Environmental concerns about the effects of effluents from wastewater treatment plants in tourist areas of the Alps: toxicity in aquatic microorganisms

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ABSTRACT

Are the effluents of wastewater treatment plants in high mountains of concern for aquatic biodiversity? To answer this question, we carried out an experimental study testing the short-term toxicity of some Pharmaceutical Active Compounds (PhACs) in the effluents of a plant in a mountain valley of the Italian Alps sampled during the high tourist season (*i.e.*, the ski season) when PhACs contamination is higher. We used different tools, taking as a model the bacterium *Aliivibrio fischeri:* the "whole-mixture approach" (Microtox test), "component-based approach", predictive models "concentration addition (CA)", "independent action (IA)", and combination index (CI)". We investigated the nature of interactions potentially occurring among seven selected PhACs (clarithromycin, naproxen, acetaminophen (paracetamol), ibuprofen, diclofenac, carbamazepine, and amoxicillin). This study showed that anti-inflammatory ibuprofen and diclofenac have higher short-term toxicity (IC_{50} <100 mg L^{-1}) for *A. fischeri* compared with antibiotics, whose toxic effects are expected to become visible in the long term. Furthermore, based on the CI method, the seven PhACs seem not to interact in a synergistic or antagonistic way, but the final effect of their mixture seems to be equal to the sum of their individual effects. Notwithstanding the high tourist pressure, the Microtox test reported an overall toxicity of only 21%, which drops to 7% in the receiving water body, the Vermigliana stream. These values, besides the predictions by CA and IA, are not alarming *per se*, *i.e.*, the treated effluent of the plant in the period of maximum tourist pressure can be considered no harmful to aquatic microorganisms. However, based on other studies highlighting negative effects of the diluted treated effluent of the same plant on macroinvertebrate community structure, we suggest that other model organisms be considered, including algae, insects, and fish, to assess the real ecological risk to wildlife of an effluent. The experimental tests on *A. fischeri* are useful for fast, preliminary information on the level of risk for freshwater ecosystems, but they should be combined with field studies and experiments on the wild populations to increase the ecological realism.

INTRODUCTION

Effluents from wastewater treatment plants (WTPs) are recognised as a significant source of emerging contaminants in freshwaters (Daughton and Ternes, 1999; Carballa *et al.*, 2005; Gosset *et al.*, 2021). Among these, a fraction (30-90%) of pharmaceutical active compounds (PhACs) in drugs is not metabolized and is excreted unchanged in the urine and faeces into the sewage (Monteiro and Boxall, 2010). Here, PhACs can persist and contaminate the WTP-receiving water bodies (Biel-Maeso *et al.* 2019; Gosset *et al.*, 2021). Due to their widespread use and continuous discharge, PhACs become pseudo-persistent contaminants in the aquatic environment (Daughton, 2005). Hundreds of different kinds of drug residues are found in water bodies worldwide at concentrations ranging from ng L^{-1} to µg L^{-1} (O'Flynn *et al.*, 2021). Even environments notoriously considered uncontaminated, such as Alpine waters, can be affected by this type of pollution (Alpine Convention, 2009). Indeed, high PhACs concentrations can be measured in surface water of mountain localities downstream of WTPs (Villa *et al.*, 2020), often in association with intense tourist activities, above all during the winter ski season (Mandaric *et al.*, 2017; Villa *et al.*, 2020). WTPs of high mountain localities are often designed to serve a small resident population. In some periods of the year, typically from Christmas to Easter and to a lesser extent in mid-summer, these localities are crowded by tourists and the treatment plants can become overloaded (Alpine Convention, 2009). In winter, this greater wastewater discharge is exacerbated by low dilution capacity of alpine rivers in the low-flow winter period (Chiogna *et al.*, 2016). This period is also the most critical for the benthic fauna living in the receiving streams exposed to a mixture of hundreds of PhACs and other emerging contaminants as well as to the negative effects of nutrient inputs (Lencioni *et al.*, 2020).

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To date, no information has been published on the toxic effects on microorganisms of the effluents of WTPs in a mountain valley with high tourist activity. Within this context, we used the Microtox® test in a series of laboratory tests to assess the whole-mixture toxicity of the treated effluent from the WTP of a famous ski resort at the Tonale Pass (Trentino, Eastern Italian Alps). This test is frequently used to perform a first screening of the treated effluent toxicity using *Aliivibrio fischeri* (formerly *Vibrio fischeri*; bacterium) as a bioindicator (Deprez *et al.*, 2012; Weltens *et al.*, 2012, 2014). This test is fast, easy to perform, highly reproducible, standardised and inexpensive (Parvez *et al.*, 2006). Notwithstanding its easy application, it does not allow us to know the contribution of each contaminant simultaneously present in the effluent in a complex, undefined mixture (Groten *et al.*, 2001), *i.e.* which contaminants drive the overall toxicity, or if overall toxicity depends on chemical-biological interactions among contaminants (Kortenkamp *et al.*, 2009). To answer these questions, we used the "whole-mixture approach" followed by the "component-based approach" that links the results obtained from a specific mixture to single chemical components (van Gestel *et al.*, 2011). This link allows generalising of results to different exposure scenarios, becoming the basis of the toxicity predictions (van Gestel *et al.*, 2011). According to the component-based approach, it is possible to estimate their overall toxicity, starting from information on the individual components of the environmental mixture (*e.g.*, which chemical components are present, what is their mode of action, and what effects they exert as individual agents) (Kortenkamp *et al.*, 2009).

