Toxicological interactions of ibuprofen and triclosan on biological activity of activated sludge

Georgiana Amariei, Karina Boltes, Roberto Rosal, Pedro Letón, Toxicological interactions of ibuprofen and triclosan on biological activity of activated sludge, Journal of Hazardous Materials, 334, 193-200, 2017, http://doi.org/10.1016/j.jhazmat.2017.04.018.

Toxicological interactions of ibuprofen and triclosan on biological activity of activated sludge

Georgiana Amariei[1], Karina Boltes[1,2,*], Roberto Rosal[1,2], Pedro Letón[1,2]

[1] Department of Chemical Engineering, University of Alcalá, E-28871 Alcalá de Henares, Madrid, Spain

[2] Madrid Institute for Advanced Studies of Water (IMDEA Agua), Parque Científico Tecnológico, E-28805, Alcalá de Henares, Madrid, Spain

* Corresponding author: karina.boltes@uah.es

Abstract

The growing use of pharmaceutical and personal care products increases their concentrations in the wastewater entering treatment plants and their levels into biological reactors. The most extended biological wastewater treatment is the activated sludge process. The toxicity of ibuprofen and triclosan, individually and combined, was studied by tracking the biological activity of the activated sludge measuring oxygen uptake rate and the inhibition of the esterase activity. Short-term exposure produced significant inhibition in oxygen uptake, with lower damage to enzymatic activity. Median effect values for oxygen uptake inhibition were 64 ± 13 mg L^{-1} and 0.32 ± 0.07 mg L^{-1} for ibuprofen and triclosan respectively using 125 mg L^{-1} activated sludge. For the inhibition of enzymatic activity values were 633 ± 63 mg L^{-1} for ibuprofen and 1.94 ± 0.32 mg L^{-1} for triclosan. Results indicated that oxygen uptake, related to primary activity of microorganisms, was more strongly affected than the enzymatic activity associated to energy consumption. Toxicity interactions were determined using the Combination Index-isobologram method. Results showed antagonism at lower values of affected population, after which the mixtures tended to additivity and synergism. For the case of enzymatic activity, the antagonism was less marked and the additivity range was higher.

Keywords: PPCP; Mixtures; Toxicity; Respirometry; Enzymatic activity.

1. Introduction

Emerging contaminants present significant research interest due to their systematic detection in wastewater [1, 2]. The contamination originated from wastewater discharges impacts surface water quality due to the incomplete removal of many polar contaminants by conventional wastewater treatments [3]. Their toxicity to aquatic organisms has been frequently reported but there is still considerable lack of information on the effects of mixtures of these compounds on environments [4]. The occurrence of emerging pollutants in complex mixtures is another knowledge gap often recognized as a problem affecting ecosystems and drinking water supplies [5, 6]. Among these compounds pharmaceutical and personal care products (PPCP) constitute a large and diverse set of chemicals, which include drugs and daily personal care products, widely used in large amounts throughout the world [7, 8]. Additionally, many emerging compounds from these groups display pseudo-persistent exposure characteristics because their environmental dissipation rates are lower than discharge rates from effluent loadings, but higher than hydrologic retention times [9].

Among PPCP, ibuprofen (IBU) and triclosan (TCS) are particularly relevant compounds. The non-steroidal anti-inflammatory drug IBU is one of the most used active pharmaceutical ingredients worldwide [10, 11]. TCS is a broad-spectrum antimicrobial agent widely used as antiseptic, disinfectant and preservative in many consumer products including cosmetics, household cleaning products and materials such as medical devices, textiles and plastic ware [12]. The occurrence of IBU and TCS is well-documented in influents and effluents of wastewater treatment plants (WWTP). The analgesic ibuprofen was detected at near 2 μg L^{-1} in hospital wastewaters [13]. Kumar et al. [14] indicated a remarkably high concentration of TCS in the influent of a WWTP of 86.2 μg L^{-1} in Savannah, Pal et al. [15] reported a maximum value of 7.1 μg L^{-1} for IBU in WWTP effluents in Europe, with lower values in America and Asia. Martínez-Bueno et al. [16] reported maximum values for IBU and TCS in effluents of WWTP of 2.4 μg L^{-1} and 3.7 μg L^{-1} respectively. Rosal et al. (2010) [17] found maximum values of 4.1 μg L^{-1} for IBU and 2.4 μg L^{-1} for TCS in the influent of a WWTP. Both compounds have also been detected in freshwater compartments reaching the microgram per liter level [12, 18]. The range at which IBU and TCS were detected in wastewater is typically in the tens of micrograms per liter and order of magnitude lower respectively, although both compounds have also been detected in freshwater compartments reaching the microgram per liter level.

Toxicant concentrations usually found in aquatic environments are at levels of μg/L or lower but considering the industrial environment of a wastewater treatment plant, the scenario is different. In the biological process of wastewater treatment, many

dissolved contaminants are transferred to the solid phase, biomass or sludge, which is also an active microbial population responsible of pollutant removal. It was recognized that organic micro-pollutant concentrate in this phase up to 3 or 4 orders of magnitude with respect to aqueous media [19]. The risk for environmental compartments can be properly estimated by environmental risk assessment (ERA), which evaluates the probability of a compound to cause undesired environmental effects. In this sense, Ortiz de Garcia et al. [4] predicted the risk of 26 PPCPs in wastewater treatment plants and in the aquatic environment using the US EPA ecological structure–activity relationship (ECOSAR). Based on ecotoxicity data that included respirometry, the authors classified IBU as harmful and TCS as toxic to aquatic organisms.

Several studies highlighted the potential of IBU and TCS to promote adverse effects on aquatic organisms [20-24]. Despite the abundance of toxicological studies of PPCP using environmental organisms, surprisingly little attention has been paid to the microorganisms forming the activated sludge (AS) community [25]. Pasquini et al. [26] found that TCS at concentration as low as 0.1 μg L^{-1} induced the overexpression of extracellular polymeric substances on AS, which is considered as stress response. The toxicity of pollutants to AS can be assessed by a number of methods apart from the simple monitoring of biomass growth or the counting of colony forming units. Oxygen uptake using respirometry, nitrification inhibition or adenosine triphosphate luminescence methods have been used for that purpose [27, 28]. The inhibition of the activity of several enzymes such as dehydrogenases, phosphatases, glucosidases and esterases are also routinely used to measure specific alterations due to toxicants [29]. According to references [4, 30-32], respirometry is the most realistic ecotoxicity test to evaluate the risk in a wastewater treatment plant and to detect the malfunction of biological reactors because they are direct indicators of the performance of the active biomass. The results are dependent on the particular characteristics of biomass, in turn affected by operational parameters, but they are essential to clarify the role of toxicants on sludge bioactivity. Additionally, esterase activity refers to a specific cellular function that requires intact membrane integrity [33] This biological reaction depends on the hydrolysis of FDA to fluorescein, by non-specific esterases present in different microbes [34], being a simple method to evaluate mixed populations such as those of activated sludge. The esterase activity is usually considered a measurement of cell viability, due to their direct relation with membrane integrity.

