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Personal care product preservatives: risk assessment and mixture toxicities with an industrial wastewater

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Abstract

The aquatic toxicity of eight preservatives frequently used in personal care products (PCPs) (iodopropynyl butylcarbamate, bronopol, diazolidinyl urea, benzalkonium chloride, zinc pyrithione, propylparaben, triclosan and a mixture of methylchloroisothiazolinone and methylisothiazolinone) was assessed by means of two different approaches: a battery of bioassays composed of single species tests of bacteria (*Vibrio fischeri* and *Pseudomonas putida*) and protozoa (*Tetrahymena thermophila*), and a whole biological community resazurin-based assay using activated sludge. The tested preservatives showed considerable toxicity in the studied bioassays, but with a marked difference in potency. In fact, all biocides except propylparaben and diazolidinyl urea had EC_{50} values lower than 1 mg L^{-1} in at least one assay. Risk quotients for zinc pyrithione, benzalkonium chloride, iodopropynyl butylcarbamate and triclosan as well as the mixture of the studied preservatives exceeded 1, indicating a potential risk for the process performance and efficiency of municipal sewage treatment plants (STPs). These four single biocides explained more than 95% of the preservative mixture risk in all bioassays. Each individual preservative was also tested in combination with an industrial wastewater (IWW) from a cosmetics manufacturing facility. The toxicity assessment was performed on binary mixtures (preservative + IWW) and carried out using the median-effect principle, which is a special case of the concept of Concentration Addition (CA). Almost 70% of all experiments resulted in EC_{50} values within a factor of 2 of the values predicted by the median-effect principle (CI values between 0.5 and 2). The rest of the mixtures whose toxicity was mispredicted by CA were assessed with the alternative concept of Independent Action (IA), which showed higher predictive power for the biological community assay. Therefore, the concept used to accurately predict the toxicity of mixtures of a preservative with a complex industrial wastewater depends on the degree of biological complexity of the bioassay.

Keywords: Sewage Treatment Plant; Single Species Tests; Activated Sludge; Median-effect principle; Concentration Addition; Independent Action.

1. Introduction

The activated sludge process is widely used in sewage treatment plants (STPs), based on the development of a heterogeneous community composed of bacteria, protozoa, fungi and rotifers in an aeration tank. The activity and population of these organisms are crucial for proper system operation, and the presence of toxic substances in the influent may result in the depletion of the biomass activity and a lower performance of the STP (Dazell et al., 2002; Ricco et al., 2004).

STPs often receive industrial wastewater discharges, which are partially treated or even untreated. In fact, the failure of the effective operation of sewage works is usually attributed to the presence of certain pollutants of industrial origin that are toxic to the activated sludge organisms (Soupilas et al., 2008). Therefore, the continuous monitoring of potential toxic influent is essential in order to ensure effluent quality, reduce operating costs and increase reliability. Conventional chemical analyses have been found inadequate to ensure that the influent is not negatively influencing STP performance (Soupilas et al., 2008). The use of bioassays provides a holistic approach that allows the toxicity assessment of all components in any given complex mixture. The evaluation of industrial effluent toxicity should include a battery of bioassays composed of representative species of different trophic levels present in the activated sludge. However, although single species tests are fast, simple to perform, cost-effective and reliable, they have significant shortcomings such as not taking into account the interaction among species, the use of species that are not indigenous to the activated sludge with genetically homogeneous populations and the fact that tests are usually conducted under experimental conditions very different from an aeration tank (Selivanovskaya et al., 2004). Thus, toxicity assessment should be monitored using indigenous microbial population from an activated sludge process under conditions of forced aeration in order to provide a more accurate indication on the effects of STP influents on biological systems. These effects can be followed by

changes in metabolic activity of the activated sludge population using a resazurin-based assay, which has been shown to be a reliable and cost-effective manner to monitor the performance of STP (McNicholl et al., 2007).

An industrial sector of special concern for STP management is the cosmetics industry, which generates wastewater with an elevated concentration of biocides including many preservatives used in cosmetic formulations to avoid the development of microorganisms in the final product (Chapman, 2003; Russell, 2003). As a consequence of their biological activity, preservatives are of particular interest as they can potentially affect and harm activated sludge biomass. In addition to industrial wastewater, the regular usage of pharmaceuticals and personal care products (PPCPs) also contributes to the discharge of large quantities of ualtered preservatives (Ternes et al., 2004). Preservatives have been detected in concentrations of up to mg·L-1 and μg·L-1 in industrial effluents and STP influents, respectively (Kummerer et al., 1997; Magner et al., 2006; Norstrom et al., 2008; Kasprzyk-Hordern et al. 2009; Kumar et al., 2010; Poberznik et al., 2011). However, in comparison with other PPCPs such as antibiotics, relatively little is known about occurrence and toxicity of preservatives (Brausch and Rand, 2011). Even less attention has been paid to their risk towards microorganisms on activated sludge, which determines whether a particular preservative or mixture has the potential to cause harmful effects in order to protect the process performance and efficiency of an STP (van Leeuwen and Vermeire, 2007).

The co-occurrence of preservatives with other components of industrial wastewater is another case for concern due to the potential interactive effects, such as synergistic or antagonistic toxicity, that may occur from complex mixtures (Kolpin et al., 2002) in the STP influents. Therefore, it is essential to study the interactions of a preservative with industrial wastewater in order to determine the hazard that preservative spillage in cosmetics industry effluents could cause on activated sludge microorganisms. Since it would be an endless task to experimentally determine the toxicity of all relevant mixtures, predictive approaches based on the mathematical concepts of Concentration Addition (CA) and Independent Action (IA) have been proposed (Backhaus et al., 2003; Altenburger et al., 2004; Kortenkamp et al., 2009). Both predict the toxicity of a mixture based on the individual toxicity of the mixture components. Several reviews have shown that CA provides a reliable and frequently used tool for predicting and assessing the ecotoxicity of multi-component mixtures (Belden et al., 2003; Kortenkamp et al., 2009; Coors and Frische, 2011).

The study aims to assess the aquatic toxicities of eight preservatives using two different approaches: a battery of bioassays composed of single species tests of bacteria (*Vibrio fischeri* and *Pseudomonas putida*) and protozoa (*Tetrahymena thermophila*), and a whole biological community assay from activated sludge process. On the basis of toxicity data it is then assessed whether the tested preservatives might pose a risk to activated sludge process, and the nature of interactions between the preservatives and a complex industrial wastewater using CA or IA concept is studied.

2. Materials and methods

2.1 Preservatives

The preservatives used in this study belong to different classes and were selected based on their potential aquatic toxicity, their volume of consumption and their occurrence in STP influents (Table S1, Supplementary data). The following eight compounds were selected: iodopropynyl butylcarbamate (IPBC), bronopol (BNP), diazolidinyl urea (DIU), zinc pyrithione (ZPT), propylparaben (PPB) and triclosan (TCS) purchased from Sigma-Aldrich; benzalkonium chloride (BAC) purchased from Fluka, and a technical mixture of methylchloroisothiazolinone and methylisothiazolinone (CMI/MI) from Dow Chemical. The purity was IPBC ≥97%, BNP ≥98%, DIU ≥95%, TCS >97%, PPB ≥99%, ZPT~95%, BAC ≥95% (consisting of homologues of different alkyl chain lengths, mainly C12 60% and C14 40%), and CMI/MI 1.5%, which are the active ingredients of a commercial biocide KathonTM CG (CMI 1.15%, MI 0.35%, magnesium salts 23% and water to 100%). The stock solutions and the dilution series of each preservative were freshly prepared in ultrapure water obtained from a Millipore Milli-Q with a resistivity of at least 18 MΩ·cm at 25°C. The stability of preservatives under bioassay conditions was examined at the beginning and at the end of the exposure time according to OECD Guidance (OECD, 2008). The concentrations of CMI/MI, PPB, IPBC, TCS, BAC and ZPT remained 80-120% of nominal, therefore, the effect concentration was expressed relative to nominal concentrations in accordance with OECD Guidance. The concentrations of DIU and BNP did not remain within 80-120% of nominal as a result of their highly instability in aqueous solutions, which together with their low biodegradability have been previously reported (Madsen, 2001; ECHA, 2014). However, the toxicity of their degradation by-products has been shown to be comparable or higher than that of their parent compounds (Madsen, 2001; Cui et al., 2011) consequently, nominal concentrations were used in these cases as well.

