup>TM</sup> (1996). This test was conducted in accordance with OECD Guideline 201 (2002). Tests were carried out with *S. capricornutum* obtained from the Culture Collection of Algae and Protozoa (CCAP), Argyll, Scotland. All microalgae growth inhibition tests were conducted at 25 °C with continuous illumination of 10,000 lx.

The algaltoxitkit technology is based on the (rapid) measurement of the optical density (OD) of 25 mL algal cell suspensions in disposable spectrophotometric cells of 10 cm path length, called "long cells". Negative controls were incorporated for each test containing only algal growth media and algal inoculum. The initial algal density was  $1 \times 10^4$  cells/mL and the density of each replicate was measured at 670 nm after 72 h using a spectrophotometer. Average of specific growth and percentage of inhibition for each concentration were calculated by comparison with standard condition. Test validity criteria required algal growth in control flasks to increase by a factor of 16 within the 72 h test period and pH not to have varied by more than 1.5 units relative to the initial pH in the growth medium. The reference chemical



potassium dichromate ( $K_2Cr_2O_7$ ) was employed as a positive control to ensure the validity of the test method.

### 2.7. Microtox® assay: *Vibrio fischeri*

Microtox® Reagent (*V. fischeri* NRRL-B 11177) from AZUR Environmental (Carlsbad, CA, USA) was reconstituted and bacteria were cultured, harvested and frozen in cryoprotectant. Bioluminescence inhibition was evaluated using the basic test protocol and was determined at 5-, 15-, and 30-min intervals (Azur Environmental, 1998). The thawed bacterial suspension was used for the toxicity tests. The number of viable bacteria in the photobacterial luminescence inhibition tests was  $\sim 10^6$  bacteria per mL of test solution, and the toxicity testing was performed at 15 °C. The measurements were made by using a 1253 Luminometer (Labsystems, Helsinki, Finland) connected to a personal computer and the calculation of inhibitory % was performed by means of standard software (Labsystems, Helsinki, Finland). A basic test was also conducted with the reference standard phenol for each fresh vial of bacteria opened to ensure the validity of the test method.

### 2.8. Statistical analysis

All experiments were independently performed three times under the same conditions and results were calculated in median values with standard deviation (SD). As the distribution of cell viability and SCGE assays deviated from normality, data following the Kruskal–Wallis test, a non-parametric analog of the Dunnett's procedure for multiple comparisons was used. Values were considered significant (\*) at  $p < 0.05$  and (\*\*) at  $p < 0.01$ .

The statistical significance of values from standardized bioassays (microtox®; algaltoxkit F™) was checked using a one-way analysis of variance ANOVA test using Statistica 9.0 package software. Post-hoc comparisons between control and samples were made using Fisher's test to determine which values significantly differed at  $p < 0.05$  (\*).

### 2.9. Interaction analysis

To address the interaction of binary (CIP + TAM) or ternary mixtures (CIP + TAM + CP) the effects were compared to the expected effects that were calculated from single compound toxicity obtained in the same experiments. The two reference model of concentration addition (CA) and the independent action (IA) were used to derive patterns of joint effects of mixture (Chou, 2006, 2011). If the interaction is strictly additive  $[1 - E(Ca)] \times [1 - E(Cb)] = 1 - E(Cmix)$ , where  $E(Ca)$  is the effect induced by the compound a alone;  $E(Cb)$  the effect induced by the compound b alone and  $E(Cmix)$  is the effect observed when the compounds are mixed. In other word if the ratio expected value (E)/observed value (O) = 1 the interaction is strictly additive, if ratio > 1, the interaction is synergistic and when the ratio < 1 the interaction is antagonistic.