The co-occurrence of wastewater pollutants causes concern due to the potential interactive effects, such as synergistic or antagonistic toxicity, that may occur in the complex mixtures discharged with WWTP influents [5, 35]. The interaction of several wastewater pollutants beyond the additivity described by the concentration addition (CA) method has been often neglected at low dose levels [36, 37]. CA assumption has a clear practical value but there are clear evidences of non-additive behavior in many cases [38]. It has been recognized that non-additive behavior is concentration- and effect-level dependent, so experimental designs addressing this point are required [39]. For it, we used the combination index (CI)-isobologram equation method established by Chou and Talalay [40], a method that quantitatively assesses mixture effects over a wide range of effect levels. Using this method, our previous works showed that non-additive interactions occur in different mixtures with a general tendency to antagonism at low effect levels [41-44].

The aim of this study was to investigate the toxic effects of IBU and TCS on the AS from a WWTP by tracking oxygen uptake and the enzymatic esterase activity. TCS and IBU were selected in view of their widespread occurrence and the well-known negative effects exerted on aquatic organisms. The focus has been paid to the toxicological interactions between IBU and TCS using the combination index (CI)-isobologram method. To the best of our knowledge, it is the first work studying the combined action of organic micropollutants on AS bioactivity.

2. Materials and methods

2.1. Chemicals

Triclosan (TCS, $C_{12}H_7O_2Cl_3$, CAS No. 3380-34-5) and ibuprofen sodium salt (IBU, $C_{13}H_{17}NaO_2$, CAS No. 31121-93-4) both with 98% of purity were purchased from Sigma-Aldrich. The rest of chemical were also analytical grade and acquired from Sigma-Aldrich. Ultrapure water was generated from a Direct-Q™ 5 Ultrapure Water Systems from Millipore (Bedford, MA, USA) with a specific resistance of 18.2 MΩ cm. Stock and working solutions of TCS, IBU and their mixtures were prepared in phosphate buffer and stored at 4 °C until use.

2.2. Activated sludge acclimatization and maintenance conditions

The bubble column reactor consisted of a transparent Plexiglass tube (12 cm inner diameter, 115 cm height and 4.5 L working volume) with a settler for sludge recirculation. The inoculum for the reactor start-up consisted of 500 mL collected from a municipal WWTP (Seville, Spain). Prior to exposure assays, the reactor operated continuously for 120 days under pseudo-steady state in order to achieve a homogeneous and stable composition of biomass. The activated reactor operated with Synthetic Sewage Feed, based on OCDE 209 standard medium: peptone, 160 mg L^{-1}; meat extract, 110 mg L^{-1}; urea, 30 mg L^{-1}; NaCl, 7 mg L^{-1}; $CaCl_2 \cdot H_2O$, 4 mg L^{-1}; MgSO4·7H2O, 2 mg L^{-1} and K_2HPO_4, 28 mg L^{-1}. The synthetic influent was fed to the reactor continuously by a fixed speed peristaltic pump (hydraulic retention time 24 h). The air was supply thought a fine bubble diffuser and the dissolved

oxygen (DO) concentration in the reactor was maintained at 3 mg L^{-1} (measured using OD sensor, OXI 45+ CRISON). The reactor performance was monitored using the concentration of total suspended solids (TSS), chemical oxygen demand (COD) and ammonium nitrogen (NH_4-N). All the analyses were determined according to Standard Methods. Temperature was maintained at about 20 °C and pH was maintained at 8.0 ±0.1, close to the pK_a of TCS reported as 8.1 [45]. Acclimatization was considered to be reached when no significant changes in chemical parameters were observed.

2.3. Toxicity tests

After acclimatization, the required amount of AS was collected from the sedimentation tank of the bubble column reactor and used to perform inhibition tests according to a modified form of the Standard Guideline OECD Test Guide 209 [46]. Exposure experiments were conducted in 100 mL Erlenmeyer flasks containing a predefined concentration of AS, phosphate buffer and standard medium. The experiments for IBU and TCS were performed using five different concentrations in the range 75–1200 mg $L^{-1}$and 0.5–6.5 mg L^{-1}, respectively. These concentrations were selected as multiples and dividers of the median effective concentration, EC_{50}. The mixture of IBU + TCS was assayed using concentration selected on the basis of the EC_{50} value of the individual components as indicated in Table 1. Five different concentrations of AS (measured as TSS) were used for each IBU + TCS mixture as indicated in Table 1. The experimental design was based on EC_x ratios [47]. Each set of experiments was replicated three times, including blank assays (without toxicant) and positive controls using the reference toxicant copper (II) sulphate pentahydrate as stated by OECD Test Guide 209 (mean EC_{50} 65.2 ± 3.2 mg L^{-1}, which is inside the 53–155 mg L^{-1} range indicated by the guideline for total respiration using activated sludge). Positive response to inhibitor was tested on the day before exposure assays. The samples were incubated in an orbital shaker (Busen AO-400) at 200 rpm and 20 °C for 1 hour. Immediately after incubation, both respiration rate and enzymatic activity were recorded simultaneously for each exposure condition.

Table 1. Experimental design for determining toxicological interactions of IBU and TCS and their binary combinations for respirometry and enzymatic activity tests.

Single toxicants		Dilutions	¼ EC_{50}	½ EC_{50}	EC_{50}	2 EC_{50}	4 EC_{50}
		IBU (mg L^{-1})	75	150	300	600	1200
		TCS (mg L^{-1})	0.5	1.0	2.0	4.0	6.5
Binary combinations	125 mg L^{-1} AS	Dilutions	¼ EC_{20}	½ EC_{20}	EC_{20}	2 EC_{20}	4 EC_{20}
		IBU (mg L^{-1})	16	32	64	128	256
		TCS (mg L^{-1})	0.24	0.47	0.94	1.88	3.76
	250 mg L^{-1} AS	Dilutions	¼ EC_{20}	½ EC_{20}	EC_{20}	2 EC_{20}	2.5 EC_{20}
		IBU (mg L^{-1})	23.8	47.7	95.3	190.6	238.3
		TCS (mg L^{-1})	0.50	1.04	2.10	4.20	5.25
	500 mg L^{-1} AS	Dilutions	¼ EC_{20}	½ EC_{20}	EC_{20}	2 EC_{20}	2.5 EC_{20}
		IBU (mg L^{-1})	54.3	108.6	217.3	434.6	537.2
		TCS (mg L^{-1})	0.60	1.20	2.45	4.90	6.12
	1000 mg L^{-1} AS	Dilutions	¼ EC_{20}	½ EC_{20}	EC_{20}	2 EC_{20}	2.5 EC_{20}
		IBU (mg L^{-1})	118.6	237.3	474.5	949.0	1186
		TCS (mg L^{-1})	0.65	1.25	2.50	5.00	6.25
	2000 mg L^{-1} AS	Dilutions	¼ EC_{20}	½ EC_{20}	EC_{20}	*	*
		IBU (mg L^{-1})	159.4	318.9	637.8	*	*
		TCS (mg L^{-1})	2.90	5.90	12.0	*	*

* Limited by the solubility of TCS in phosphate buffer.