We subsequently compared the toxicity of the experimental mixture with predictions obtained by applying two commonly used models, the concentration addition (CA) and the independent action (IA) (Loewe and Muischnek, 1926). Finally, we investigated the nature of interactions potentially occurring among selected PhACs by applying the combination index (CI) method. Both CA and IA are applied for mixtures of compounds having respectively the same mode of action (MoA) or different MoA against the selected endpoint (Loewe and Muischnek, 1926; Bliss, 1939). CA and IA estimate the toxicity of the mixture additively: additivity of concentrations (CA) and additivity of effects (IA). The idea behind the CA model is that if all the components of a mixture have the same or similar MoA, they behave towards the biological target as if they are the same chemical, so each could be replaced by another chemical at an equipotent concentration (*e.g.*, at EC_{50} =50% Effective Concentration), without changing the final toxic effect of the mixture. The CA model implies that a significant mixture effect can be detected even if mixture constituents are present in concentrations that do not induce individual toxicity. This phenomenon is known as the principle of *something from "nothing*" (Silva *et al.*, 2002; Thrupp *et al.*, 2018). Conversely, in the case of the IA model, the concentrations of chemical components of a mixture cannot be added, as they are assumed to have different MoAs and they cannot be considered as homogeneous quantities. In this case, the overall effect of the mixture is estimated from the sum of the effects of the individual components (Bliss, 1939; Loewe and Muischnek, 1926). The important assumption behind the concepts of CA and IA models is the absence of chemical or biochemical interactions among substances in the mixture (*i.e.*, the assumption of simple additivity) (Backhaus, 2014).

However, more realistically, interactions and/or biological interference among chemicals could occur at different levels in the environment (SCHER, SCCS, SCENIHR, 2012), leading to a higher or lower toxic effect than expected from CA or IA models , *e.g.*, more than additive or synergistic effects and less than additive or antagonistic effects, respectively (Kienzler *et al.*, 2014).

More recently, the CI method has been employed to study the joint effects of environmentally relevant mixtures of toxicants (Gonzalez-Pleiter *et al.*, 2013; Rodea-Palomares *et al.*, 2010; Di Nica *et al.*, 2016, 2017a, 2017b). This method was historically used in pharmacology to assess and quantify the nature of interactions among pharmaceuticals, revealing the occurrence of synergism or antagonism (Chou and Talalay, 1983). The CI concept has the great advantage of application without a priori knowledge of the MoA of chemicals (Chou, 2006).

Overall, this study, based on experimental and modelling data, gives new insights into the toxicity of treated effluents from WTPs and selected PhACs and their potential hazard for freshwater populations, which can be generalized to a larger territorial scale and to other mountain stream ecosystems.

METHODS

Water sampling, selection of pharmaceuticals and mixture preparation

The treated effluent was collected from the Tonale Pass WTP (1799 m asl; (http://adep.heidix.net/plant.html?PT), downstream of the Tonale Pass village, a famous ski resort in the Italian Alps (46°N, 10°E, 1884 m asl, Trentino Province). This WTP is equipped with conventional treatments (oxidation, secondary sedimentation) and represents a source of PhACs for the receiving stream (the Vermigliana stream), especially in winter due to the high number of tourists. This WTP was designed to serve 10,000 equivalent inhabitants but the population increases from 239 local inhabitants in 2021 (http://italia.indettaglio.it/ita/trentinoaltoadige/trento_vermiglio_passo-

tonale.html) up to, every year as average, about 400,000 total and 190,000 respectively during the winter and the summer high tourist seasons (ISPAT - Annuario on-line, www.statweb.provincia.tn.it).

The sampling of the effluent was carried out on 6 March of 2017, the day after the end of the highest tourist pressure occurring during the Carnival holidays. Three samples were collected in sterile 1 L graduated polypropylene (PP) water bottles (from LP Italiana, Milan, Italy), kept refrigerated during the transport, and subsequently frozen at -20°C within 2 h from sampling.

Before performing the acute toxicity tests, the pH of samples was measured and adjusted in the pH range between 6.0 and 8.5 following the DIN EN ISO 11348-3 procedures for the assessment of bioluminescence inhibition effects of aqueous samples on *A. fischeri.* In addition, to reproduce the minimum osmotic pressure necessary for the survival of the bacterium, the osmolarity of the sample was adjusted by adding 2% NaCl (DIN EN ISO 11348-3).