## 3. Results

### 3.1. HepG2 cell viability

Viability of HepG2 cells after exposure to drugs alone or mixed was detected by the MTS cell proliferation assay. The data (Figs. 2 and 3) are expressed as percentage of viable cells compared to non-exposed cells. Each value corresponds to the median value of 15 measurements (3 experiments, five replicates). Whatever the exposure time of hepatic cells to CP, a slight but significant ( $p < 0.05$ ) decrease of cell viability was only observed with the highest dose tested (10  $\mu\text{g/L}$ ) (Fig. 2A and B). Exposure of cells to the lowest concentration (10 ng/L) of CIP or TAM significantly ( $p < 0.01$ ) reduced cell viability (Fig. 2A) and the inhibition was time dependent (Fig. 2B). With higher doses of CIP (100 ng/L–10  $\mu\text{g/L}$ ), the cytotoxicity is lower than with 10 ng/L and was not dose dependent. Exposure to a ten-fold higher concentration of CIP (100  $\mu\text{g/L}$ ), significantly ( $p < 0.01$ ) decreases the cell viability. The percentages of viable cells exposed to 100  $\mu\text{g/L}$  of CIP were 90% and 80% respectively after 24 and 72 h of exposure. When cells are exposed to TAM whatever the time of exposure a U-shaped dose–response curve was observed.

Fig. 3 shows the combined effects of CIP + TAM or of the three drugs (CTC) on HepG2 cell viability. The concentrations of exposure are described in Table 1 (cf. Section 2.3). Whatever the length of cell exposure to CIP + TAM a U-shaped dose–response curve was observed.

The inhibition of cell growth is lower than expected if a strictly additive effect will occur (Table 2). When CP was added in the mixture the antagonistic effect was higher compared to a binary mixture, with almost no more impact on cell viability. Whatever the relative concentrations, the type of mixture (binary or ternary mixture) and the time of exposure, the E/O ratio was always < 1 indicating that an antagonistic effect occurs when the drug are present together, even at very low doses (Table 2). The antagonism is higher in the presence of CP compared to CIP + TAM (Fig. 4).

### 3.2. DNA damage on HepG2 cells

The genotoxic effect of the three drugs on HepG2 was tested by analysis of DNA single strand break formation using the comet assay. In the range of concentrations tested (10 ng/L–10  $\mu\text{g/L}$ ) exposure of cells to individual compounds did not induce any DNA damage, whatever the exposure time (Fig. 5). Even exposure of cells to 10 more CIP concentration did not induce any DNA breaks.

Simultaneous exposure to increasing concentrations of the three drugs, whatever the time of exposure, significantly increased DNA damages (Fig. 6). DNA breaks are two folds higher when cells are exposed to 10  $\mu\text{g/L}$  of CP + 10  $\mu\text{g/L}$  TAM + 100  $\mu\text{g/L}$  CIP compared to 10  $\mu\text{g/L}$  TAM + 100  $\mu\text{g/L}$  CIP (condition 5; Fig. 5A). The increase of DNA damage was dose dependent when the three drugs are present simultaneously (Fig. 6) and was significantly higher compared to exposure to binary mixture. Although DNA breaks decreased with the time of exposure,

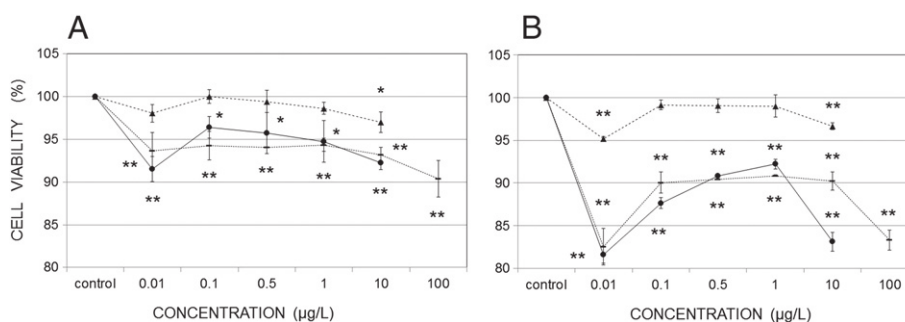
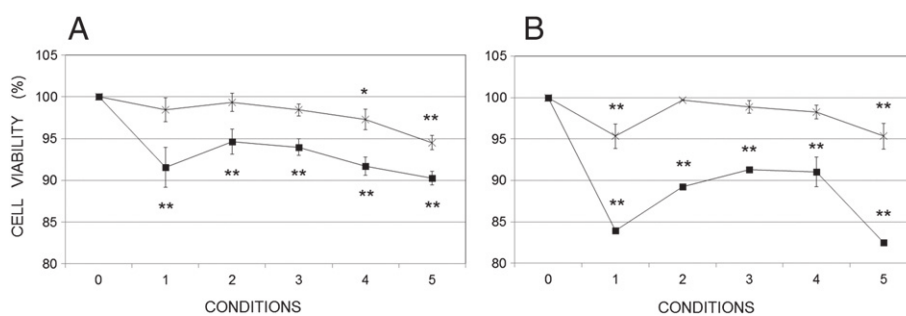


Fig. 2. Survival rate of HepG2 cells exposed to CP (▲); CIP (-); TAM (◆) during 24 h (A) to 72 h (B). Results are expressed as median value  $\pm$  SD of the percentage of viable cells compared to control condition. Statistically significant at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*) compared to control cell.