2.3.1. Inhibition of aerobic microbial activity

Respirometry assays were carried out using Oxygraph System (Hansatech System, Germany) connected to a computer. The reaction chamber included a Clark-type oxygen electrode in an enclosed cell equipped with magnetic stirring and thermostated at 30 °C. The cell was filled with 2 mL of sample taken from the 100 mL exposition flask. The evolution of the concentration of dissolved oxygen (DO) was registered and that, total oxygen uptake rate (OUR) was determined as a measure of whole aerobic biomass activity. The inhibition in respiration activity was calculated as the difference between the measured OUR in blank samples and OUR in presence of target compounds.

2.3.2. Inhibition of microbial enzymatic activity

In order to investigate the effect of toxicants on microbial enzymatic activity, we used fluorescein diacetate (FDA) staining. FDA is a non-fluorescent molecule that diffuse into cells and are hydrolyzed by intracellular non-specific esterases o many microorganisms and is a well-known indicator for the total esterase activity of AS. A stock solution was prepared in dimethyl sulfoxide with a concentration of 2 mg FDA mL^{-1} and stored at −20 °C until use. For

exposure assays, 5 µL of the stock solution were added to 195 µL of each sample in 96-well black microplates. Each plate was then incubated at 29.9 °C for 5 min into a Fluoroskan Ascent FL Fluorimeter/Luminometer. Fluorescence readings were taken during 30 min, at intervals of 5 min with excitation at 485 nm and emission at 528 nm. According to OECD Test Guide 209, synthetic sewage must be used for sludge feeding. We checked that the components of culture medium do not interfere with fluorescence measurements; consequently, the medium can be used [48]. The effect of the medium used on the measured signal of fluorescence was also checked, including three replicates of synthetic feed medium as a control without microorganisms for each series of Table 1. Background fluorescence was subtracted from the fluorescence recorded in each assay with AS and the inhibition percentage was calculated from the difference with the fluorescence value of blank runs.

2.3.3. Stability of exposure concentration

The stability and biodegradability of pollutants under the test conditions were tested according to OECD guideline for testing chemicals [49]. Briefly, toxicant concentrations were added to 2 g L^{-1} AS and incubated during 1 hour in the same conditions used in batch toxicity tests. Blank and control runs were performed in the absence of microorganisms and using sterilized sludge (previously autoclaved) in order to evaluate the sorption of target pollutants. Each assay condition was replicated three times. Sorption and other physicochemical phenomena were estimated by measuring the concentration of each contaminant in the liquid fraction at the beginning and at the end of the exposure time (1 h). From blank runs it was evaluated the effect of light and hydrolysis of compounds into aqueous reaction media. The biodegradation was assessed comparing the concentrations measured in the presence of sterile and nonsterile sludge samples. The sorption capacity of the sludge was calculated from the difference between the concentrations measured in assays with sterile sludge and blank runs performed in the absence of any kind of sludge. For the adsorption data, aqueous phase concentration (C_w) and sorbed concentration (C_s) were fitted to Freundlich equation, $C_s = K_f C_w^n$, in order to estimate K_f and n. TCS and IBU were analysed by high-performance liquid chromatography HPLC-UV using a 1200 Series Agilent Technologies equipment with Promosil C18 (4.6 × 150 mm, 60 mm) column. The elution was performed using 1 ml min^{-1} of 70:30 acetonitrile/water at pH 2 adjusted with phosphoric acid.

2.4. Equations for the evaluation of toxicity parameters

Toxicity parameters were calculated for individual pollutants and their binary combination, using the median-effect/combination index (CI)-isobologram equation, proposed in [40], which is based on the median-effect principle:

$$\frac{f_a}{1-f_a} = \left(\frac{D}{D_m}\right)^m \quad (1)$$

D is a concentration of toxicant that affects a population fraction f_a. D_m is the median effective concentration. The parameter m accounts for the sigmoidicity of the dose–effect curve. The combined effect was assessed by using combined doses over a wide range of effect levels. The combination index (CI) values were obtained according to the combination index equation, valid for n-chemical combination at x-percentage inhibition [50]:

$$(CI)_x^n = \sum_j^n \frac{(D)_j}{(D_x)_j} = \sum_j^n \frac{(D_x)_{1-n}\{[D]_j/\sum_1^n[D]\}}{(D_m)_j\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}} \quad (2)$$

Where $\mathrm{CI}(CI)_x^n$ is the combination index for n chemicals at a certain x inhibition, $[D]_j/\sum_1^n[D]$ is the ratio of a given (j) chemical inducing a x inhibition in combination and $(D_m)_j\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the dose of each compound alone producing the same effect. CI indicates additivity (CI = 1), synergism (CI < 1) or antagonism (CI > 1). The calculations were performed using CompuSyn software [51].

3. Results and discussion

3.1. Experiments for stability control under assays conditions

In this study, nominal concentrations of the chemicals were used for dose–effect parameter calculations because only small differences were found for both chemicals during the experiments performed; changes were always lower than 10% of initial concentration. The comparison between data using activated and sterilized sludge proved that neither TCS nor IBU were biodegraded in 1 h. The absence of IBU biodegradation was stated elsewhere for short contact [52]. Freundlich affinity coefficients, K_f, reflect the adsorption affinity and allow comparing activated and non-activated sludge samples. The values obtained were 0.35 ± 0.02 $mg^{(1-n)}$ L^n kg^{-1} for IBU and 1.36 ± 0.16 $mg^{(1-n)}$ L^n kg^{-1} for TCS irrespective of using sterile or nonsterile sludge. Freundlich linearity index (n) were 1.08 ± 0.13 and 0.63 ± 0.07 for IBU and TCS respectively. The amount of IBU adsorbed was determined as 17.3 ± 2.7% without significant differences between experiments using activated or sterilized sludge and runs with IBU alone or in mixture with TCS. The same figures for TCS amounted to 86.1 ± 5.5%. Orvos et al. [47] determined a mean percent of the TCS chemical adsorbed to the suspended solids of an AS of 53.7%. The difference is probably a consequence of the different pH (6.5–7.0 instead of 8.0) as the pKa of TCS is 8.1 as noted before. The similar results obtained for activated and non-activated sludge showed that no significant biodegradation took place during the 24 h period used for measuring equilibrium concentrations. TCS and IBU partition between the

biosolids and the liquid fraction. In any case, the concentration used for determining toxicity endpoints was the nominal one, which represented the sum of both once excluded biodegradation as responsible for significant depletion of both pollutants during bioassays.