We assessed the toxicity on *A. fischeri* of a relevant mixture of PhACs potentially discharged in the water body, starting from the list of PhACs detected by Villa *et al.* (2020) in the Vermigliana stream downstream of the selected WTP. Generally, the number of chemicals discharged into surface waters is extremely high, and it would not be reasonable to consider all of them in the toxicity assessment of the resulting environmental mixture (Backhaus and Karlsson, 2014). It has been empirically observed that in realistic mixtures only a limited number of substances are responsible for almost all the toxicity of the entire mixture (Price *et al.*, 2012; Backhaus and Karlsson, 2014). For instance, Backhaus and Karlsson (2014) proposed the analysis of the Toxicity Units (TU) distribution of compounds in a complex mixture as a tool to classify the substances present in order of "ecotoxicological importance". The TU is defined as the ratio of the concentration of an active substance (as) measured in the environment (*e.g.*, surface waters) (measured environmental concentration - MEC) and the toxicity (IC_{50}) shown against a defined biological endpoint (*e.g. A. fischeri*) (eq. 1)

$$TU = \frac{MECsw}{IC50} \quad \text{(eq. 1)}$$

Where MEC is the measured environmental concentration of the PhACs in surface water; the IC_{50} is the 50% inhibition concentration value measured on *A. fischeri*. According to Backhaus and Karlsson (2014), almost 90% of the overall toxicity of a complex mixture is caused by about the first five compounds in order of TU; whereas the first 10 compounds are responsible for about 100% of the overall toxicity. To select the PhACs potentially responsible for the overall toxicity of the receiving environmental mixture, we compared the MEC values of PhACs found downstream the WTP (data from Villa *et al.*, 2020, see Tab. 1) with the acute toxicity values (IC50) exerted as single chemicals against A. fischeri (data from the literature, see Tab. 1). Seven PhACs, were selected among those detected in the Vermigliana stream having the highest TU values (Tab. 1):

non-steroidal anti-inflammatory drug (NSAID, including three drugs: ibuprofen, diclofenac, naproxen), antibiotics (including clarithromycin, amoxicillin),one analgesic (acetaminophen) and one anticonvulsant (carbamazepine). According to Backhaus and Karlsson (2014) these PhACs could be responsible for the 90-100% of the overall toxicity for aquatic microorganisms.

To obtain the information (*i.e.*, concentration-response relationship of single substances that compose the mixture) necessary for the application of the predictive models, some single PhACs (clarithromycin, naproxen and acetaminophen) were tested to define their toxicity curve parameters (IC_{50} and slope) on *A. fischeri*. The IC_{50} value and the parameters of the concentration-response curves (slope) of ibuprofen, diclofenac, carbamazepine and amoxicillin curves were available from literature (Di Nica *et al.*, 2017a). These data besides other information on the single PhACs (*e.g.*, mode of action) were included in the predictive models of the mixture toxicity and to investigate how they interact with each other (*i.e.*, in a synergic or antagonist way).

Tab. 1. Toxicity Unit values (TU = MEC/IC_{50}) for *A. fischeri* of the selected pharmaceuticals (PhACs) detected in the Vermigliana stream, downstream of the Tonale Pass WTP. PhACs are listed for decreasing values of TU.

PhACs	MEC (ng L^{-1})	IC_{50} (mg L^{-1})	TU
Ibuprofen	619.0[a]	18.3[b]	3.38E-05
Clarithromycin	80.37[a]	12.1[c]	6.65E-06
Naproxen	91.25[a]	17.9[c]	5.09E-06
Diclofenac	41.90[a]	15.9[b]	2.63E-06
Acetaminophen	744.0[a]	567.5[d]	1.31E-06
Carbamazepine	5.14[a]	94.0[b]	5.47E-08
Amoxicillin	79.70[a]	2845[b]	2.80E-08

MEC, measured environmental concentration (ng L^{-1}); IC_{50}, inhibition concentration of the bioluminescent of *A. fischeri* (mg L^{-1}) from literature: [a]Villa *et al*. (2020), [b]Di Nica *et al*. (2017a); [c]Ortiz de Garcia *et al*. (2014); [d]Kim *et al*. (2007).

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Tab. 2. Non-linear regression models selected for each treatment group.