2.2 Industrial wastewater

Wastewater was obtained from a cosmetics manufacturing facility located in Madrid (Spain) before further treatments. The physico-chemical characteristics of untreated IWW are shown in Table 1. Analytical determinations were carried out using standard methods (APHA, 1998). Trace metal concentrations were determined by Agilent 7700x ICP-MS. Industrial effluent samples gave high values for COD, total surfactants, total phenols and low for BOD5/COD, indicating that IWW

was largely loaded by non-biodegradable organic matter. Low concentration of AOX and heavy metals, which Zn represents near 95%, were detected. Wastewater samples were filtered using 0.45 μm glass-fiber filters and their pH adjusted to 7.0 ± 0.2 before conducting toxicity bioassays.

Table 1 Main physico-chemical parameters of the cosmetics industry wastewater

pH	4.1
Conductivity ($\mu S \cdot cm^{-1}$)	1473
TSS ($mg \cdot L^{-1}$)	167
COD ($mg \cdot L^{-1}$)	21280
BOD_5 ($mg \cdot L^{-1}$)	77
Chloride ($mg \cdot L^{-1}$)	206
Fluoride ($mg \cdot L^{-1}$)	ND
Sulphate ($mg \cdot L^{-1}$)	39.8
Sulfide ($mg \cdot L^{-1}$)	0.37
Sodium ($mg \cdot L^{-1}$)	359
Potassium ($mg \cdot L^{-1}$)	19.3
Magnesium ($mg \cdot L^{-1}$)	9.0
Calcium ($mg \cdot L^{-1}$)	66.5
Total phosphorous ($mg\ P \cdot L^{-1}$)	4.89
Total nitrogen ($mg\ N \cdot L^{-1}$)	26.4
Total surfactants ($mg \cdot L^{-1}$)	288
Anionic surfactants ($mg \cdot L^{-1}$)	179
Cationic surfactants ($mg \cdot L^{-1}$)	0.32
Non-ionic surfactants ($mg \cdot L^{-1}$)	109
AOX ($mg\ Cl \cdot L^{-1}$)	0.26
Total phenols ($mg \cdot L^{-1}$)	13.6
Arsenic ($\mu g \cdot L^{-1}$)	7.93
Cadmium ($\mu g \cdot L^{-1}$)	0.30
Chromium ($\mu g \cdot L^{-1}$)	7.62
Nickel ($\mu g \cdot L^{-1}$)	14.5
Mercury ($\mu g \cdot L^{-1}$)	0.16
Lead ($\mu g \cdot L^{-1}$)	14.1
Selenium ($\mu g \cdot L^{-1}$)	0.71
Copper ($\mu g \cdot L^{-1}$)	16.8
Zinc ($\mu g \cdot L^{-1}$)	841
ND = not detected	

2.3. Aquatic toxicity bioassay

The aquatic toxicities of the aforementioned compounds were assessed using a battery of bioassays composed of standard tests of the two main groups present in the activated sludge, namely the bacteria *V. fischeri* and *P. putida* and the protozoa *T. thermophila*, as well as an activated sludge biological community assay.

V. fischeri acute test measures the decrease in bioluminescence induced in cell metabolism. The bioassay was carried out according to ISO 11348-3 standard protocol (ISO, 2007) using the BioFix®Lumi test (*V. fischeri*, NRRL-B 11177 from Macherey-Nagel, Germany). The test was carried out in 96-well white polypropylene microplate. 100 mL of test solution (2% w/v NaCl and pH 7.0 ± 0.5) was transferred into each well, which was supplemented with 100 mL of bacterial suspension. Light was measured at 15 ± 1 ºC after 30 min by means of a Fluoroskan Ascent FL microplate luminometer (Thermo Scientific). *P. putida* test determines the inhibitory effect of a substance on the bacteria (*P. putida*, NCIB 9494 from CECT, Spain) by means of cell growth inhibition. The bioassay was performed according to ISO 10712 guideline (ISO, 1995). Bacterial cultures were exposed to test solutions at 23 ± 1 ºC for 16 h in glass incubation vials which were constantly shaken in darkness. The cell growth was determined by optical density (λ = 600 nm) in 96-well clear microplate (200 mL test suspension per well) using a Rayto RT-2100C microplate reader. Growth inhibition assay with the ciliate protozoa *T. thermophila* was performed according to the Standard Operational Procedure Guideline of Protoxkit F™ (1998). The test is based on the turnover of substrate into ciliate biomass. Substrate was purchased from MicroBioTest Inc. (Belgium) whereas *T. thermophila* (SB 210) was kindly supplied by D. Cassidy-Hanley (Tetrahymena Stock Center, Cornell University, USA). Ciliates were incubated with water samples and food suspension in test vessels at 30 ± 1 ºC for 24 h in darkness. Growth inhibition was determined on the basis of turbidity changes (OD at λ = 440 nm). $ZnSO_4 \cdot 7H_2O$ for *V. fischeri* (EC_{50} between 17 and 22 mg L^{-1}), 3,5-dichlorophenol for *P. putida* (EC_{50} between 10 and 30 mg L^{-1}) and $K_2Cr_2O_7$ for *T. thermophila* (EC_{50} between 15 and 24 mg L^{-1}) were used as reference substances in order to check each test procedure.

Activated sludge bioassay was carried out by evaluating the effect of water samples on activated sludge metabolic activity using the resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) method under the experimental conditions described in OECD Method 209 (OECD, 2010). Briefly, resazurin, blue and non-fluorescent in its oxidized stated, is reduced by metabolically active microorganisms to a pink fluorescent derivative (resorufin) by means of a dehydrogenase enzyme (McNicholl et al., 2007). Fresh activated sludge was collected from the aeration tank of an STP located in Guadalajara (Spain). Activated sludge was characterized by determining physico-chemical parameters (Table S2, Supplementary data). Inoculum (3.0 g L^{-1} of MLSS) supplemented with synthetic sewage feed was exposed to tested water samples at 20 ± 2 ºC for 3 h in glass vials which were constantly shaken (200 rpm) in darkness under conditions of forced aeration (0.5-1.0 L min^{-1}) (OECD, 2010). After exposure, biomass metabolic activity was measured in 96-well black polypropylene microplate by adding 200 mL test suspension and 20 mL of resazurin (100 mg L^{-1}) to each well. Resazurin reduction was measured after 20 min incubation using a Fluoroskan Ascent FL microplate fluorometer (Excitation 542 nm, Emission 592 nm). The suitability of each batch of activated sludge biomass was determined using $CuSO_4 \cdot 5H_2O$ as reference substance (EC_{50} between 15 and 35 mg L^{-1}).

2.4. Experimental design

Solutions of preservatives were tested singly and in binary mixtures with the industrial wastewater (preservative + IWW). The compounds were mixed relative to their potency (according to their EC_{50} values). Five to seven dilutions of each toxicant and combination, control and a reference substance were tested in three independent experiments with duplicate samples as described elsewhere (Rodea-Palomares et al., 2010).

2.5. Data treatment for determining individual and mixture toxicities

The description of the concentration-response curve for each substance and mixtures were estimated using the median effect equation based on the mass-action law (Chou and Talalay, 1984):

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

where D is the dose [concentration], D_m is the dose [concentration] for 50% (EC_{50}), f_a is the fraction affected by dose [concentration] D (e.g. 0.75 if growth is inhibited by 75%), fu is the fraction unaffected (i.e., $f_u = 1 - f_a$) and m is the coefficient of the sigmoidicity of the concentration-response curve: m = 1, >1, and <1 indicate hyperbolic, sigmoidal and flat sigmoidal concentration-response curve, respectively (Chou, 2006). Therefore, the method takes into account both potency (D_m) and shape (m) parameters. Eq. (1) may be rearranged as follows:

$$D = D_m \left(\frac{f_a}{1 - f_a}\right)^{1/m} \quad (2)$$

The D_m and m values for each individual compound or mixture were determined by the median-effect plot: x = log(D) versus y = log(f_a/f_u) which is based on the logarithmic form of Eq. (1). In the median-effect plot, m is the slope and $D_m = 10^{-(\text{y-intercept})/m}$. The conformity of the data to the median-effect principle can be readily assessed by the linear correlation coefficient (r) of the fitting to Eq. (2) (Chou, 2006).