3.2. Effect of individual pollutants on activated sludge

The inhibition tests were performed using AS after acclimatization, as stated at the beginning of Section 2.3. Table 2 summarizes the toxicity values obtained for IBU and TCS individually and in binary combinations for both toxicity assays. In cases in which the EC_{50} concentration was not reached, the calculation of Eqs. (1) and (2) was based on EC_{20} values. The inhibition of oxygen uptake due to the effect of individual pollutants decreased in the presence of relatively low amounts of TCS, in the few mg L^{-1} range. For a concentration of AS of 125 mg L^{-1}, the presence of 0.32 ± 0.07 mg L^{-1} of TCS led to a 50% decrease in the oxygen uptake rate, which increased with AS concentration up to a EC_{20} value of 4.93 ± 1.83 for 2 g L^{-1} AS. The effect of IBU was much less marked, with a concentration causing 50% reduction in oxygen consumption of 64 ± 13 mg L^{-1} for 125 mg L^{-1} AS. Over 125 mg L^{-1} AS, EC_{50} fell out of the 75–1200 mg L^{-1} experimental range for IBU concentrations. Concerning the enzymatic esterase activity, the toxicity profile was similar, with higher EC_{20} or EC_{50} values for increasing AS concentration. The sensitivity towards IBU and TCS was lower with EC_{50} values for IBU as high as 633 ± 63 mg L^{-1} for 125 mg L^{-1} AS. The EC_{50} values for TCS were in the 1.94-5.34 mg L^{-1} range for AS concentrations in the 125–500 mg L^{-1} range. Considering the obtained EC_{50} values, TCS should be considered toxic and IBU non-toxic to the microbial population of AS at TSS below 1 g L^{-1}, according to EU directives 93/67/EC and 67/548/EEC, which classified a compound as toxic when 1 mg L^{-1} > EC_{50} > 10 mg $L^{-1}$and non-toxic when having an EC_{50} > 100 mg L^{-1}.

Table 2. Dose-effect relationship parameters for IBU, TCS and their binary mixtures for oxygen uptake and enzymatic activity test. CI for different values of f_a have been obtained using CompuSyn software (Chou and Martin, 2005).

Oxygen uptake (respirometry)								
	IBU				TCS			
AS (mg L^{-1})	*EC_{20} (mg/L)*	*EC_{50} (mg/L)*	*m*	*r*	*EC_{20} (mg/L)*	*EC_{50} (mg/L)*	*m*	*r*
125	-	64 ± 13	0.42 ± 0.09	0.931	-	0.32 ± 0.07	0.45 ± 0.13	0.892
250	-	373 ± 53	0.46 ± 0.07	0.960	-	2.25 ± 0.40	0.40 ± 0.08	0.933
500	4.3 ± 1.6	-	0.21 ± 0.06	0.885	-	1.86 ± 0.14	0.60 ± 0.05	0.988
1000	197 ± 44	-	0.38 ± 0.05	0.975	-	5.49 ± 0.93	0.50 ± 0.08	0.958
2000	1032 ± 67	-	1.04 ± 0.13	0.977	4.93 ± 1.83	-	0.24 ± 0.03	0.973
	IBU + TCS				Combination index (CI)			
AS (mg L^{-1})	*EC_{20} (mg/L)*	*EC_{50} (mg/L)*	*m*	*r*	f_a = 0.2	f_a = 0.4	f_a = 0.6	f_a = 0.8
125		33.5 ± 4.0	0.89 ± 0.11	0.975	-	3.1 ± 0.8	1.2 ± 0.2	0.43 ± 0.16
250		63.1 ± 7.5	0.73 ± 0.11	0.970	-	1.6 ± 0.3	0.71 ± 0.11	0.26 ± 0.09
500		109 ± 22	0.57 ± 0.12	0.934	-	2.4 ± 0.1	1.01 ± 0.09	0.37 ± 0.28
1000	361 ± 24		1.13 ± 0.08	0.992	8.4 ± 1.6	2.8 ± 0.4	-	-
2000	131 ± 18		1.51 ± 0.35	0.915	5.2 ± 1.4	-	-	-
Enzymatic activity (fluorimetry)								
	IBU				TCS			
AS (mg L^{-1})	*EC_{20} (mg/L)*	*EC_{50} (mg/L)*	*m*	*r*	*EC_{20} (mg/L)*	*EC_{50} (mg/L)*	*m*	*r*
125	165 ± 16	633 ± 63	0.96 ± 0.10	0.984	-	1.94 ± 0.32	0.87 ± 0.17	0.950
250	255 ± 32	-	1,47 ± 0.18	0.978	1.61 ± 0.10	4.51 ± 0.28	1.34 ± 0.08	0.993
500	206 ± 68	-	0.65 ± 0.15	0.929	2.20 ± 0.14	5.34 ± 0.33	1.56 ± 0.20	0.975
1000	501 ± 167	-	0.62 ± 0.11	0.950	2.52 ± 1.20	-	0.95 ± 0.21	0.930
2000	764 ± 211	-	0.50 ± 0.07	0.966	-	-	0.85 ± 0.11	0.974
	IBU + TCS				Combination index (CI)			
AS (mg L^{-1})	*EC_{20} (mg/L)*	*EC_{50} (mg/L)*	*m*	*r*	f_a = 0.2	f_a = 0.4	f_a = 0.6	f_a = 0.8
125	26.5 ± 2.2	73.2 ± 6.0	1.36 ± 0.16	0.979	1.26 ± 0.23	0.85 ± 0.09	0.62 ± 0.12	0.42 ± 0.14
250	83.7 ± 5.9	147 ± 8	2.27 ± 0.19	0.989	1.38 ± 0.08	1.04 ± 0.08	0.83 ± 0.07	0.62 ± 0.08
500	119 ± 24	508 ± 101	0.94 ± 0.18	0.947	1.66 ± 0.37	1.24 ± 0.12	1.02 ± 0.39	-
1000	488 ± 30	-	1.02 ± 0.06	0.993	1.96 ± 0.20	1.46 ± 0.26	-	-
2000	-	-	1.72 ± 0.38	0.975	1.79 ± 0.42	-	-	-

The different toxicity of IBU and TCS was consistent with the data available for other aquatic organisms. The EC_{50} of TCS has been reported in the 2–390 μg L^{-1} range for the marine bacterium Vibrio fischeri, the crustacean Daphnia magna, the fish *Pimephales promelas* and some microalgae [41, 42, 53-56]. The reported toxicity to IBU is much lower, with EC_{50} in the 2–342 mg L^{-1} range for different species of algae and bacteria [47, 57-59]. The work of Orvos et al. [53] is particularly relevant as the authors measured the oxygen consumption of an AS exposed to TCS for 3 h. The authors reported a median inhibitory concentration of 20 mg L^{-1} for respiration using sludge concentrations of 2340 mg L^{-1} TSS. Ortiz et al. [4] reported EC_{50} values for IBU to the respiration activity of an AS of 937 mg L^{-1} for biomass concentration between 2000 to 4000 mg L^{-1}. The same authors reported for TCS a surprising EC_{50} of 1089 mg L^{-1} under the same conditions. These values are higher than the median effect oxygen uptake inhibition reported here for both contaminants. The differences can be due to the microbial diversity of the biomass used, the lower sludge concentration, the shorter exposure time and the fact that no carrier solvent was used in our assays.