**Fig. 3.** Survival rate of HepG2 cells exposed to CIP + TAM (□); CTC (×); during 24 h (A) to 72 h (B). The numbers 0–5 refer to the mixture condition described in the M & M part (Table 1). Results are expressed as median value  $\pm$  SD of the percentage of viable cells compared to control (condition 0). Statistically significant at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*) compared to control cell.

even after 72 h, the amount of DNA damages observed when the cells are exposed to the three drugs is significantly higher ( $p < 0.01$ ) compared to treatment with CIP + TAM (Fig. 6B) and compared to control.

### 3.3. Growth inhibition of microalgae *S. capricornutum*

TAM significantly ( $p < 0.05$ ) stimulated the growth of microalgae (Fig. 7). CIP has no effect on microalgae. The dose effect induced by CP exhibited a U-shape form. With the lowest dose a slight but significant inhibition of algae growth was observed ( $p < 0.05$ ); the highest CP dose tested induced a proliferation.

When microalgae were exposed to the mixture (CIP + TAM or CTC) whatever the exposure time, the algae growth was dose-dependently inhibited (Fig. 8). There is no difference between exposure to binary mixture (CIP + TAM) compared to mixture of the three drugs.

### 3.4. Extinction of bioluminescence of bacteria *V. fischeri*

Except with CIP at the highest dose (100  $\mu\text{g/L}$ ), none of these drugs inhibited *V. fischeri* bioluminescence (Fig. 9).

In contrast, exposure of bacteria to the mixture induced a non-monotone dose–response (U-shape curve), mainly with CIP + TAM (Fig. 10). The addition of CP in the mixture induced an antagonist effect. For the lowest doses in the mixture, the inhibition of bacteria bioluminescence was much higher when the three drugs were present compared to only CIP + TAM. For the other conditions, the inhibitions were lower in the presence of the three drugs compared to CIP + TAM (Fig. 10).

## 4. Discussion

Recent studies show that among many environmental contaminants, pharmaceutical and personal care product (PPCP) residues in the environment are of emerging concern. Cytostatic drugs and human metabolites are directly discharged into the sewage system without any specific control after being administered in the hospitals. Household discharge by out-patients presents another pathway of cytostatics to the environment (Bound and Voulvoulis, 2006). Due to their highly potent mechanism of action (cytotoxicity, genotoxicity, mutagenicity and teratogenicity), cytostatic drugs could induce adverse effects on any growing eukaryotic organism and bacteria (Besse et al., 2012; Johnson et al., 2008). Another point is also the deleterious effects of these drugs for the hospital staff (Bouraoui et al., 2011; Burgaz et al., 1999, 2002; Fucic et al., 1998).

In fact, some cytostatics have been detected in hospital wastewaters and even influent wastewaters at concentrations ranging from ng/L to  $\mu\text{g/L}$  (Buerge et al., 2006; Johnson et al., 2008; Negreira et al., 2013a, 2013b; Rowney et al., 2009). Although environmental concentrations are below human therapeutic doses, the persistent release of these drugs may lead to chronic effects at low concentration.

Mass fluxes alone are insufficient to evaluate the risk stemming from pharmaceuticals; their ecotoxic potential needs to be considered. Despite recent large efforts to increase the database on ecotoxicological effects of pharmaceuticals (PhACT Database, 2006), the implications of chronic low dose exposure of aquatic fauna and humans to environmental discharge of these drugs remain largely unknown (Crane et al., 2006). The lack of chronic toxicity data for drugs hinders the ability to predict effects. In addition, using only physico-chemical analysis, it is impossible to predict the toxic and/or genotoxic properties of complex

**Table 2**

Comparison between expected additive values and results obtained in percentage (%) from HepG2 cell exposed during 24 h (A) and 72 h (B) to CIP + TAM and CTC. E/O is the ratio between expected values (E) compared to observed values (O).