These parameters were then used to calculate doses [concentrations] of individual compounds and their mixtures required to produce various effect levels according to Eq. (1). For each effect levels, Combination Index (CI) values were then calculated according to the general Combination Index equation for n-chemical combination at x% inhibition (Chou, 2006):

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

where $^{n}(CI)_x$ is the Combination Index for n chemicals at x% inhibition (e.g., growth inhibition); $(D_x)_{1\text{-}n}$ is the sum of the dose [concentration] of n chemicals that exerts x% inhibition in combination, $\{[D_j]/\Sigma[D]\}$ is the proportionality of the dose [concentration] of each n chemicals that exerts x% inhibition in combination, and $(D_j)\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the dose of each drug alone that exerts x% inhibition.

Combination Index is a special case of the more general concept of Concentration Addition (CA) (Backhaus, 2014), which is based on the assumption that all components in the mixture behave as if they are simple dilutions of one another, which is often taken to mean that CA describes the joint action of compounds with an identical mechanism of action (Kortenkamp et al., 2009). For a mixture of n components, the CA concept can be mathematically expressed as:

$$\sum_{i}^{n} \frac{c_i}{EC_{x_i}} = 1 \quad (4)$$

where c_i denotes the concentration of compound i in a mixture that is expected to cause x% effect, and EC_{xi} gives the concentration at which the compound i alone causes the same x% effect. If a mixture is accurately predicted by CA then the sum of fraction c_i/EC_{xi} equals 1, in the same way as in Combination Index CI = 1. Thus, two-fold deviation was applied as a threshold to denote compliance between the observed and the predicted mixture toxicity by median-effect principle (i.e., CI values between 0.5 and 2). Toxicity mixtures mispredicted by CA (i.e., CI values out of range 0.5-2) were assessed with the alternative concept of Independent Action (IA).

IA assumes that the resulting combined effect can be calculated from the effects caused by the individual mixture components, which is often taken to mean that IA describes the joint action of compounds with a dissimilar mechanism of action (Kortenkamp et al., 2009). The expected mixture effect can hence be calculated according to the joint probability of statistically independent events as:

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \quad (5)$$

where $E(c_{mix})$ is the total expected effect of the mixture, n is the number of mixture components and $E(c_i)$ is the effect that the i th component would cause if applied singly in concentration c_i.

2.5. Risk assessment

In order to estimate and assess the potential risk that preservatives could cause on activated sludge microorganisms, risk quotients (RQs) for microbial activity in municipal STP are calculated for the worst case scenario, the maximum preservative concentrations measured in STP influents.

The toxic units (TUs, EnvConc/EC50) of single preservatives were first calculated for each bioassay. Multiplying TUs by the assessment factor (AF), 10 for single species tests (highly relevant *P. putida* and *T.*

thermophila, and with limited relevance for STP process *V. fischeri*) and 100 for the activated sludge bioassay (ECHA, 2008), were calculated RQ for each single preservative. On this basis, the expected joint risk of the preservative mixture is then estimated using the strategy for the compound-based risk assessment of chemical mixtures (Backhaus and Faust, 2012), which is primarily based on the mixture toxicity concept of CA. In fact, the sum of toxic units (STU) was calculated in a first step for each bioassay. The final RQ for the mixture then equals the STU of the most sensitive bioassay (single species and activated sludge tests) multiplied by the corresponding AF (ECHA, 2008). RQ higher than 1 suggests that preservative risk would be inadequately controlled for the microorganisms present in an STP.

The application of CA to the preservative mixtures violates a main assumption: similar mode or mechanism of action. Hence, the maximum error that occurs by ignoring IA can be estimated as follows (Junghans et al., 2006):

$$\frac{EC_{50}^{IA}}{EC_{50}^{CA}} \leq \frac{\sum_{i}^{n} \frac{c_i}{EC_{50_i}}}{\max_{i \in (1,\ldots,n)} \left(\frac{c_i}{EC_{50_i}} \right)} \qquad (6)$$

Under these circumstances a maximum possible ratio by which CA may predict a higher mixture toxicity than IA equals the number of mixture components (n) (Faust, 1999). Given the uncertainty of the hazard and exposure estimates of the individual preservatives, a possible maximum error of less than 2 might be considered acceptable (Backhaus and Karlsson, 2014).

3. Results

3.1 Toxicity of single preservatives

All tested preservatives showed considerable toxicity in the studied bioassays, but with a marked difference in potency. Table 2 provides EC_{50} values together with the shape parameter *m* used for curve fitting to the observed data by means of the median-effect equation. Linear regression correlation coefficients of the median-effect plot were >0.95 in all cases (data not shown), indicating the agreement of the experimental data with the mass-action law. In order to show the quality of both observed data and curve fitting, as an example, concentration-response curves for CMI/MI in the studied bioassays are represented in Fig. 1 (for the rest of preservatives see Supplementary data, Fig. S1).

The single species tests were highly sensitive to selected preservatives without significant differences to previously published data (Table S3). All biocides, except PPB and DIU, displayed EC_{50} values lower than 1mg L^{-1} in at least one assay. This fact is in line with their classification as hazardous to the aquatic environment according to Regulation (EC) No. 1272/2008, which harmonises the provisions and criteria for the classification and labelling of substances, mixtures and certain specific articles within the European Union (EU Parliament and the Council, 2008). In fact, IPBC, CMI/MI, BNP, BAC, ZPT and TCS have already been classified into the acute aquatic hazard category as "very toxic to aquatic life" (H400, EC_{50} < 1 mg L^{-1} for algae, crustacean or fish), while the toxicities of DIU and PPB are characterized as "conclusive but not sufficient for classification" (ECHA, 2014). It should be noticed that ZPT and CMI/MI were the most toxic studied preservatives, showing EC_{50} values < 1 mg L^{-1} for the three organisms. The data also indicated the relative nonsensitivity of *P. putida* to preservatives that present a phenol moiety: TCS and PPB. High-level intrinsic resistance to TCS and PPB of Pseudomonas due to use degradative enzymes has been previously shown by Russell (1991) and Schweizer (2001), in agreement with the results presented in this study.

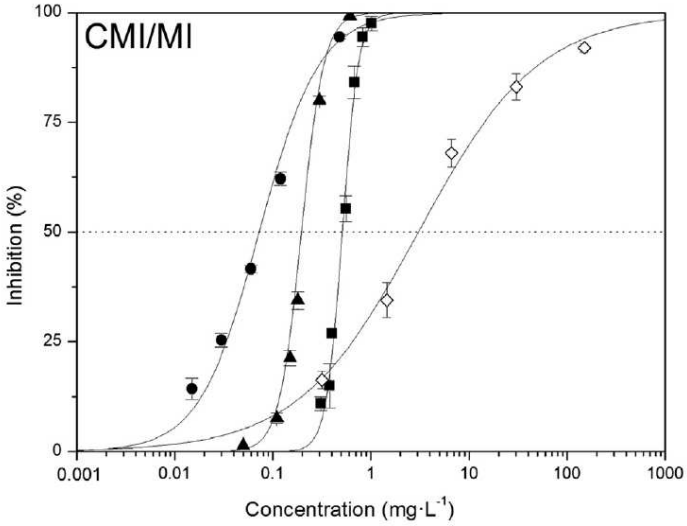

Figure 1. Concentration-response curves of CMI/MI for the bioassays: *Vibrio fischeri* (●), *Pseudomonas putida* (■), *Tetrahymena thermophila* (▲) and activated sludge test (◊) (mean ± 95% confidence interval).

In general, the same toxicity pattern displayed in single species tests was observed in the activated sludge assay. ZPT, CMI/MI and BAC are powerful biocides as demonstrated by their EC_{50} values lower than 4 mg L^{-1}, whereas low toxicity values were found for DIU and PPB. It is worth mentioning that the aquatic toxicity of preservatives for activated sludge based on oxygen consumption showed EC_{50} values in line or slightly higher than those obtained in this study despite the different endpoint used (Table S3). When comparing the toxicity of studied preservatives in biological community assay with single species tests, it becomes evident that the compounds show a comparatively lower toxicity towards activated sludge. The higher tolerance observed should be expected, considering the heterogeneity and more variable growth environment of the microorganisms used (Ricco et al., 2004), as well as the numerous mechanisms of resistance to biocides of activated sludge biomass as consequence of floc structure (Russell, 2003; Henriques and Love, 2007). The *V. fischeri* test was the most sensitive bioassay to the studied preservatives in line with previously published

Table 2. Concentration-response parameter values of the studied preservatives for each bioassay (mean ± 95% confidence interval).