Treated group	Mathematical model		
Treated effluent MixMEC Clarithromycin	Two parameters Weibull (W2.2)	$f = 1 - esp(-exp(\beta 2) - lnln(c) - lnln(\beta 1)))$	(eq. 2)
Acetaminophen Naproxen	Two parameters Log Normal (LN.2)	$f = \varphi(\beta 2(lnln(c) - lnln(\beta 1)))$	(eq. 3)

Stock solutions and testing procedure

Clarithromycin, naproxen, and acetaminophen were from Sigma-Aldrich laboratories at the highest degree of purity. Clarithromycin and naproxen, having a low solubility in the saline water, were pre-dissolved in dimethyl sulfoxide (DMSO) and then in saline water. The concentration of DMSO in the tested solutions was less than 1% (v/v), as requested by the OECD guidance document (2000) and according to Villa *et al.* (2018a, 2018b). The pH of the final solutions was adjusted in the range between 6.0 and 8.5 using a pH meter (model 250; Denver Instrument) by adding NaOH 0.1 M (DIN EN ISO 11348-3, 2007).

A stock solution of acetaminophen was prepared directly in the saline water solution (2% NaCl) used for the tests, according to the method indicated by Vighi *et al.* (2009).

The multicomponent mixture (mixMEC) was prepared by combining the seven PhACs according to a fixed concentration ratio corresponding to the respective MECs value (MEC are from Villa *et al.*, 2020). To describe the entire concentration-response curve, the total concentrations of the mixture were systematically varied using an appropriate dilution factor and keeping constant the concentration ratio among the single compounds in the mixture.

Tests were performed following the operating protocol and the conditions provided by the instruction manual of the Microtox® system (Acute toxicity test, Azur Environmental, 1998; "whole effluent toxicity") procedure for the effluent, and "basic test" procedure for single PhACs and their mixture). The Microtox® test is based on the measurement of the bioluminescence inhibition of the bacterium *A. fischeri* exposed to toxic agents or environmental samples. The inhibition of bioluminescence was measured in the acute mode using a Microtox® model 500 analyzer. The freeze-dried bacteria and the reconstitute solution were acquired from Ecotox LDS S.r.l. (Milan, Italy). Bacteria were exposed to the test samples (treated effluent, single chemicals and their mixture) for 5 and 15 min at the temperature of 15°C. Only the inhibition data obtained after the 15-min of exposure were used for the final elaborations, due to the very negligible difference with results obtained after 5 min. The inhibition of the bacterial bioluminescence concerning the control provides the toxic effect (IC_x) of the samples.

Toxicity tests of each group tested (treated effluent, single chemicals and their mixture) were performed in a duplicate of one control and nine different dilutions obtained with serial dilutions in a 2% NaCl solution (dilution factor used was of 1.5 and 2). The aliquots of the tested treated effluent ranged from 100% to 0.4% (Tab. S1). The aliquots of WTP output water were tested in six replicates. For each chemical and the mixture, tests were performed twice for a total of four replicates for each concentration. In single chemical tests and their mixture, the nominal concentration was considered.

Statistical analyses

The probability of the occurrence of a toxicity event IC_x (bioluminescence inhibition), as a function of the chemical concentration *c* (or fraction of the environmental sample), and a vector of parameters ($\beta 1$; $\beta 2$) were obtained using non-linear regression models. The most appropriate model to fit the concentration-response relationship for each group of treatment (effluent, single chemicals and their mixture) was obtained by analysing the goodness of fit of the different mathematical models (*i.e.*, Weibull; Log-Normal; Logistic and Log Logistic with two, three and four parameters) through the functions log likelihood and Akaike Information Criterion (AIC). The final mathematical models selected for each group of treatment are indicated in Tab. 2 (eq. 2 and eq. 3).

The statistical parameters of the estimated regression coefficients for both of the above-reported models were analysed to check their goodness of fit to the data obtained from each treatment group.

The analysis of the residues of the models was also carried out using the Kolmogorov-Smirnov test. All statistical analysis and the IC_x values with 95% confidence intervals were obtained using the R software (R Core Team, 2021).

To reach a deep knowledge about the MoA of the tested chemicals on *A. fischeri*, the QSAR (Quantitative Structure Activity Relationship) equations developed by Vighi *et al.* (2009) for the prediction of the effect on *A. fischeri* of non-polar narcotic (eq. 4) and polar narcotics compounds (eq. 5) were applied:

$$Log1/C_{narcotic\ (mmol\ L^{-1})} = 0.94\ logKow - 2.61 \quad (n = 23;\ R^2 = 0.92) \qquad \text{(eq. 4)}$$

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$Log1/C_{polarnarcotic\ (mmol\ L^{-1})} = 0.502\ logKow + 0.294$
$(n = 23;\ R^2 = 0.81)$ (eq. 5)

Both equations are based on the octanol-water partition coefficient (logKow) as a measure of the hydrophobicity of the molecules. Both models describe the so-called baseline toxicity of substances: *i.e.*, the narcosis (expressed by Eq. 4) described as the toxic effect of a chemical having not a specific MoA towards an organism (inert chemical), and the polar-narcosis (expressed by eq. 5) described as the toxic effect of molecules that also have not a specific MoA but that exerts a slightly stronger toxic effect than non-polar narcosis (less inert chemicals) (Verhaar *et al.*, 1992). In addition to these two primary processes, other two mechanisms of action causing the toxicity of a substance are identified: *i.e.*, reactivity and specificity of the MoA (Verhaar *et al.*, 1992). Reactive compounds are chemicals that exert a greater toxic effect than polar or nonpolar narcotics but they act non-selectively with chemical structures commonly found in biomolecules; whereas chemicals with a specific MoA specifically interact with a definite molecular receptor.