The use of enzymatic activity for evaluating toxicity has been frequently reported. FDA hydrolysis has been used to estimate the total microbial activity, based on the ubiquitous presence of enzymes involved in FDA hydrolysis. Cytoplasmic esterases have also been used to measure cell viability because damaged cells cannot retain the fluorescent product from FDA cleavage, so viable cells are bright while nonviable cells are dim or non-fluorescent [60]. However, in the AS process, microbial communities use both intracellular and extracellular enzymes to hydrolyze nutrients with exoenzymatic activity usually localized in the particulate flocs as immobilized enzymes. Moreover, it has been noted the presence of enzymes in wastewater influents adsorbed on the extracellular matrix [61]. In our case, FDA cleavage was not intended to directly assess viability, but used as an expression of the secondary metabolic activity of biomass [62]. In our study, the EC_{20} and EC_{50} values obtained for IBU and TCS were systematically higher when measuring enzymatic activity than those recorded in oxygen uptake tests, which indicated a lower degree of damage. The reason for respirometric activity to be more strongly affected than enzymatic activity is that oxygen uptake is an indicator of the primary activity of microorganisms, closely linked to cellular respiratory processes, whereas esterase activity is representative of secondary metabolic processes linked to the consumption of energy reserves [63]. However, up to our knowledge, no data have been previously reported for the inhibition of the enzymatic activity of AS upon exposure to IBU or TCS or its comparison with respirometric measurements. Both methods yield different endpoints and are complementary. The lower values of ECx obtained in respirometry bioassays suggest that oxygen uptake is primarily impaired upon exposure to IBU or TCS.

3.3. Combined toxicity of ibuprofen and triclosan mixtures

The toxic effect of binary mixtures was obtained using the CI-isobologram methodology, which led to CI values that indicates synergism (CI < 1) or antagonism (CI > 1) when significantly, deviates from the unity. The advantage of using this approach is the relative simplicity to assess additivity over a wide range of concentrations and effect levels. Table 2 shows the EC_{20} (or EC_{50} when appropriate) for IBU + TRI mixtures at different AS concentration. The values (of equipotent mixtures) lay between the individual median effect values of IBU and TCS. Computed CI values are also shown in Table 2 for different values of affected population fraction, f_a, in the 0.2–0.8 range. For the lower effects and irrespective of using oxygen uptake or enzymatic activity as endpoints, the CI values were systematically higher than the unity, indicating antagonism. The concentration of AS did not influence the additive or non-additive behavior. Fig. 1, which refers to oxygen uptake experiments, shows the whole range of CI-f_a values for the different concentrations of AS used in this work. The lines represent the theoretical values computed using the algorithm of Chou and Martin [51], while the markers correspond to the actual experimental points with their corresponding 95% confidence intervals. IBU + TCS binary combinations showed a clear antagonism at low values of the population affected fraction up to about $f_a = 0.4$, after which the mixtures were additive up to about $f_a = 0.8$. For higher toxic effects, IBU + TCS mixtures were synergistic, with lowest CI clearly below 0.5. Considering additive behavior in the 2 < CI < 0.5

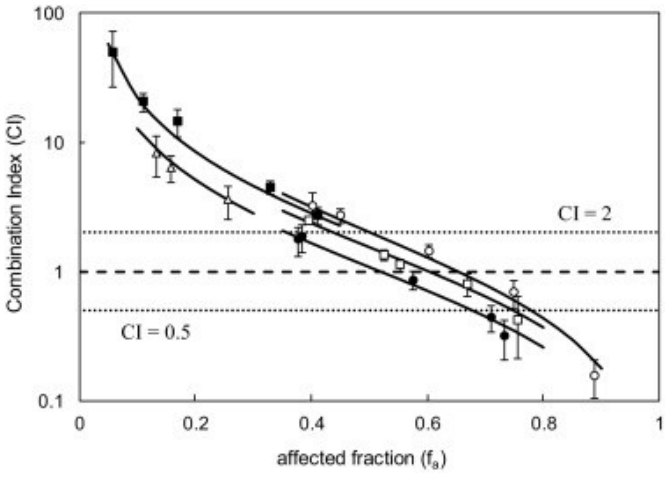

Figure 1. Combination index plot (CI-f_a) for the binary combinations IBU+TCS in oxygen uptake experiments with different concentrations of AS: 0.125 mg L^{-1} (○), 0.250 mg L^{-1} (●), 0.500 mg L^{-1} (□), 1.00 g L^{-1} (■) and 2.00 g L^{-1} (△). CI values are plotted as a function of the oxygen uptake inhibition by computer simulation CompuSyn within the experimental range of every assay. The vertical bars indicate 95 % confidence intervals.

interval, only the region close to the median effect value of mixtures can be deemed additive. The fact that at low effect levels, which corresponded to low IBU + TCS dosages, the behavior of mixtures was clearly antagonistic, with CI values over 50 is not a surprising observation. The tendency of mixtures moving from antagonism towards additivity and synergism as effect level increased has been reported previously in other systems [42, 43, 64].

Fig. 2 shows the CA-f_a plot for enzymatic activity measurements. The general trend is similar to that depicted from oxygen uptake inhibition except because the antagonism is restricted to the region of lower f_a values. For higher effect levels, IBU + TCS mixtures moved to additivity ($0.2 < f_a < 0.8$) with a slight tendency to synergy for the higher concentrations tested. Again, there were no clear differences depending on the AS concentration. Most CI for $f_a < 0.2$ correspond to clearly antagonistic mixtures (CI > 2), but their values were considerably lower than those obtained from oxygen uptake runs.