Drugs (in mixture)	Condition				
	1	2	3	4	5
<b>A.</b>					
CIP + TAM	91.52 $\pm$ 2.39	94.64 $\pm$ 1.51	93.97 $\pm$ 0.96	91.67 $\pm$ 1.09	90.23 $\pm$ 0.80
CIP + TAM <sub>exp.</sub>	85.71	90.88	90.35	88.34	83.43
E/O	0.94	0.96	0.96	0.96	0.92
CTC	98.57 $\pm$ 1.42	99.34 $\pm$ 1.10	98.44 $\pm$ 0.73	97.28 $\pm$ 1.21	94.52 $\pm$ 0.87
CTC <sub>exp.</sub>	84.04	90.91	89.84	87.14	80.94
E/O	0.85	0.92	0.91	0.90	0.86
<b>B.</b>					
CIP + TAM	83.93 $\pm$ 0.11	89.22 $\pm$ 0.15	91.29 $\pm$ 0.38	91.03 $\pm$ 1.77	82.51 $\pm$ 0.18
CIP + TAM <sub>exp.</sub>	67.34	78.92	82.51	83.19	69.24
E/O	0.780	0.88	0.90	0.91	0.81
CTC	95.33 $\pm$ 1.46	99.72 $\pm$ 0.07	98.87 $\pm$ 0.78	98.24 $\pm$ 0.82	95.36 $\pm$ 1.54
CTC <sub>exp.</sub>	64.12	78.26	81.72	82.38	66.91
E/O	0.67	0.78	0.83	0.84	0.70

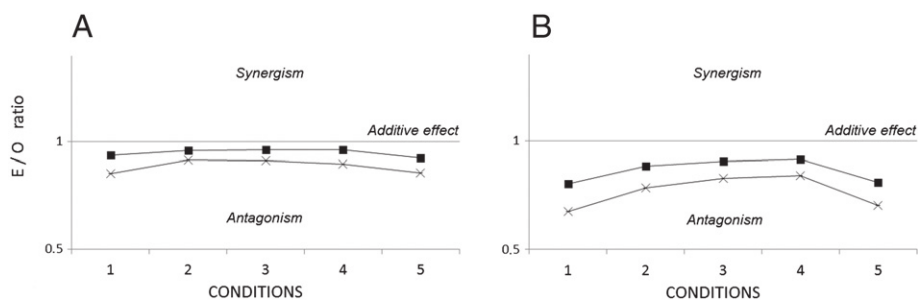


Fig. 4. Drug combination plot. Ratio expected (E)/observed (O) versus concentration. CIP + TAM (□); CTC (×), at 24 h (A) and 72 h (B).

water samples, especially if synergistic, antagonistic or potentiating effects between the components occur.

Our study focused on the cytotoxic and genotoxic effects of three drugs, from different chemical classes (ciprofloxacin (CIP), a fluoroquinolone antibiotic; cyclophosphamide (CP), a neoplastic chlorinated compound; tamoxifen (TAM), an estrogenic compound used as an anti-cancer agent). Considering that these drugs can co-occur in water, the toxic effects of mixtures (CIP + TAM and CTC) were compared to their individual effects.

Toxicology has long been dominated by an emphasis on very high doses and the assessment of toxic responses. In comparison with human health risk assessment, ecological risk assessment is poorly understood. In the environment, it is important to know what happens with a low dose, corresponding to real water contamination. The concentrations chosen were based on the range reported in the literature, although the concentrations of PPCPs vary at each site with pharmaceutical usage patterns, the size of the surrounding population, the specific wastewater treatment available, and the discharge location. Thus, the concentrations are not meant to be an exact match to what is found at one municipal wastewater treatment plant, or field site, but to represent concentrations that are environmentally reasonable. The high concentration tested was near the highest reported concentrations found in hospital wastewater up to 150 µg/L (Martins et al., 2008; Negreira et al., 2013a, 2013b; Santos et al., 2013).

Table 3 summarized and compared the four bioassays.