In this study, we applied both the QSAR models (in the case of ionisable PhACs, LogDow (distribution ration) is used instead of the LogKow). The LogDow values were chosen at the pH values close to that of the solution used for the Microtox tests. In Tab. S2 of the Supporting information, the LogDow values of the selected PhACs are reported together with other relevant physical-chemical parameters.

To estimate the effects on *A. fischeri* of the tested mixture (IC_{xmix}), two predictive mathematical models were applied. For a multi-component mixture, the mathematical concept of CA can be expressed as follows (Berenbaum, 1985) (eq. 6):

$$\sum_i^n \frac{C_i}{EC_{xi}} = 1 \qquad \text{(eq. 6)}$$

where n is the number of chemicals in the mixture that cause the x % of the overall effect; c_i is the concentration of the i^{th} component in the mixture, and EC_{xi} is the concentration of the i^{th} component which provokes the same effect when considered individually.

The c_i/EC_{xi} fraction is the Toxicity Unit (TU) of each chemical component (Sprague, 1970). The concentration of each component of the mixture is represented as an equi-effective fraction of the individual concentration, consequently, the behaviour of the mixture is additive if the sum of all the TUs is 1.

Conversely, by applying the IA model, the overall effect of the mixture is predicted by summing the effects of the individual components, according to the statistical concept of independent random events. The toxic effect of each component is not influenced by the toxic effects of the other components of the mixture, as they act differently. Thus, the single toxic effects are added, and the probability that the components act simultaneously on the same target is subtracted. The IA mathematical concept can be expressed as follows:

$$E(C_{mix}) = 1 - \prod_{i=1}^{n}(1 - E(C_i)) \qquad \text{(eq. 7)}$$

where $E(C_{mix})$ is the effect estimated for the total concentration of the mixture; C_{mix} is the total concentration of the mixture, and $E(C_i)$ is the effect that the i^{th} component would have if applied individually.

To investigate and quantify the nature of the interactions potentially occurring among the substances in the mixture, the CI method was applied (Chou, 1976, 2006; Chou and Talalay, 1984). For n chemicals the equation CI can be expressed as follows (eq. 8):

$$n(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n}\left\{\frac{[D]_j}{\sum_1^n [D]}\right\}}{(D_m)_j\left\{\frac{(fa_x)_j}{[1-(fa_x)_j]}\right\}^{1/m_j}} CI =$$

$$\frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = \frac{(D)_1}{(D_m)_1[fa/(1-fa)]^{1/m_1}} + \frac{(D)_2}{(D_m)_2[fa/(1-fa)]^{1/m_2}}$$

(eq. 8)

where $^n(CI)_x$ is the resulting CI value for n chemicals at x% of inhibition; $(D_x)_{1-n}$ is the sum of the concentrations of n chemicals that exert in combination the x% of inhibition; $\{([D]_j/\Sigma[D]\}$ is the proportionality of the concentration of each of the n components which exerts in inhibition of $x\%$; $(D_m)_j\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the concentration of each component that alone exerts the x%; of inhibition, D_m is the concentration of the median effect; f_{ax} is the fractional inhibition at x% inhibition; m is the slope of the median concentration-effect curve.

From eq. 7, the presence of synergistic, additive and antagonistic effects can be calculated.

The CompuSyn program (Compusyn Inc., Paramus, NJ, USA) (Chou and Martin, 2005) was used to calculate the CI values for the mixMEC mixture at different ranges of effect levels. The *Fa*_CI graph was obtained (graph of CI values of the mixMEC mixture as a function of the different *Fa*).

RESULTS AND DISCUSSION

Toxic effect of treated effluent

The bioluminescence inhibition values (IC_x) achieved for *A. fischeri* at different dilutions of the treated effluent are indicated in Tab. 3, together with the statistical parameters describing the goodness of the mathematical model

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applied. Fig. 1 shows the concentration-response relationship obtained (relative bioluminescence inhibition as a function of the % of effluent).

The acute toxicity on *A. fischeri* of the effluent discharged from the Tonale Pass WTP before the dilution into the Vermigliana stream (100% of the treated effluent) achieved a mean value of 21%. According to hydrological parameters calculated for the Vermigliana stream downstream from the WTP, the site-specific dilution factor in the water during the winter season was about 40 (Villa *et al.*, 2020). This means that the realistic percentage of treated effluent in the stream in winter was about 2.5%. The presence in the water of this percentage of treated effluent would lead to a bioluminescence inhibition of about 7% (IC_{07}) (Tab. 3). These results revealed a modest toxicity of the effluent on aquatic microorganisms over the short term.