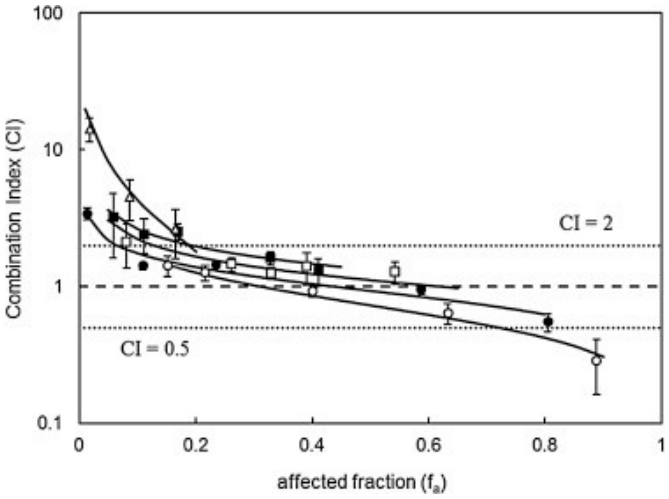

Figure 2. Combination index plot (CI-f_a) for the binary combinations IBU+TCS in enzymatic activity inhibition experiments with different concentrations of AS: 0.125 mg L^{-1} (○), 0.250 mg L^{-1} (●), 0.500 mg L^{-1} (□), 1.00 g L^{-1} (■) and 2.00 g L^{-1} (△). CI values are plotted as a function of the oxygen uptake inhibition by computer simulation CompuSyn within the experimental range of every assay. The vertical bars indicate 95 % confidence intervals.

Deviations from additivity are a complex issue but highly relevant for regulatory measures. The prediction of the effect of anthropogenic chemical mixtures has been studied in a high number of works. The results led to establishing an additivity paradigm, according to which, chemicals with common mode of action are described by concentration addition (CA), while independent action is commonly used for toxicants with different modes of action [37, 65]. Both, CA and IA, predict mixture toxicity based on the effect of individual components and there it is usually considered that deviations from additivity (antagonism and synergy) are considered toxicologically irrelevant and correspond to unusual situations. A common criticism to studies showing non-additive interactions is that they are often limited to a phenomenological description and do not offer an explanation of the underlying causes [39]. Certainly, there must be an underlying mechanism, unclear up to date in most cases although most probably based on dispositional or receptor antagonisms. On the other hand, the additive behavior of completely different substances at concentrations very different to that used to calculate their potency also lacks from a sound explanation and is entirely based on phenomenological observations. Our results show that even in mixtures approaching additivity near their EC_{50}, the deviations from additivity can be highly relevant for lower or higher effects. To the best of our knowledge, the present study is the first one to report the combined toxicity data and toxicological interaction of IBU and TCS to activated sludge. Consequently, for this microorganism community, no comparison with previous studies can be made.

4. Conclusions

We studied the toxicological interactions of IBU and TCS upon contact with AS. We found no significant biodegradation during this period and an extent of adsorption amounting to 17.3 ± 2.7% for IBU and 86.1 ± 5.5% for TCS.

IBU and TCS were respectively non-toxic and toxic with median effective concentrations of 64 ± 13 mg L^{-1} and 0.32 ± 0.07 mg L^{-1} for the reduction of respirometric activity.

The inhibition of enzymatic activity was measured from esterase FDA cleavage. The effect on enzymatic esterase activity was lower, with considerably higher EC_x values for both compounds. The oxygen uptake was more strongly impaired than enzymatic one because it was an indicator of the primary activity of microorganisms, whereas esterase activity is linked to the metabolic consumption of energy reserves.

The toxic effect of binary mixtures IBU + TCS was measured from the CI methodology. The mixtures showed a clear antagonism for oxygen uptake inhibition at low values of affected fraction, up to about $f_a = 0.4$, after which the mixtures behaved additively. Contrary to the commonly accepted assumption, strong mixture effect existed at low effect values, corresponding to the lower range of assayed concentrations. The antagonism was higher for oxygen uptake than for enzymatic activity and there were no differences depending on the AS concentration.

Acknowledgements

Financial support for this work was provided by the Regional Government of Madrid through program S2013/MAE-2716-REMTAVARES-CM. GA thanks the University of Alcalá for the award of predoctoral grant.

References

[1] A. Jurado, E. Vázquez-Suñé, J. Carrera, M. López, E. Pujades, D. Barceló, Emerging organic contaminants in groundwater in Spain: A review of sources, recent occurrence and fate in a European context, Sci. Total Environ. 440 (2012) 82–94.

[2] R. Loos, R. Carvalho, D.C. António, S. Comero, G. Locoro, S. Tavazzi, B. Paracchini, M. Ghiani, T. Lettieri, L. Blaha, B. Jarosova, S. Voorspoels, K. Servaes, P. Haglund, J. Fickd, R.H. Lindberg, D. Schwesig, B.M. Gawlik, EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents,Water Res. 47 (2013) 6475-6487.

[3] D.W. Kolpin, M. Skopec, M.T. Meyer, E.T. Furlong, S.D. Zaugg, Urban contribution of pharmaceuticals and other organic wastewater contaminants to streams during differing flow conditions, Sci. Total Environ. 328 (2004) 119–130.

[4] S.A. Ortiz de García, G. Pinto Pinto, P.A. García-Encina, R. Irusta-Mata, Ecotoxicity and environmental risk assessment of pharmaceuticals and personal care products in aquatic environments and wastewater treatment plants, Ecotoxicology 23 (2014) 1517–1533.

[5] M.C. Celander, Cocktail effects on biomarker responses in fish, Aquat. Toxicol. 105S (2011) 72– 77.

[6] M. Schriks, M.B. Heringa, M.M.E. van der Kooi, P. de Voogt, A.P. van Wezel, Toxicological relevance of emerging contaminants for drinking water quality, Water Res. 44 (2010) 461-476.

[7] J.L. Liu, M.H. Wong, Pharmaceuticals and personal care products (PPCPs): A review on environmental contamination in China, Environ. Int. 59 (2013) 208–224.

[8] C. Corada-Fernández, J. Jiménez-Martínez, L. Candela, E. González-Mazo, P.A. Lara-Martín, Occurrence and spatial distribution of emerging contaminants in the unsaturated zone. Case study: Guadalete River basin (Cadiz, Spain), Chemosphere 119 (2015) S131-S137.

[9] D. Mackay, D.M. Hughes, M.L. Romano, M. Bonnell, The role of persistence in chemical evaluations, Integr. Environ. Assess. Manag. 10 (2014) 588-594.

[10] G. Huschek, P.D. Hansen, H.H. Maurer, D. Krengel, A. Kayser, Environmental risk assessment of medicinal products for human use according to European commission recommendations, Environ Toxicol. 19 (2004) 226–240.

[11] M. Carballa, F. Omil, J.M. Lema, Comparison of predicted and measured concentrations of selected pharmaceuticals, fragrances and hormones in Spanish sewage, Chemosphere 72 (2008) 1118–1123.

[12] G. Bedoux, B. Roig, O. Thomas, V. Dupont, B. Le Bot, Occurrence and toxicity of antimicrobial triclosan and by-products in the environment, Environ. Sci. Pollut. Res. Int. 19 (2012) 1044–1065.