In the range of doses tested (ng/L–10 µg/L), the dose–response of cell viability observed after exposure of HepG2 cells to TAM, CIP and CP, mainly after 72 h of exposure, exhibited a U-shape. This type of curve was also observed when microalgae were exposed to CP. These kinds of non-linear dose–response relationships are referred to hormetic responses and have been described across a wide range of organisms (from bacteria to vertebrates), in response to exposure to at least 1000 different chemicals and environmental stressors. Hormesis is connected with both acclimation and phenotypic plasticity, and may play an important role in allowing animals to adjust to changing environments (Costantini et al., 2010). Deviations from linearity are favored by the occurrence of multiple concurrent and sequential events in toxicological responses. Mechanisms contributing to hormetic responses at low doses included overcompensation to a disruption in homeostasis;

adaptive responses based on inducible repair processes; interactions among cell proliferation, cell-cycle delay, apoptosis, and DNA damage; enhancement of gap junction intercellular communication at low doses but inhibition at high doses; etc. (for a review see Hoffmann, 2009). Hormesis is a biphasic dose–response that often results from the actions of partial agonists and partial antagonists. TAM is an endocrine disruptor due to its agonist and antagonist effect on estrogen receptor (Coezy et al., 1982; Vandenberg et al., 2012). The low dose stimulating hormetic responses could lead to undesirable effects e.g. proliferation of harmful bacteria (Randall et al., 1947) or enhancement of proliferation of tumor cells by antitumor drugs (Calabrese, 2005, 2013). In the case of chemotherapeutic agents, the low dose stimulation appears to reflect an adaptive response to tumor tissue. To address the interaction of binary (CIP + TAM) or ternary mixtures (CIP + TAM + CP) the effects were compared to the expected effects that were calculated from single toxin toxicity obtained in the same experiments. The two reference models of concentration addition (CA) and the independent action (IA) were used to derive patterns of joint effects of mixture (Chou, 2006, 2011). The CA model assumes that mixed chemicals have the same mode of action (MoA), whereas the IA model is based on the idea of a dissimilar action of mixture components, usually to answer the question whether the probability of toxicity from exposure to no chemical is independent from the probability to another chemical (Altenburger et al., 2003; Jonker et al., 2004, 2005).

Data modeling allowed us to infer that all three toxins exerted interactive effects in HepG2 cell viability; bacteria viability (microtox®); algae proliferation (algaltoxkit F™) and genotoxicity (comet assay). A U-shape dose–response was observed for the cell viability test and microtox® test with both kinds for the mixture. Both of these latter tests reflect a mainly acute toxic effect. The application of the CA model showed that the observed cytotoxic effects were lower than expected, and an antagonistic pattern was identified.

Concerning the algaltoxkit F™ test and the comet assay, reflecting a chronic toxic effect, both mixtures have a synergistic effect. While individually, the three drugs would not be expected to cause DNA breaks or inhibition of algae growth at these low doses, the drugs in the mixture lead to a dose-dependent increase of effects. In general these effects are more pronounced after a 72 h exposure compared to lower exposure time confirming a chronic effect, and an adaptive mechanism.

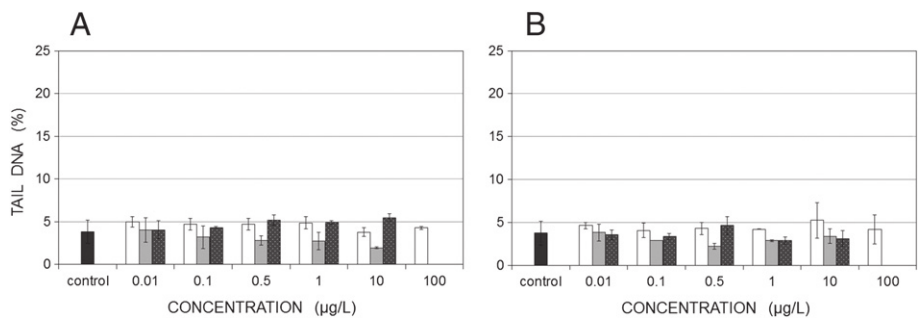
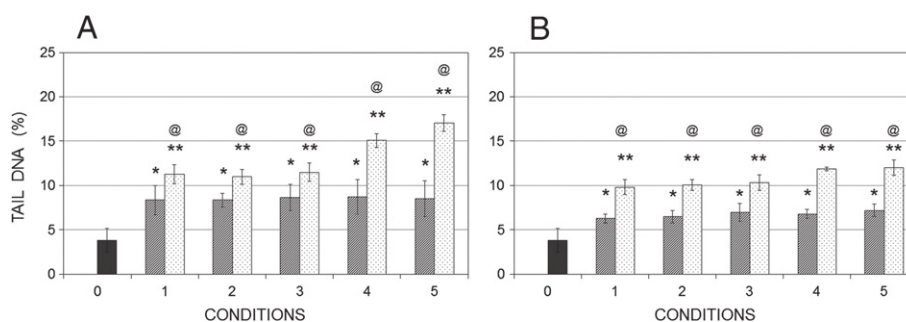