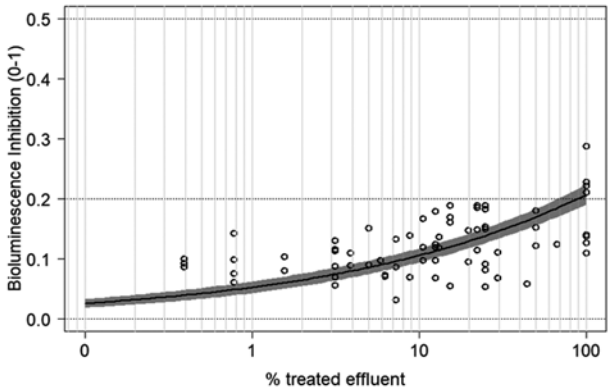

Fig. 1. Concentration-response curve for the treated effluent from the Tonale Pass wastewater treatment plant. The percentages of treated effluent in the tested sample are on the horizontal axis, and the relative values of bioluminescence inhibition (0-1) are on the vertical axis. The relative inhibition values of 0.5, 0.2 and 0.0 are indicated by horizontal lines.

Effects of individual chemicals on *A. fischeri*

The IC_{10} and IC_{50} values obtained from the exposure of *A. fischeri* to the single PhACs are reported in Tab. 4,

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Tab. 3. Bioluminescence inhibition values (IC_x) of the treated effluent (% of effluent in the tested sample), with 95% confidence intervals in brackets and statistical parameters of the selected regression model.

IC_x	% of treated effluent	Weibull model parameters		SE	t-value	p-value	n-1
IC_{07} IC_{10}	2.5% (1.3%-3.8%) 8.3% (5.1%-11.5%)	*β1*	3.16E-01	2.06E-02	15.36	<2.2e-16	69
IC_{20} IC_{21}	89% (64%-114%) 100% (71%-129%)	*β2*	1.03E+04	3.74E+03	2.7631	0.007333	

SE, standard error.

Tab. 4. Acute toxicity values (IC_{10} and IC_{50}, mg L^{-1}) for *A. fischeri* for acetaminophen, clarithromycin, naproxen, the mixMEC, amoxicillin, carbamazepine, diclofenac, and ibuprofen [95% mean confidence intervals in brackets]; the statistical parameters (β_1, β_2) of the selected regression models are shown.

	Mod.	IC_{10}	IC_{50}	Stat. par.	SE	t-value	p-value	n-1
Diclofenac*	W1.4	5.9 [5.03;6.74]	15.9 [14.59;17.26]	β_1 11.75 β_2 -1.21	0.4919 0.0856	23.89 -14.082	<0.001 <0.001	50
Ibuprofen*	W2.2	1.4 [0.99;1.72]	18.3 [15.96; 20.58]	β_1 30.30 β_2 0.72	2.0776 0.0346	14.5865 20.93	<0.001 <0.001	26
MixMEC	W2.2	3.15 [2.10;4.21]	66.9 [56.10; 77.70]	β_1 121.20 β_2 0.62	12.199 0.0371	9.9354 16.629	<0.001 <0.001	40
Carbamazepine*	W2.2	4.2 [2.85;5.47]	94 [82.03; 105.91]	β_1 172.35 β_2 0.60	36.045 0.0156	3.753 3.483	<0.001 <0.001	68
Naproxen	LN.2	4.72 [3.99;5.45]	131.06 [106.5; 155.6]	β_1 131.10 β_2 0.39	12.029 0.016	10.895 24.123	<0.001 <0.001	31
Clarithromycin	W2.2	28.65 [19.95; 37.35]	302.54 [171.10; 433.97]	β_1 478.50 β_2 0.80	122.42 0.08703	3.909 9.184	<0.001 <0.001	33
Acetaminophen	LN.2	74.13 [67.86; 80.40]	504.1 [483.6; 524.5]	β_1 504.10 β_2 0.67	9.743 0.0142	51.734 47.217	<0.001 <0.001	18
Amoxicillin*	W2.2	717.6 [537.5; 897.7]	>1702	β_1 3719.40 β_2 1.37	552.232 0.1935	6.7351 7.0689	<0.001 <0.001	16