	Vibrio fischeri		*Pseudomonas putida*		*Tetrahymena thermophila*		Activated sludge	
	EC_{50}	m	EC_{50}	m	EC_{50}	m	EC_{50}	m
IPBC	3.87±0.29	1.12±0.17	105±16	1.65±0.18	0.119±0.021	3.52±0.21	25.8±5.2	0.851±0.033
CMI/MI	0.063±0.004	1.34±0.15	0.509±0.065	5.29±0.09	0.195±0.033	3.85±0.18	3.04±0.66	0.710±0.040
BNP	0.171±0.012	1.38±0.09	1.25±0.18	3.47±0.21	4.66±0.56	3.08±0.21	12.1±2.3	0.604±0.051
DIU	51.4±1.9	1.25±0.03	171±12	5.72±0.13	37.0±4.1	5.61±0.08	335±33	0.618±0.073
BAC	0.259±0.08	1.50±0.12	8.40±0.52	4.27±0.18	4.28±0.43	2.33±0.12	3.43±0.58	0.620±0.055
ZPT	0.072±0.012	1.12±0.11	0.128±0.020	2.95±0.11	0.039±0.007	5.01±0.10	1.84±0.26	0.754±0.047
PPB	5.57±0.18	1.03±0.02	-	-	10.0±1.4	3.99±0.11	414±36	0.944±0.075
TCS	0.228±0.034	3.79±0.18	-	-	1.33±0.16	3.08±0.19	13.6±0.56[a]	0.328±0.029

[a] Estimation of EC_{50} value outside the concentration range. Maximum inhibition of 44% inhibition at 6.0 mg L^{-1}.

Table 3. Risk quotient (RQ) of the studied preservatives assuming a worst case scenario (maximum concentration detected in STP influents) to microorganism in STP. RQ higher than one are emphasized in bold.

	Occurrence in STP effluent (µg/L)	RQ	*Vibrio fischeri*		*Pseudomonas putida*		*Tetrahymena thermophila*		*Activated sludge*	
			TU	RQ	TU	RQ	TU	RQ	TU	RQ
IPBC	130[a]	**11**	0.034	0.34	0.001	0.01	1.092	**11**	0.005	0.50
CMI/MI	1.33[b]	0.21	0.021	0.21	0.003	0.03	0.007	0.07	0.000	0.04
BNP	ND (LOD=0.1)[c]	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000	0.00
DIU	-	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000	0.00
BAC	280[d]	**11**	1.081	**11**	0.033	0.33	0.065	0.65	0.082	**8.2**
ZPT	17[e]	**4.4**	0.236	**2.4**	0.133	**2.4**	0.436	**4.4**	0.009	0.92
PPB	8.29[f]	0.01	0.001	0.01	-	-	0.001	0.01	0.000	0.00
TCS	86.2[g]	**3.8**	0.378	**3.8**	-	-	0.065	0.65	0.006	0.63
MIXTURE		**18**	1.75	**18**	0.170	**1.7**	1.67	**17**	0.102	**10**

a Norstrom, K., Remberger, M., Kaj, L., Wiklund, P., Brorstrom-Lunden, 2008. Results from Swedish National Screening Programme 2008. Subreport 1. Biocides: 3-Iodo-2-propynyl butyl carbamate (IPBC) and 2,2-dibromo-2-cyanoacetamide (DBNPA). IVL Swedish Environmental Research Institute Ltd. IVL report B1889.

b Rafoth, A., Gabriel, S., Sacher, F., Brauch, H.J., 2007. Analysis of isothiazolinones in environmental waters by gas chromatography-mass spectrometry. J. Chromatogr. A. 1164, 74–81.

c Dye, C., Schlabach, M., Green, J., Remberger, M., Kaj, L., Palm-Cousins, A., Brorstrom-Lunden, E., 2007. Bronopol, resorcinol, m-cresol and triclosan in the Nordic environment. TemaNord 585 Available online at: http://www.norden.org/en/publications/publications/2007-585 (Verified October 15, 2014).

d Clara, M., Scharf, S., Scheffknech, C., Gans, O., 2007. Occurrence of selected surfactants in untreated and treated sewage. Water Res. 41, 4339–4348.

e Magner, J., Kaj, L., Brorstrom-Lunden, E., 2012. Results from the Swedish National Screening Programme 2006. Subreport 3: Zinc pyrithione and Irgarol 1051. IVL Swedish Environmental Research Institute Ltd. IVL Report B1764.

f Kasprzyk-Horden, B., Dinsdale, R.M., Guwy, A.J., 2009. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving water. Water Res. 43, 363–380.

g Kumar, K.S., Priya, S.M., Peck, A.M., Sajwan, K.S., 2010. Mass loading of triclosan and triclocarban from four wastewater treatment plants to three rivers and landfill in Savannah, Georgia, USA. Arch. Envrion. Contam. Toxicol. 58, 275–285.

ND = not detected; LOD = limit of detection.

results from other authors (Dalzell et al., 2002; Ricco et al., 2004). Nevertheless, the use of the *V. fischeri* test alone to assess effluent discharges to the sewer may lead to an overestimation of the toxicity effects on the biomass operating in the STP.

3.2 Preservative risk assessment

First, the potential risk for activated sludge microorganisms from the individual preservatives assuming a worst case scenario for a municipal STP is briefly assessed. Table 3 shows the maximum preservative concentrations detected in STP influents, the toxic units (TUs) calculated from toxicity data for the set of four bioassays (Table 2) and the risk quotients (RQs). RQs were calculated for each single preservative using the TUs and the corresponding assessment factor (10 for single species test and 100 for activated sludge bioassay according to ECHA, 2008). IPBC, BAC, ZPT and TCS exceed the threshold value of 1 for the protection of the activated sludge process. In all cases, RQs were based on toxicity data from the most sensitive tests: *V. fischeri* and *T. thermophila*. However, it is worth pointing out that the *V. fischeri* test has a limited relevance for the risk assessment of STP microorganisms (ECHA, 2008).

The data show that ZPT seems to be a risky preservative for both bacteria and ciliate protozoa (RQ > 1 in all single species tests), while IPBC, BAC and TCS might suppose a specific risk for one microorganism. Current published literature for STP influent concentrations is fairly extensive for some preservatives (i.e., TCS, PPB and BAC) but relatively little information is available for others (e.g., ZPT, IPBC, BNP, DIU) (Brausch and Rand, 2011). This fact constrains the calculation of their potential risk for activated sludge organisms as risk does not exist if exposure to a harmful substance or situation does not or will not occur (van Leeuwen and Vermeire, 2007).

On the basis of individual preservative TUs the expected joint risk of their mixture is then estimated and assessed, summing up the toxic units (STUs) for each bioassay according to the strategy for the compound-based risk assessment of chemical mixture proposed by Backhaus and Faust (2012). Thus, using the STUs from the four biotests and considering their assessment factor (ECHA, 2008), the results showed final RQs for single species of 1.7 for *P. putida*, 17 for *T. thermophila* and 18 for *V. fischeri*, and 10 for the activated sludge assay. That is, for all bioassays the preservative mixture poses a potential risk for the activated sludge process. However, this strategy is primarily based on the concept of Concentration Addition (CA), which can be criticized for violating its main assumption when applied to the mixture of preservatives presents in this paper: similar mode or mechanism of action of the substances of the mixture. The maximum error that occurred by simply ignoring the competing concept of Independent Action (IA) was estimated by means of the ratio between the EC_{50}s predicted by CA and IA as indicated in Eq. (6) (Junghans et al., 2006). The results show maximum errors of 1.6 for *V. fischeri*, 1.3 for *P. putida*, 1.5 for *T. thermophila* and 1.3 for activated sludge; all of them lower than 2, the value considered acceptable according to Backhaus and Karlsson (2014). This fact shows that CA can predict toxicity mixtures of dissimilarly acting substances with reasonable accuracy. Indeed, empirical evidence suggests that CA predicts with a tendency to slightly overestimate the mixture toxicity of dissimilarly acting compounds (Backhaus et al., 2010).