[13] M.J. Gómez, M. Petrovic, A.R. Fernández-Alba, D. Barceló, Determination of pharmaceuticals of various therapeutic classes by solid-phase extraction and liquid chromatography–tandem mass spectrometry analysis in hospital effluent wastewaters, J. Chromatogr A. 1114 (2006) 224–233.

[14] K.S. Kumar, S.M. Priya, A.M. Peck, K.S. Sajwan, Mass loadings of triclosan and triclocarban from four wastewater treatment plants to three rivers and landfill in Savannah, Georgia, USA, Arch. Environ. Contam. Toxicol. 58 (2010) 275–285.

[15] A. Pal, K.Y.H. Gin, A.Y.C. Lin, M. Reinhard, Impacts of emerging organic contaminants on freshwater resources: Review of recent occurrences, sources, fate and effects, Sci. Tot. Environ. 408 (2010) 6062–6069.

[16] M.J. Martínez Bueno, M.J. Gómez, S. Herrera, M.D. Hernando, A. Agüera, A.R. Fernández-Alba, Occurrence and persistence of organic emerging contaminants and priority pollutants in five sewage treatment plants of Spain: Two years pilot survey monitoring, Environ. Pollut. 164 (2012) 267-273.

[17] R. Rosal, A. Rodríguez, J.A. Perdigón-Melón, A. Petre, E. García-Calvo, M.J. Gómez, A. Agüera, A.R. Fernández-Alba, Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation, Water Res. 44 (2010) 578–588.

[18] W.C. Li, Occurrence, sources, and fate of pharmaceuticals in aquatic environment and soil, Environ. Pollut. 187 (2014) 193-201.

[19] P. Verlicchi, E. Zambello, Pharmaceuticals and personal care products in untreated and treated sewage sludge: Occurrence and environmental risk in the case of application on soil — A critical review, Sci. Tot. Environ. 538 (2015) 750–767

[20] H. Ishibashi, N. Matsumura, M. Hirano, M. Matsuoka, H. Shiratsuchi, Y. Ishibashi, Y. Takao, K. Arizono, Effects of triclosan on the early life stages and reproduction of medaka *Oryzias latipes* and induction of hepatic vitellogenin, Aquat. Toxicol. 67 (2004) 167-179.

[21] L.H. Yang, G.G. Ying, H.C. Su, J.L. Stauber, M.S. Adams, M.T. Binet, Growth inhibiting effects of 12 antibacterial agents and their mixtures on the freshwater microalgae *Pseudokirchneriella subcapitata*, Environ. Toxicol. Chem. 27 (2008) 1201–1208.

[22] Y. Hayashi, L.H. Heckmann, A. Callaghan, R.M. Sibly, Reproduction recovery of the crustacean

Daphnia magna after chronic exposure to ibuprofen, Ecotoxicology 17 (2008) 246–251.
[23] S. Han, K. Choi, J. Kim, K. Ji, S. Kim, B. Ahn, J. Yun, K. Choi, J.S. Khim, X. Zhang, J.P. Giesy, Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*, Aquat. Toxicol. 98 (2010) 256–264.
[24] M. Ragugnetti, M.L. Adams, A.T.B. Guimaraes, G. Sponchiado, E.C. de Vasconcelos, C.M.R. de Oliveira, Ibuprofen genotoxicity in aquatic environment: an experimental model using *Oreochromis niloticus*, Water Air Soil Pollut. 218 (2011) 361–364.
[25] J.B. Carbajo, J.A. Perdigón-Melón, A.L. Petre, R. Rosal, P. Letón, E. García-Calvo, Personal care product preservatives: Risk assessment and mixture toxicities with an industrial wastewater, Water Res. 72 (2015) 174–185.
[26] L. Pasquini, C. Merlin, L. Hassenboehler, J.F. Munoz, M.N. Pons, T. Görner, Impact of certain household micropollutants on bacterial behavior. Toxicity tests/study of extracellular polymeric substances in sludge, Sci. Total Environ. 463–464 (2013) 355-365.
[27] D.J.B. Dalzell, S. Alte, E. Aspichueta, A. de la Sota, J. Etxebarria, M. Gutierrez, C.C. Hoffmann, D. Sales, U. Obst, N. Christofi, A comparison of five rapid direct toxicity assessment methods to determine toxicity of pollutants to activated sludge, Chemosphere 47 (2002) 535-545.
[28] A.S. Stasinakis, D. Mamais, N.S. Thomaidis, E. Danika, G.Gatidou, T.D. Lekkas, Inhibitory effect of triclosan and nonylphenol on respiration rates and ammonia removal in activated sludge system, Ecotox. Environ. Saf. 70 (2008) 199–206.
[29] M. Molina-Muñoz, J.M. Poyatos, B. Rodelas, C. Pozo, M. Manzanera, E. Hontoria, J. González-López, Microbial enzymatic activities in a pilot-scale MBR experimental plant under different working conditions, Bioresource Technol. 101 (2010) 696–704.
[30] A. Guisasola, J.A. Baeza, J. Carrera, C. Casas, J. Lafuente, An off-line respirometric procedure to determine inhibition and toxicity of biodegradable compounds in biomass from an industrial WWTP, Water Sci. Technol. 48 (2004) 267-275.
[31] S. Ren, Assessing wastewater toxicity to activated sludge: recent research and developments, Environ. Int. 30 (2004)1151–1164.
[32] A.M. Polo, M. Tobajas, S. Sanchis, A.F. Mohedano, J.J. Rodríguez, Comparison of experimental methods for determination of toxicity and biodegradability of xenobiotic compounds, Biodegradation 22 (2011) 751–761.
[33] D.M. Coder, Assessment of cell viability, in: Current Protocols in Citometry, J Wiley & Sons, Inc, 1997, 9.2.1-9.2.14. DOI: 10.1002/0471142956.cy0902s15
[34] N. Brower, J. Kohen, J. Jamie, S. Vemulpadet, Modification of the fluorescein diacetate assay for screening of antifungal agents against *Candida albicans*: Comparison with the NCCLS methods, J. Microbiol. Meth. 66 (2006) 234– 24.
[35] D.W. Kolpin, E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, H.T. Buxton, Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: A national reconnaissance, Environ. Sci. Technol. 36 (2002) 1202–1211.
[36] A. Kortenkamp, T. Backhaus, M. Faust, State of the art report on mixture toxicity. Final report to the European Commission, 2009.
[37] European Commission Toxicity and assessment of chemical mixtures; Scientific Committee on Health and Environmental Risks (SCHER), Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and Scientific Committee on Consumer Safety (SCCS), Brussels, 2011.
[38] C. Marx, V. Mühlbauer, P. Krebs, V. Kuehn, Environmental risk assessment of antibiotics including synergistic and antagonistic combination effects, Sci. Total Environ. 524–525 (2015) 269-279.
[39] T. Backhaus, Medicines, shaken and stirred: a critical review on the ecotoxicology of pharmaceutical mixtures, Philos. Trans. R. Soc. Lond. B Biol. Sci. 369 (2014) 20130585.
[40] T.C. Chou, P. Talalay, Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors, Adv. Enzyme Regul. 22 (1984) 27–55.
[41] I. Rodea-Palomares, A.L. Petre, K. Boltes, F. Leganés, J.A. Perdigon-Melón, R. Rosal, F. Fernández-Piñas, Application of the combination index (CI)- isobologram equation to study the toxicological interactions of lipid regulators in two aquatic bioluminescent organisms, Water Res. 44 (2010) 427–438.
[42] R. Rosal, I. Rodea-Palomares, I., K. Boltes, F. Fernández-Piñas, F., Leganés, A. Petre, Ecotoxicological assessment of surfactants in the aquatic environment: combined toxicity of docusate sodium with chlorinated pollutants, Chemosphere 81 (2010) 288–293.
[43] K. Boltes, R. Rosal, E. García-Calvo, Toxicity of mixtures of perfluorooctane sulphonic acid with chlorinated chemicals and lipid regulators, Chemosphere 86 (2012) 24–29.
[44] I. Rodea-Palomares, F. Leganés, R. Rosal, F. Fernández-Pinas, Toxicological interactions of perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) with selected