*SE, standard error; Mod., mathematical model; PhACs are listed for increasing values of IC_{50}; *data from Di Nica et al. (2017a).*

along with the statistical parameters of the selected regression models. In Fig. 2 the concentration-response curves of the single PhACs fitted by the selected regression models are shown. The three PhACs tested in this study (acetaminophen, clarithromycin, naproxen) showed an IC_{50} value greater than 100 mg L^{-1}. The 50% inhibition concentration value was not reached for the antibiotic clarithromycin at the maximum concentration that it was possible to test; we estimated an IC_{50} value of 302.5 mg L^{-1} from the application of the non-linear regression function. Similarly, the IC_{50} value found by Di Nica *et al.* (2017a) was not reached for the other antibiotic, amoxicillin. This is because antibiotics have a specific mechanism of action against *A. fischeri*, exerting their toxic effect mainly in the long term (*e.g.*, after 24 h) rather than the short term (*e.g.*, 15 min) (Backhaus and Grimme, 1999). This was expected because antibiotics such as clarithromycin that inhibit the biosynthesis of proteins show a delayed toxic effect over time (Backhaus and Grimme, 1999). On the contrary, the toxic effect exerted by compounds not having a specific mechanism of action (*i.e.*, compounds having baseline toxicity that interfere in a predominant way with processes associated with membranes) is almost comparable over time (*i.e.*, in the short and long terms) (Backhaus and Grimme, 1999).

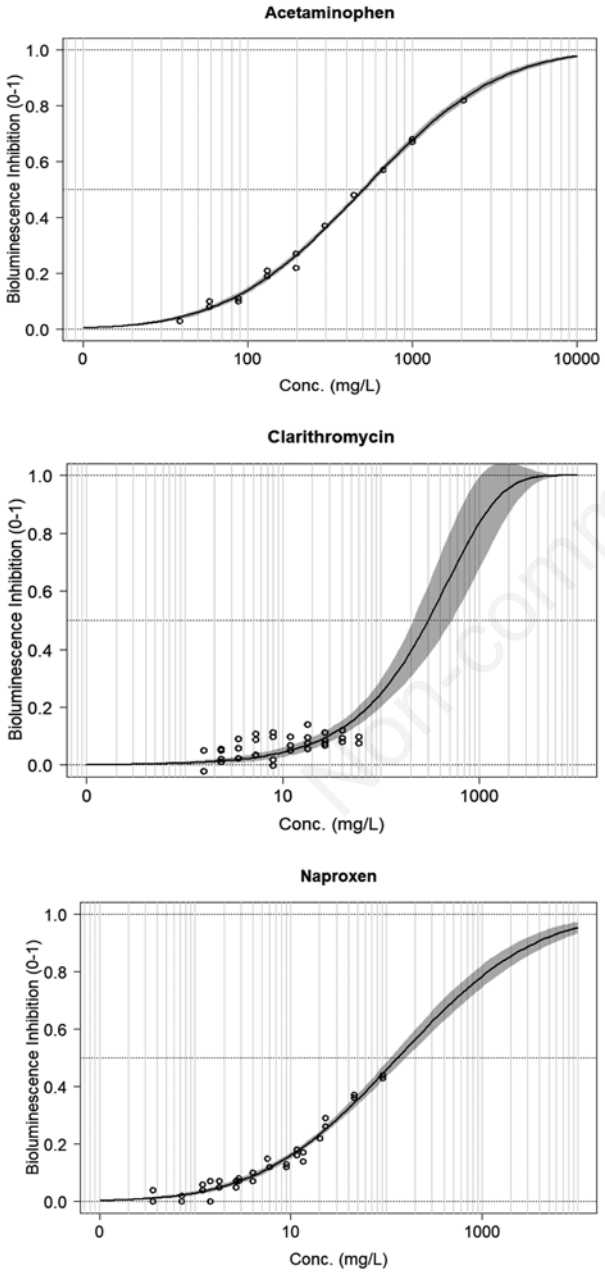

Fig. 2. Concentration-response curves for the pharmaceuticals: acetaminophen, clarithromycin, and naproxen.

According to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (United Nations, 2013), based on the IC50 estimated in this study (for the NSAID naproxen, the antibiotic clarithromycin and the analgesic acetaminophen) or taken from the literature (for the NSAID diclofenac and ibuprofen, the antibiotic amoxicillin and the anticonvulsant carbamazepine), the anti-inflammatory ibuprofen and diclofenac were most toxic in the short term on *A. fischeri*, whereas the antibiotics were the least toxic.

In particular, based on GHS, diclofenac, ibuprofen, and carbamazepine are classified as harmful to aquatic microorganisms, having an IC50 between 10 mg L^{-1} and 100 mg L^{-1}a nd the other four PhACs as not acutely harmful, having an IC50 greater than 100 mg L^{-1}.

Experimental and predicted mixture toxicity

The experimental values of IC_{10} (3.2 mg L^{-1}) and IC_{50} (66.9 mg L^{-1}) of the mixMEC mixture were between the highest and the lowest toxicity values of each of the components of the mixture (Tab. 4).