The distribution of the relative TUs is shown in Fig. 2 for the used bioassays. The plot clearly shows the uneven distribution of the toxic units in the mixture. BAC, ZPT and TCS contribute more than 95% to the overall STUs in all cases except for *T. thermophila*, for which IPBC explains a 66% of the total mixture toxicity. In fact, these four preservatives contribute most to the overall STUs, while the rest of the compounds has only a negligible contribution. It is also important to note that the main ecotoxicity risk driver depends on the bioassay: IPBC for *T. thermophila*, ZPT for *P. putida* and BAC for *V. fischeri* and activated sludge. In the same way, Backhaus and Karlsson (2014) have previously shown that the riskiest compound for pharmaceuticals in STP effluents depends on the species group under consideration

3.3. Interaction of preservatives with industrial wastewater

3.3.1. Industrial wastewater toxicity

Industrial wastewater (IWW) is studied by a whole-mixture approach, which is based on the direct ecotoxicological assessment of a given effluent. This approach allows analysing the real industrial effluent as if it was a single chemical (Backhaus et al., 2010). The tested IWW was highly toxic to *V. fischeri* and *T. thermophila* with EC_{50} of 0.051 and 0.318%, respectively. High toxicity of cosmetics industry effluents for single species has been previously reported (Perdigon-Melon et al., 2010; Pliego et al., 2012; de Melo et al., 2013), in which the elevated amount of toxicants present (surfactants, phenol derivatives, dyes, preservatives, etc.) and the possible mixture effects result in high toxicities. Particularly, de Melo et al. (2013) determined that surfactants were the main source of toxicity in a cosmetics industry effluent, whose concentrations in the presently studied wastewater were 288 mg L^{-1} (Table 1). The occurrence of 0.84 mg L^{-1} of Zn could also contribute to its toxicity due to the low EC_{50} values reported for this metal: 0.41-4.6 mg L^{-1} for *V. fischeri* (Dalzell et al., 2002; Teodorovic et al., 2009) and 3.6e6.7 mg L^{-1} for *T. thermophila* (Gallego et al., 2007; Mortimer et al., 2010). On the contrary, low toxicity was detected for *P. putida* (EC_{50} = 64.4%), a fact that may be due to its different metabolic pathways, including the ability of this microorganism to degrade organic pollutants and solvents (Hafner, 2004). The activated sludge assay was not especially sensitive to the tested IWW either, showing a 50% inhibition at 11.8%.

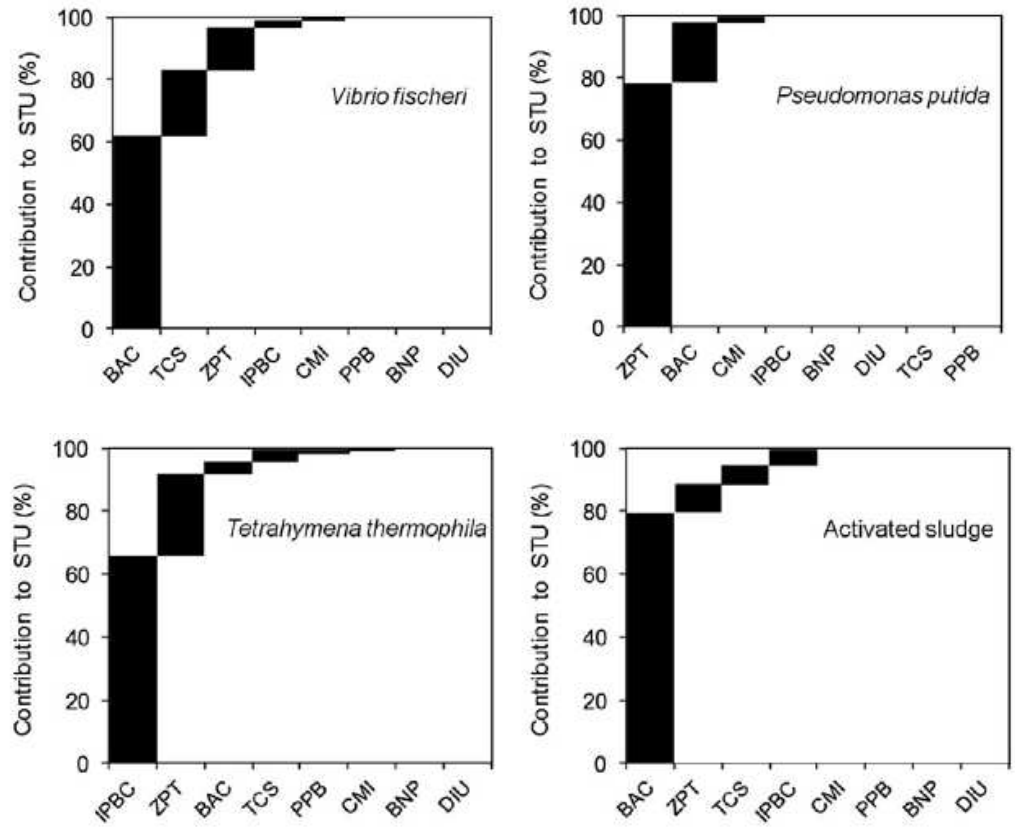

Figure 2. Distribution of toxic units of the studied preservatives present in the worst case scenario (maximum measured concentration in STP influents) for each bioassay.

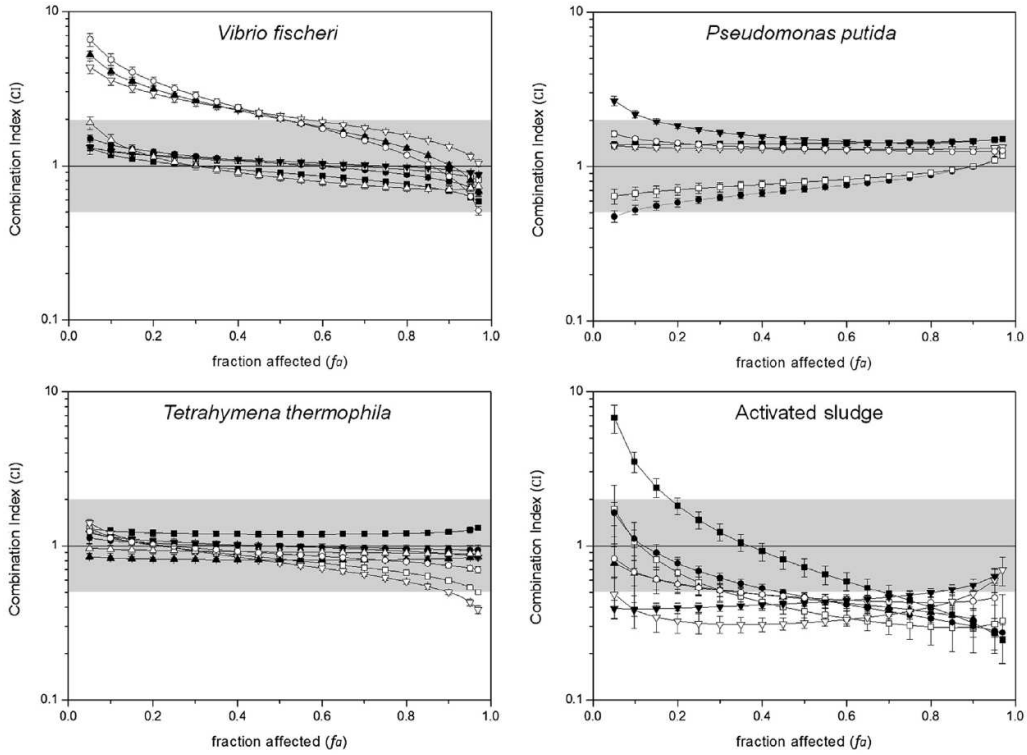

Figure 3. Combination Index plot (f_a-CI plot) for the studied bioassays of binary mixtures (preservative + IWW): IPBC + IWW (▼), CMI/MI + IWW (●), BNP + IWW (□), DIU + IWW (■), BAC + IWW (▽), ZPT + IWW (○), PPB + IWW (▲), TCS + IWW (△). CI values are plotted as function of the fractional inhibition of bioluminescence/growth/metabolic activity (f_a) by computer simulation (CompuSyn). CI between 0.5 and 2 indicate compliance between observed and predicted mixture toxicity by median-effect principle (CA). The vertical bars indicate 95% confidence intervals for CI values based on SDA (Sequential Deletion Analysis) (Chou and Martin, 2005).

3.3.2. Binary mixtures

The EC_{50} (D_m) and *m* values from IWW and single preservative, and their binary combinations (preservative + IWW) were used to quantify the predictive accuracy of median-effect law by Combination Index (CI) equation (Chou, 2006). The ratio between observed and predicted mixture toxicities was expressed as CI. A two-fold deviation has been applied as a threshold to denote compliance between observed and predicted mixture toxicity in the present study in accordance with previous studies (Belden et al., 2007; Coors and Frische, 2011).