Fig. 5. Comparison of DNA breaks induced on HepG2 exposed to CIP (white), TAM (gray) or CP (black) during 24 h (A) to 72 h (B). The data are expressed as median value ± SD.



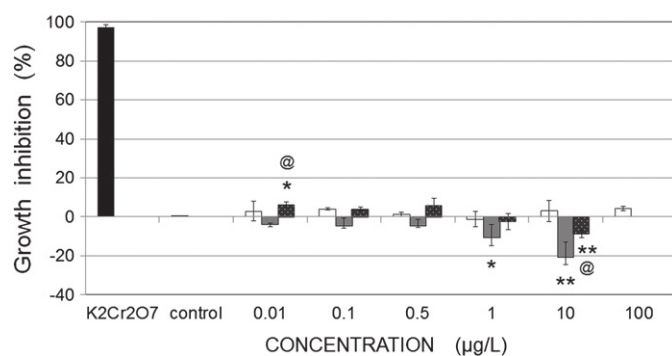


**Fig. 6.** Comparison of DNA breaks induced on HepG2 exposed to CIP + TAM (hatched) or CTC (stippling) during 24 h (A) to 72 h (B). The data are expressed as median value  $\pm$  SD. The numbers 1 to 5 refer to the mixture conditions explained in the M & M part (Table 1). Statistically significant at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*) compared to control cell; @ significant difference between exposure to three drugs (CTC) compared to CIP + TAM.

This is in line of the data obtained by Yusuf et al. (2000), even though the doses used in their study were much higher.

The evidence suggests that if one measures biological effects too early, the response may appear to fit a threshold or linear model rather than hormesis because insufficient time has elapsed for the requisite gene expression for an adaptive response. If one makes measurements too late, the system may have already returned from the induced state to the ground state.

Comparison between impact of both mixtures (CIP + TAM versus CTC) shows that CP increased the antagonistic effect on cell viability and bacteria bioluminescence; and the synergistic effect on DNA breaks formation (comet assay). These data show clearly that the three drugs have a different mode of action. In the case of binary mixture TAM + CIP, interaction could be due to the impact of TAM on membrane efflux. Indeed, Darvari and Boroujerdi (2004) showed that tamoxifen biphasically modulated the membrane efflux of doxorubicin (an antibiotic like CIP). For all the three drugs the interaction could be due to interference on biotransforming enzymes. Tamoxifen must be activated by the cytochrome P450 system to exert its effects via anti-estrogenic metabolites that are more potent than the parent compound (Coezy et al., 1982). The main metabolites of tamoxifen are N-desmethyltamoxifen (formed by CYP3A), and 4-hydroxytamoxifen and endoxifen (formed by CYP2D6) (Desta et al., 2004). CP is not cytotoxic by itself but undergoes activation through a metabolic step *in vivo*. Numerous metabolites are known (Kerbusch et al., 2001) and the major ones are chloroacetaldehyde, acrolein and 4-hydroxycyclophosphamide produced by the mixed function of cytochrome P450 oxidase (CYP) present in hepatic microsomes (Huang et al., 2000). The active metabolite (4-hydroxy derivative) is produced mainly by CYP2B6. These metabolites are excreted in the urine. N-dechloroethylation results in inactivation of anticancer activity of CP and formation of the toxic byproduct chloroacetaldehyde.