pollutants, J. Hazard. Mater. 201– 202 (2012) 209– 218.
[45] S.K. Behera, S.Y. Oh, H.S. Park, Sorption of triclosan onto activated carbon, kaolinite and montmorillonite: effects of pH, ionic strength, and humic acid, J. Hazard. Mater. 179 (2010) 684–691.
[46] OECD, Test No. 209: Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation). http://www.oecd-ilibrary.org/environment/test-no-209-activated-sludge-respiration-inhibition-test_9789264070080-en, 2010 (accessed 10.11.16).
[47] V. González-Naranjo, K. Boltes, Toxicity of ibuprofen and perfluorooctanoic acid for risk assessment of mixtures in aquatic and terrestrial environments, Int. J. Environ. Sci. Technol. 11 (2013) 1743–1750.
[48] J.M. Clarke, M.R. Gillings, N. Altavilla, A.J. Beattie, Potential problems with fluorescein diacetate assays of cell viability when testing natural products for antimicrobial activity, J. Microbiol. Methods 46 (2001) 261–267.
[49] OECD, Guidelines for the Testing of Chemicals. http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm, (accessed 10.11.2016).
[50] T.C. Chou, Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies, Pharmacol. Rev. 58 (2006) 621–681.
[51] T.C. Chou, N. Martin, CompuSyn for drug combinations: PC Software and User's Guide: a computer program for quantitation of synergism and antagonism in drug combinations, and the determination of IC50 and ED50 and LD50 values. ComboSyn, Paramus, NJ, 2005.
[52] L. Ferrando-Climent, N. Collado, G. Buttiglieri, M. Gros, I. Rodríguez-Roda, S. Rodríguez-Mozaz, D. Barceló, Comprehensive study of ibuprofen and its metabolites in activated sludge batch experiments and aquatic environment, Sci. Total Environ. 438 (2012) 404–413.
[53] D.R. Orvos, D.J. Versteeg, J. Inauen, M. Capdevielle, A. Rothenstein, V. Cunningham, Aquatic toxicity of triclosan, Environ. Toxicol. Chem. 21 (2002) 1338–1349.
[54] N. Tatarazako, H. Ishibashi, K.Teshima, K. Kishi, K. Arizono, Effects of Triclosan on Various Aquatic Organisms, Environ. Sci. 11 (2004) 133-140.
[55] M.E. DeLorenzo, J.M. Keller, C.D. Arthur, M.C. Finnegan, H.E. Harper, V.L. Winder, D.L. Zdankiewicz, Toxicity of the antimicrobial compound triclosan and formation of the metabolite methyl-triclosan in estuarine systems, Environ. Toxicol. 23 (2008) 224-232.
[56] M. Farré, D. Asperger, L. Kantiani, S. González, M. Petrovic, D. Barceló, Assessment of the acute toxicity of triclosan and methyl triclosan in wastewater based on the bioluminescence inhibition of *Vibrio fischeri*, Anal. Bioanal. Chem. 390 (2008) 1999-2007.
[57] M. Cleuvers, Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid, Ecotox. Environ. Saf. 59 [2004] 309315.
[58] H. Yamamoto, Y. Nakamura, Y. Nakamura, C. Kitani, T. Imari, J. Sekizawa, Y. Takao, N. Yamashita, N. Hirai, S. Oda, N. Tatarazako, Initial ecological risk assessment of eight selected human pharmaceuticals in Japan, Environ. Sci. 14 (2007) 177-193.
[59] M. Farré, I. Ferrer, A. Ginebreda, M. Figueras, L. Olivella, L. Tirapu, M. Vilanova, D. Barceló, Determination of drugs in surface water and wastewater samples by liquid chromatography–mass spectrometry: methods and preliminary results including toxicity studies with *Vibrio fischeri*, J. Chromatogr. A 938 (2001) 187–197.
[60] S. Johnson, V. Nguyen, D. Coder, Assessment of cell viability, Curr. Protocol. Cytom. 64 (2013) 1-9.
[61] B. Frølund, T. Griebe, P.H. Nielsen, Enzymatic activity in the activated-sludge floc matrix, Appl. Microbiol. Biotechnol. 43 (1995) 755-761.
[62] J. Barragán, J.M. Quiroga, M.D. Coello, Use of microbial activity parameters for determination of a biosolid stability index, Biores. Technol. 97 (2006) 562-568.
[63] M.D. Coello, J. Barragán, J.M. Quiroga, Enzymatic estimation of biosolids stability in aerobic digestion systems, Enzyme Microbial Technol. 36 (2005) 191-197.
[64] G. Ziglio, G. Andreottola, S. Barbesti, G. Boschetti, L. Bruni, P. Foladori, R. Villa, Assessment of activated sludge viability with flow cytometry, Water Res. 36 (2002) 460-468.
[65] M. González-Pleiter, S. Gonzalo, I. Rodea-Palomares, F. Leganés, R. Rosal, K. Boltes, E. Marco, F. Fernández-Piñas, Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: Implications for environmental risk assessment, Water Res. 47 (2013) 2050-2064.
[66] T. Backhaus, R. Altenburger, Å. Arrhenius, H. Blanck, M. Faust, A. Finizio, P. Gramatica, M. Grote, M. Junghans, W. Meyer, M. Pavan, T. Porsbring, M. Scholze, R. Todeschini, M. Vighi, H. Walter and L. Horst Grimme, Cont. Shelf Res., 2003, 23, 1757-1769.