Fig. 3 shows fa-CI plots of binary mixtures for single species and activated sludge tests. Twenty out of twenty-nine combinations that evaluated the median-effect principle observed effective concentration within a factor of 2 of predicted values (CI values between 0.5 and 2) on the f_a = 0.5 (further data Table S4, Supplementary data), where the inflexibility of the median-effect principle matters least (Backhaus, 2014). Hence, the combination effects of a given preservative in a complex industrial wastewater for almost 70% of mixtures could be approximated well by the median-effect principle, a special case of the more general concept of CA (Backhaus, 2014). Specifically, 86% of the studied binary combinations could be accurately predicted by CA concept for single species tests. These results are in line with the review on the predictive power of CA for pesticide mixtures performed by Belden et al. (2007), which demonstrated that in the majority of experiments (80% and more), mixture toxicity predictions based on CA deviated from the observed mixture toxicity by less than factor 2.

Nevertheless, the CA model was not able to correctly describe the mixture toxicity of nine of the combinations. In particular, BAC + IWW, ZPT + IWW, PPB + IWW toxicities for *V. fischeri* were overestimated with CI values higher than 2, while binary combinations of IPBC, CMI/MI, BNP, BAC, ZPT and PPB with IWW for activated sludge were underestimated, yielding CI values between 0.32 and 0.47. Toxicity mixtures mispredicted by CA were assessed with the alternative concept of IA. Fig. 4 displays the comparison for *V. fischeri* and activated sludge assays of CA- and IA-prediction with experimental data from the BAC + IWW mixture as an example. Experimental and predicted toxicity values for the rest of the tested mixtures can be found in Supplementary data (Figs. S2 and S3 for *V. fischeri* and activated sludge test, respectively). As can be seen in Fig. 4, CA and IA predict similar toxicities despite their mutually exclusive concepts, especially at low effects levels. This finding can be explained as a consequence of the similar shape and slope of the concentration-response curves of individual compounds (see Fig. S1 in Supplementary data) and do not involve any mechanistic implication (Backhaus et al., 2004). For *V. fischeri* test, IA and CA did not differ in predicting mixture toxicities at EC50 level, contrary to what is expected in most situations in which CA is considered the conservative

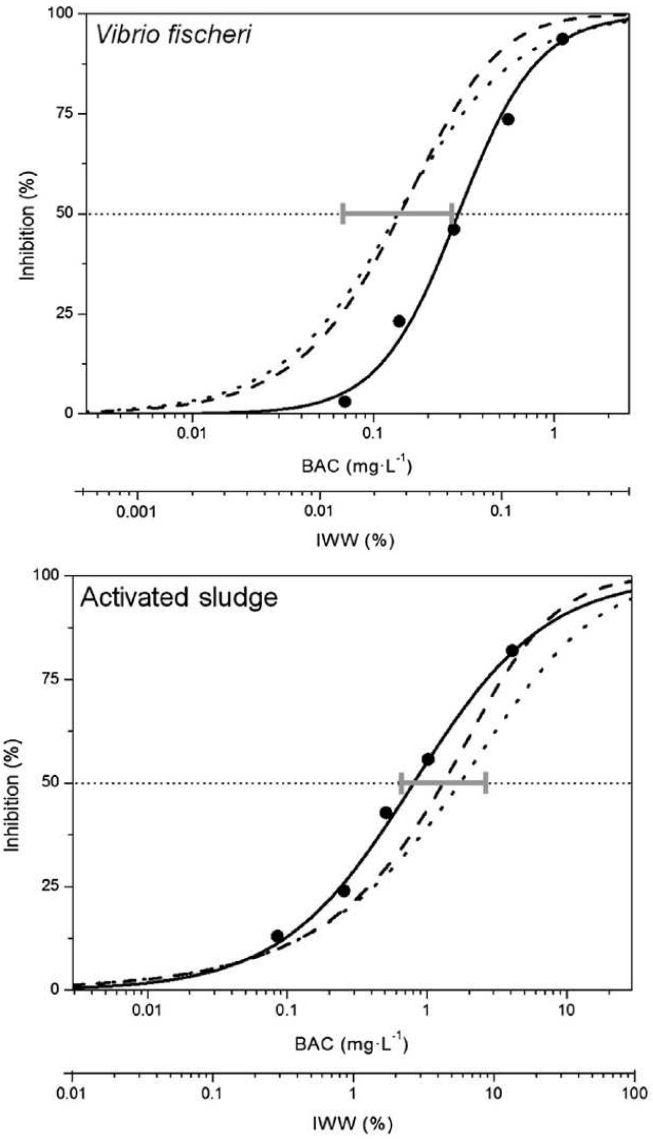

Figure 4. Predicted and observed toxicity for *Vibrio fischeri* and activated sludge test of BAC + IWW mixture. CA (⋯), IA (---) and observed effects (●). Two-fold deviation as a threshold to denote compliance between observed and predicted mixture toxicity is represented with a grey line.

model (Kortenkamp et al., 2009). This fact is due to the steepness of the concentration-response curves of single substances, which is known to be the major factor determining the relation between the EC_{50} values predicted by CA and IA (Boedeker et al., 1993; Drescher and Boedeker, 1995). Backhaus et al. (2004) showed that both concepts predict equal mixture toxicity with ratio EC_{50}/EC_{05} = 13.5, which corresponds to a median-effect parameter m = 1.13. If the steepness for the concentration-response curve of the mixture components is lower, CA predicts a lower EC_{50} for the mixture than IA and vice versa (Brosche and Backhaus, 2010). Specifically, *m* values of IWW (1.18), BAC (1.50), ZPT (1.12) and PPB (1.03) were in the interval 1.03-1.50. Thus, both CA and IA were not able to predict mixture toxicities for BAC + IWW. In this case as well as for the binary combinations of ZPT and PPB with IWW, observed mixture toxicities were slightly less toxic than those predicted by both models, displaying an antagonistic effect. The antagonism of these mixtures

might be explained considering that BAC, ZPT and PPB are membrane active agents that can be inactivated by surfactants (Rieger and Rhein, 1997). In the case of BAC, whose mechanism of action is based on the interaction of bipolar quaternary ammonium compound with the bacterial phospholipid bilayer, the occurrence of anionic surfactants in IWW (179 mg L^{-1}) may lead to ion pair formation, losing the bipolar structure and BAC bioactivity. Sutterlin et al. (2008) observed the same behaviour for *V. fischeri* exposed to mixtures of BAC and different anionic surfactants. In a similar way, the antagonism between PPB and an anionic surfactant (perfluorooctane sulfonic acid) has been reported to *Anabaena* CPB4337 (Rodea-Palomares et al., 2012). PPB can also be inactivated by non-ionic surfactants (Denyer, 1995), which were present at a significant concentration in the studied IWW (109 mg L^{-1}).

For activated sludge, it is interesting to note that IA predicts a higher toxicity than CA at EC_{50} values (Table S4). As it has already explained, this fact is due to the low steepness of the concentration-response curves of the single substances. In fact, *m* values of individual compounds are substantially smaller than the critical threshold of 1.13 ($m < 1.13$ equivalent to $EC_{50}/EC_{05} > 13.5$, Backhaus et al., 2004). Similar results were found for the prediction of mixture toxicity of antibiotic combinations on bacterial communities from artificial (STP in Christensen et al., 2006) and natural environment (lake in Brosche and Backhaus, 2010). In this study, higher predictive power was illustrated for IA than CA concept for the combination effects of preservatives in a complex industrial wastewater on a biological community. This was the case independent of whether or not these mixtures had been well approximated to CA such as DIU + IWW (Fig. S3). This fact could be explained by the complexity of IWW on a biological community. Activated sludge biomass is composed by flocs that are biological aggregates containing several levels of organization and porous structures in which cells are embedded in a polymer matrix (Henriques and Love, 2007). Reduced access of biocides to microbial cells because of the chemical interactions between extracellular polymeric substances and antimicrobial molecules is one of the proposed resistance mechanisms (Russell, 2003; Henriques and Love, 2007). However, the higher concentration of monovalent cations relative to the concentrations of divalent cations and the occurrence of poor biodegradable surfactants in the IWW (Table 1) could cause the disruption of exopolymer bridging thus, microorganisms would not adhere to each other and loose one floc resistance mechanism (Jenkins et al., 2003).