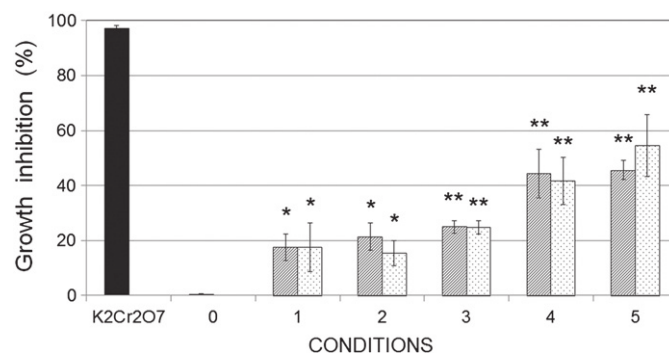
**Fig. 7.** Growth inhibition of microalgae exposed 72 h to ciprofloxacin (CIP, white); tamoxifen (TAM, gray); and cyclophosphamide (CP, black).  $K_2Cr_2O_7$  corresponds to the positive control. Results are expressed as median value  $\pm$  SD of the percentage of viable algae compared to control condition. Statistically significant at  $p < 0.05$  (\*) compared to control algae without any treatment.

This reaction is catalyzed by CYP3A4. Induction or inhibition of CYP activity mediated by concomitantly administered drugs can change the balance between the activating and inactivating metabolic pathways of cyclophosphamide (Scripture et al., 2005). Ciprofloxacin is an inhibitor of cytochrome P450 3A activity *in vitro* and *in vivo* (Xie et al., 2003). Ciprofloxacin inhibits the CYP which mediates CP biotransformation leading to reduction in the metabolic ratio and a decrease in the levels of active 4-OH-CP. The immunosuppressive effect of CP is hampered by ciprofloxacin, which is detrimental to the treatment, explaining that treatment did not anymore combine these two drugs. In the same way, TAM and its metabolites N-desmethyltamoxifen and 4-hydroxytamoxifen inhibit metabolism mediated by CYP 2B6, 2C9 and 2D6 (Sridar et al., 2002) but increased CYP3A4 (Desai et al., 2002; Sane et al., 2008) and have the potential to cause drug interactions (Scripture et al., 2005). Altogether, modifications of the metabolic pathway lead in the enhancement of the formation of genotoxic metabolites of CP such as acrolein. The dG-acrolein adduct has been reported to be considerably more mutagenic in mammalian cells than in *Escherichia coli*. Mammalian polymerase has been reported to bypass the dG-acrolein lesion in the presence of proliferating cell nuclear antigen (Kanuri et al., 2002).

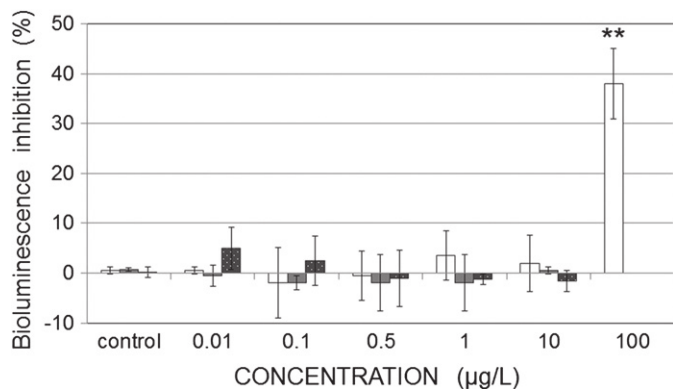
To confirm the link between DNA adduct formations and DNA breaks (comet assay) experiments are underway in our laboratory, DNA breaks can reflect two different events, either a direct attack of DNA or the reparation of DNA damaged by a bulky adduct. In general after repair cells will proliferate, whereas after DNA damage and inaccurate repair cells can die.

## 5. Conclusion

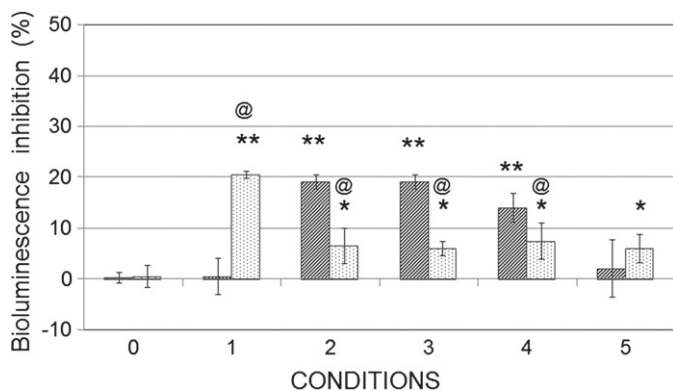
Only a battery-based approach, including in one hand biomarkers and on other hand an acute and chronic aquatic toxic test on whole organisms, investigating the causal relationship between exposures and environmental pollutants will aid to assess water quality. Two



**Fig. 8.** Comparison of microalgae growth inhibition induced by exposure to CIP + TAM (hatched) or CTC (stippling) during 72 h. The data are expressed as median value  $\pm$  SD. The numbers 1 to 5 refer to the mixture conditions explained in the M & M part (Table 1).