Therefore, the application of CA as the first predictive approach to mixture whose toxicity is actually better described by IA would hence lead to a slight overestimation of the mixture toxicity, and it was therefore suggested to apply CA as a somewhat conservative default approach for the predictive assessment of mixture toxicity in general (Backhaus and Faust, 2012). However, the toxicity of preservatives with a complex industrial wastewater towards intricate microbial communities might be an exception from this pattern, as the vast differences in sensitivity of the involved species seems to lead to extraordinary flat concentration-response curves.

4. Conclusions

The studied preservatives (iodopropynyl butylcarbamate, bronopol, diazolidinyl urea, benzalkonium chloride, zinc pyrithione, propylparaben, triclosan and a mixture of methylchloroisothiazolinone and methylisothiazolinone) showed considerable aquatic toxicity in single species tests and biological community activated sludge assay, but with a marked difference in potency. In fact, benzalkonium chloride, zinc pyrithione, iodopropynyl butylcarbamate and triclosan as well as the mixture of the tested preservatives displayed a potential risk to municipal STP performance. Among them, benzalkonium chloride is shown as the most problematic compound as is the risk driver of the mixture toxicity of preservatives in the activated sludge assay.

The degree of biological complexity of the used bioassays influences on the more suitable concept to predict the joint toxicity of the tested compounds with a cosmetic industry wastewater. In spite of the mixture toxicity in single species tests can be accurately predicted by Concentration Addition (CA), in the biological community activated sludge assay the prediction power is lower, and the alternative concept of Independent Action (IA) should be considered.

These results highlight that the toxic effects of preservatives towards activated sludge need to be carefully evaluated and that the potential risk management options should be studied. Special attention may be placed on benzalkonium chloride, on which should be assessed whether there is a need to perform mitigation measures such as source control by targeted restrictions or wastewater pretreatment before activated sludge process.

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Abbreviations

AF, Assessment factor;

BAC, Benzalkonium chloride;

BNP, Bronopol;

CA, Concentration Addition;

CI, Combination Index;

CMI/MI, Methylchloroisothiazolinone and methylisothiazolinone;

DIU, Diazolidinyl urea;

f_a, Fraction affected;

IA, Independent Action;

IPBC, Iodopropynyl butylcarbamate;

IWW, Industrial wastewater;

MLSS, Mixed liquor suspended solids;

PPB, Propylparaben;

PPCPs, Pharmaceuticals and personal care products;

RQs, Risk quotients;

STP, Sewage treatment plant;

STUs, Sum of Toxic Units;

TCS, Triclosan;

TUs, Toxic Units;

ZPT, Zn pyrithione

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.12.040.

References

Altenburger, R., Walter, H., Grote, M., 2004. What contributes to the combined effect of a complex mixture? Environ. Sci. Technol. 38, 6353e6362.

APHA, AWWA, WEF, 1998. Standard Methods for the Examination of Water and Wastewater, twentieth ed. American Water Works Association, Water Environment Federation, Washington, DC, USA.

Backhaus, T., Altenburger, R., Arrhenius, A., Blanck, H., Faust, M., Finizio, A., Gramatica, P., Grote, M., Junghans, M., Meyer, W., Pavan, M., Porsbring, T., Scholze, M., Todeschini, R., Vighi, M., Walter, H., Grimme, L.H., 2003. The BEAM-project: prediction and assessment of mixture toxicities in the aquatic environment. Cont. Shelf Res. 23, 1757e1769.

Backhaus, T., Faust, M., Scholze, M., Gramatica, P., Vighi, M., Grimme, L.H., 2004. Joint algal toxicity of phenylurea herbicides is equally predictable by concentration addition and independent action. Environ. Toxicol. Chem. 23, 258e264.

Backhaus, T., Faust, M., Blanck, H., 2010. Hazard and Risk Assessment of Chemical Mixtures under REACH. State of the Art, Gaps and Options for Improvement. Swedish Chemicals Agency report PM 3/10.

Backhaus, T., Faust, M., 2012. Predictive environmental risk assessment of chemical mixtures: a conceptual framework. Environ. Sci. Technol. 46, 2564e2573.

Backhaus, T., Karlsson, M., 2014. Screening level mixture risk assessment of pharmaceuticals in STP effluents. Water Res. 49, 157e165.

Backhaus, T., 2014. Medicines, shaken and stirred: a critical review on the ecotoxicology of pharmaceutical mixtures. Philos. Trans. R. Soc. B 369, 20130585.

Belden, J.B., Gilliom, R.J., Lydy, M.J., 2007. How well can we predict the toxicity of pesticide mixtures to aquatic life? Int. Environ. Assess. Manag. 3, 364e372.

Brausch, J.M., Rand, G.M., 2011. A review of personal care products in the aquatic environment: environmental concentrations and toxicity. Chemosphere 82, 1518e1532.

Boedeker, W., Drescher, K., Altenburger, R., Faust, M., Grimme, L.H., 1993. Combined effects of toxicants: the need and soundness of assessment approaches in ecotoxicology. Sci. Total Environ. 134, 931e939.

Brosche, S., Backhaus, T., 2010. Toxicity of five protein synthesis inhibiting antibiotics and their mixture to limnic bacterial communities. Aquat. Toxicol. 99, 457e465.

Chapman, J.S., 2003. Biocide resistance mechanisms. Int. Biodeterior. Biodegrad. 51, 133e138.

Chou, T.C., Talalay, P., 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. 22, 27e55.

Chou, T.C., Martin, N., 2005. CompuSyn for Drug Combinations: PC Software and User's Guide: a Computer Program for Quantification of Synergism and Antagonism in Drug Combinations and the Determination of IC_{50} and ED_{50} and LD_{50} Values. ComboSyn, Inc., Paramus, NJ.

Chou, T.C., 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. 58, 621e681.

Christensen, A.M., Ingerslev, F., Baun, A., 2006. Ecotoxicity of mixtures of antibiotics used in aquacultures. Environ. Toxicol. Chem. 25, 2208e2215.

Clara, M., Scharf, S., Scheffknech, C., Gans, O., 2007. Occurrence of selected surfactants in untreated and treated sewage. Water Res. 41, 4339e4348.

Coors, A., Frische, T., 2011. Predicting the aquatic toxicity of commercial pesticide mixtures. Environ. Sci. Eur. 23, 22e40.

Cui, N., Zhang, X., Xie, Q., Wang, S., Chen, J., Huang, L., Qiao, X., Li, X., Cai, X., 2011. Toxicity profile of labile preservative bronopol in water: the role of more persistent and toxic transformation product. Environ. Pollut. 159, 609e615.

Dalzell, D.J.B., Alte, S., Aspichueta, E., de la Sota, A., Etxebarria, J., Gutierrez, M., Hoffmann, C.C., Sales, D., Obst, U., Christofi, N., 2002. A comparison of five rapid direct toxicity assessment

methods to determine toxicity of pollutants to activated sludge. Chemosphere 47, 535e545.
de Melo, E.D., Mounteer, A.H., Leao, L.H.S., Bahia, R.C.B., Campos, I.M.F., 2013. Toxicity identification evaluation of cosmetics industry wastewater. J. Hazard. Mater. 244e245, 329e334.
Denyer, S.P., 1995. Mechanisms of action of antibacterial biocides. Int. Biodeterior. Biodegrad. 227e245.
Drescher, K., Boedeker, W., 1995. Assessment of the combined effects of substances e the relationship between concentration addition and independent action. Biometrics 51, 716e730.
Dye, C., Schlabach, M., Green, J., Remberger, M., Kaj, L., Palm-Cousins, A., Brorstrom-Lunden, E., 2007. Bronopol, Resorcinol, m-Cresol and Triclosan in the Nordic Environment. Nordic Council of Ministers. TemaNord 585.
ECHA (European Chemical Agency), 2008. Characterisation of dose [concentration]-response for environment (Chapter R. 10). Guideline on Information Requirements and Chemical Safety Assessment.
ECHA (European Chemical Agency), 2014. Information on Chemicals. European Chemical Agency. Available online at: http://echa.europa.eu/information-on-chemicals (verified 15.10.14.).
EU Parliament and the Council, 2008. Regulation (EC) 1272/2008 of the European Parliament and of the Council of 16 December 2008 on Classification, Labeling and Packaging of Substances and Mixtures, Amending and Repealing Directives 67/548/EEC and 199/45/EC, and Amending Regulation (EC) No 1907/2006.
Faust, M., 1999. Kombinationseffekt von Schadstoffen Auf Aquatische Organismen: Prüfung Der Vorhersagbarkeit Am Beispeil Einzelliger Grünalgen. GCA-Publishers, Herdecke.
Gallego, A., Martin-Gonzalez, A., Ortega, R., Gutierrez, J.C., 2007. Flow cytometry assessment of cytotoxicity and reactive oxygen species generation by single and binary mixtures of cadmium, zinc and copper on populations of the ciliated protozoan *Tetrahymena thermophila*. Chemosphere 68, 647e661.
Hafner, C., 2004. *Pseudomonas putida* growth inhibition test. In: Moser, H., Römbke, J. (Eds.), Ecotoxicological Characterization of Waste. Results and Experiences of an International Ring Test. Springer, New York.
Henriques, I.D.S., Love, N.G., 2007. The role of extracellular polymeric substances in the toxicity response of activated sludge bacteria to chemical toxins. Water Res. 41, 4177e4185.
ISO (International Organization for Standardization), 1995. Water Quality - *Pseudomonas putida* Growth Inhibition Test (*Pseudomonas* Cell Multiplication Inhibition Test). EN ISO 10712:1995.
ISO (International Organization for Standardization), 2007. Water Quality e Determination of the Inhibitory Effect of Water Samples on the Light Emission of *Vibrio fischeri* (Luminescent Bacteria Test). ISO 11348-3:2007.
Jenkins, D., Richard, M.G., Daigger, G.T., 2003. Manual on the Causes and Control of Activated Sludge Bulking, Foaming, and Other Solids Separation, third ed. IWA Publishing, London.
Junghans, M., Backhaus, T., Faust, M., Scholze, M., Grimme, L.H., 2006. Application and validation of approaches for the predictive hazard assessment of realistic pesticide mixtures. Aquat. Toxicol. 76, 93e110.
Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2009. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving water. Water Res. 43, 363e380.
Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999e2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202e1211.
Kortenkamp, A., Backhaus, T., Faust, M., 2009. State of the Art Report on Mixture Toxicity. Final report to the European Commission. Contract no. 070307/2007/485103/ETU/D.1.
Kumar, K.S., Priya, S.M., Peck, A.M., Sajwan, K.S., 2010. 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, 275e285.
Kummerer, K., Eitel, A., Braun, U., Hubner, P., Daschner, F., Mascart, G.,Milandri, M.,Reinthaler, F.,Verhoef, J., 1997.Analysis of benzalkonium chloride in the effluent from European hospitals by solid-phase extraction and high-performance liquid chromatography with post-column ion-pairing and fluorescence detection. J. Chromatogr. A 774, 281e286.
Madsen, T., Boyd, H.B., Nylen, D., Pedersen, G.I.R., Simonsen, F., 2001. Environmental and Health Assessment of Substances in Household Detergents and Cosmetic Detergent Products. Danish Environmental Protection Agency. Environmental project no. 615.
McNicholl, B.P., McGrath, J.W., Quinn, J.P., 2007. Development and application of a resazurin-based biomass activity test for activated sludge plant management. Water Res. 41, 127e133.
Mortimer, M., Kasemets, K., Kahru, A., 2010. Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*. Toxicology 269, 182e189.
Norstrom, K., Remberger, M., Kaj, L., Wiklund, P., Brorstrom-Lunden, 2008. Results from Swedish National Screening Programme 2008. Subreport 1.

Biocides: 3-Iodo-2-Propynyl Butyl Carbamate (IPBC) and 2,2-Dibromo-2-Cyanoacetamide (DBNPA). IVL Swedish Environmental Research Institute Ltd.. IVL report B1889.

OECD (Organisation for Economic Co-operation and Development), 2010. Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation), Method 209. In: OECD Guidelines for Testing of Chemicals on Aquatic Toxicity. OECD, Paris.

OECD (Organisation for Economic Co-operation and Development), 2008. Testing of Difficult Substances and Mixtures, No. 23. In: OECD Guidelines for Testing of Chemicals on Aquatic Toxicity. OECD, Paris.

Perdigón-Melón, J.A., Carbajo, J.B., Petre, A.L., Rosal, R., García-Calvo, E., 2010. Coagulation-Fenton coupled treatment for ecotoxicity reduction in highly polluted industrial wastewater. J. Hazard. Mater. 181, 127e132.

Pliego, G., Zazo, J.A., Blasco, S., Casas, J.A., Rodríguez, J.J., 2012. Treatment of highly polluted hazardous industrial wastewaters by combined coagulation-adsorption and high temperature Fenton oxidation. Ind. Eng. Chem. Res. 51, 2888e2896.

Poberznik, M., Bauman, M., Ros, M., Lobnik, A., 2011. Reduction of biocide-polluted wastewater using O3 and H_2O_2/O_3 oxidation processes. Ozone-Sci. Eng. 33, 31e36.

Protoxkit F™, 1998. Freshwater Toxicity Test with a Ciliate Protozoan, Standard Operational Procedure. Creasel, Deinze.

Rafoth, A., Gabriel, S., Sacher, F., Brauch, H.J., 2007. Analysis of isothiazolinones in environmental waters by gas chromatography-mass spectrometry. J. Chromatogr. A. 1164, 74e81.

Ricco, G., Tomei, M.C., Ramadori, R., Laera, G., 2004. Toxicity assessment of common xenobiotic compounds on municipal activated sludge: comparison between respirometry and Microtox®. Water Res. 38, 2013e2110.

Rieger, M., Rhein, L.D., 1997. Surfactants in Cosmetics, second ed. Markel Dekker, Inc., New York.

Rodea-Palomares, I., Leganés, F., Rosal, R., Fernandez-Piñas, F., 2012. Toxicological interactions of perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) with selected pollutants. J. Hazard. Mater. 30, 209e218.

Rodea-Palomares, I., Petre, A.L., Boltes, K., Leganés, F., Perdigón-Melón, J.A., Rosal, R., Fernandez-Piñas, F., 2010. Application of the combination index (CI)-isobologram equation to study the toxicological interactions of lipid regulators in two aquatic bioluminescent organisms. Water Res. 44, 427e438.

Russell, A.D., 1991. Mechanisms of bacterial resistance to nonantibiotics: food additives and food and pharmaceutical preservatives. J. Appl. Bacteriol. 71, 191e201.

Russell, A.D., 2003. Similarities and differences in the responses of microorganisms to biocides. J. Antimicrob. Chemother. 52, 750e763.

Schweizer, H.P., 2001. Triclosan: a widely used biocide and its link to antibiotics. FEMS Microbiol. Lett. 202, 1e7.

Selivanovskaya, S.Y., Latypova, V.Z., Stepanova, N.Y., Hung, Y.T., 2004. Bioassay of industrial waste pollutants. In: Wang, L.K., Hung, Y.T., Lo, H.H., Yapijackis, C. (Eds.), Handbook of Industrial and Hazardous Wastes Treatment, second ed. Marcel Dekker, Inc., New York.

Soupilas, A., Papadimitriou, C.A., Samaras, P., Gudulas, K., Petridis, D., 2008. Monitoring of industrial effluent ecotoxicity in the greater Thessaloniki area. Desalination 224, 261e270.

Sutterlin, H., Alexy, R., Kummerer, K., 2008. The toxicity of the quaternary ammonium compound benzalkonium chloride alone and in mixtures with other anionic compounds to bacteria in test systems with *Vibrio fischeri* and *Pseudomonas putida*. Ecotoxicol. Environ. Saf. 71, 498e505.

Teodorovic, I., Planojevic, I., Knezevic, P., Radak, S., Nemet, I., 2009. Sensitivity of bacterial vs. acute *Daphnia magna* toxicity tests to metals. Cent. Eur. J. Biol. 4, 482e492.

Ternes, T., Joss, A., Siegrist, H., 2004. Scrutinizing pharmaceuticals and personal care products in wastewater treatment. Environ. Sci. Technol. 38, 393Ae399A.

van Leeuwen, C.J., Vermeire, T.G., 2007. Risk Assessment of Chemicals. An Introduction, second ed. Springer, Dordrecht.

Woldegiorgis, A., Remberger, M., Kaj, L., Green, J., Ekheden, Y., Cousins, A.P., Brorstrom-Lunden, E., Dye, C., Aspmo, K., Vadset, M., Schlabach, M., Langford, K., 2007. Results from the Swedish National Screening Programme 2006. Subreport 3: Zinc pyrithione and Irgarol 1051. IVL Swedish Environmental Research Institute Ltd, IVL report B1764.