**Fig. 9.** Bioluminescence inhibition of bacteria exposed to ciprofloxacin (CIP, white); tamoxifen (TAM, gray); and cyclophosphamide (CP, black). Results are expressed as median value  $\pm$  SD of the percentage of inhibition compared to control condition. Statistically significant at  $p < 0.05$  (\*) compared to control algae without any treatment.



**Fig. 10.** Comparison of bioluminescence inhibition induced by exposure to CIP-TAM (hatched) or CTC (stippling). The numbers 1 to 5 refer to the mixture conditions explained in the M & M part (Table 1). Results are expressed as median value  $\pm$  SD of the percentage of viable bacteria compared to control (condition 0). Statistically significant at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*) compared to control; @ significant difference between exposure to three drugs (CTC) compared to CIP + TAM.

endpoints (cell viability and microtox®) reflect the acute toxic effect and give similar responses. The two other endpoints (DNA breaks and algaltoxkit F™) reflect a chronic effect, and the adaptive response of organisms.

The results of the mixture experiments demonstrate that chemicals in real water samples act together in mixtures and how not only the

absolute concentrations of the mixture components are important but also their contribution to the mixture effect. Throughout this study we identified significant hormetic responses with the cell viability tests and algaltoxkit F™ test at low concentrations of drugs. The hormesis phenomenon is characterized by low dose stimulation of the test species over the control and high dose enhancement of adverse effects. These data pinpoint the difficulties of risk assessment to low concentration (subnanograms) exposure and confirm the data obtained by Hernando et al. (2006) and Zegura et al. (2009). Consequently, while individually these drugs would not be expected to cause acute toxicity in environmental conditions, this study revealed that chronic endpoints were a lot more sensitive than acute endpoints, notably when the drugs occurred simultaneously, and hence further research is needed to investigate chronic effects at environmentally relevant concentrations and in mixtures. The synergism/antagonism observed in the present study demonstrated the necessity to investigate the ecotoxicological effects of contaminants at low concentration mixtures and at different time-exposures.

Cell culture can offer several advantages over whole animal (or organism) studies, e.g., reduced labor, sample volume, variability and at least reduced cost.

#### Acknowledgment

This paper was prepared with financial support by the French National Research Agency, in the framework of the 2010 "CD2I" call program, within the project "PANACEE" and by the French company. The authors thank G.M. for English proofreading. The authors declare that there are no conflicts of interests.

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**Table 3**

Comparison of the four bioassays reflecting acute effect (cell or bacteria viability) or chronic effect (DNA damages or algae growth inhibition).

Bioassay	Cell viability (MTS)	DNA break (COMET)	Algae growth inhibition (algaltoxkit F™)	Bacteria activity (microtox®)
CIP (alone)	Hormesis/inhibition	-	-	- (except inhibition with high dose)
TAM (alone)	Hormesis/inhibition	-	proliferation	-
CP (alone)	= (Inhibition high dose)	-	Hormesis (inhibition followed by proliferation)	-
CIP + TAM	Hormesis/antagonism	+ (synergy)	inhibition	Hormesis/synergy
CTC	Hormesis/antagonism (almost return to basal level)	++ (synergy)	inhibition	Hormesis/antagonism*
Comment	Effects more pronounced after 72 h exposure.	Effects less pronounced after 72 h exposure. Reflect repair system, and chronic effect	Reflect chronic aquatic effect	Inhibition of bacteria luminescence by CIP could be explained by its antibiotic activity. *Comparison between the two mixtures condition shows an antagonist effect. Reflect acute aquatic effect

CIP = ciprofloxacin; TAM = tamoxifen; CP = cyclophosphamide; CTC = mixture of CIP + TAM + CP.

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