At the concentration corresponding to the sum of the MEC values in the Vermigliana stream (1.66E^{-03} mg L^{-1}), no acute toxicity for *A. fischeri* was observed (Fig. 3). This result is consistent with those obtained by applying the whole mixture approach, whereby an acute bioluminescence inhibition below 10% was observed at the % of treated effluent realistically found in the Vermigliana stream (about 2.5%).

The toxicity of the mixMEC mixture on *A. fischeri* was compared with the CA and IA model predictions (Fig. 3). The experimental toxicity of the tested mixture was well predicted by both models applied (CA and IA) in the lower part of the toxicity curve, while, with increasing toxicity, the prediction capability is lower. Both models seem to overestimate the real toxicity. The prediction window, *i.e.*, the distance between the CA and IA curves, was very narrow. This occurs particularly when the mixture's

chemicals have a comparable slope with an intermediate steepness of their concentration-response curves (Kortenkamp *et al.*, 2009).

In our study, no synergistic or antagonistic interaction was detected among the seven components of the mixMEC mixture. In fact, the experimental toxic effect of all the PhACs in multicomponent combinations was predicted in additive terms (both concentrations and effects).

Synergistic or antagonistic responses seem to be confined to mixtures of few compounds (2 or 3 components) and are rarely observed in multicomponent mixtures, as in this case, where a sort of buffering effect seemed to occur (Backhaus, 2014). In addition, when the mixture is composed of chemicals without a specific MoA (narcotic compounds), as in our study, the overall toxic effect tends to be additive with the increase of the number of components (Warne and Hawker, 1995). By comparing the experimental toxicity data obtained for the single PhACs (IC_{50} in Tab. 4) and the predictions obtained from the QSAR equations, we calculated the toxicity ratio values (see Tab. S3). It was not possible to apply the QSAR models for the antibiotic clarithromycin, as the experimental value of IC50 was not available. The results obtained are presented in Fig. 3. The experimental toxicity of acetaminophen and naproxen fell within the toxicity range of compounds of polar narcotic behaviour. This result is consistent with those previously obtained for all the other components of the mixture that exhibit also a polar narcotic behaviour against *A. fischeri* (Di Nica *et al.*, 2017c).

The knowledge of the MoA of the substances is necessary for the application of predictive models of mixture toxicity. An environmental mixture can be composed of substances acting according to a similar MoA, according to different MoA, or more likely by a combination of both. In our study, all the selected compounds apart from the antibiotic clarithromycin had a polar narcotic-like behaviour towards the test organism, and this can be assimilated to a common MoA. Therefore, the toxicity of the mixMEC mixture should be well predicted by applying the concept of additivity of concentration.

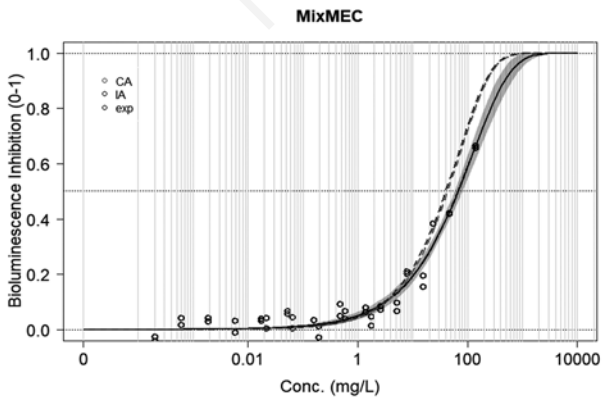

Fig. 3. Experimental (black points) and predicted (red and blue curves for the CA and IA models) concentration-response curves for the mixMEC mixture; mixMEC, ibuprofen acetaminophen, clarithromycin, naproxen, amoxicillin, carbamazepine, and diclofenac.

The CI method

The prediction values were obtained by applying the CI method at various effect levels up to the maximum concentration that was possible to test (corresponding to 65% of the effect level). The results (Fa) are reported in Tab. S4 and, for almost all the investigated effect levels, the CIs are between 0.5 and 2. These results highlight the absence of interactions among the PhACs tested in multicomponent combinations. However, for effect levels above 55% (*Fa* > 0.55), the CI values were higher than the threshold of 2, indicating the existence of a slight antagonistic effect among the mixture components. A threshold of twice the deviation from the unit is generally applied to indicate the agreement between the experimental mixture toxicity and prediction obtained through the concentration additivity principle (*i.e.*, CI values between 0.5 and 2) to indicate the absence of synergistic or antagonistic interactions (Belden *et al.*, 2007; Cedergreen, 2014).

The application of the CI model confirmed the results obtained by means of the CA and IA predictions, *i.e.*, the seven PhACs combined in a mixture have an additive type of response on *A. fischeri* at the high level of contamination (*e.g.*, at a concentration of about 40 mg L^{-1} of PhACs in mixture).

As the tested concentration increases, the PhACs start to interact, producing lower toxicity than that predicted by the additive model (additivity of concentration). From the point of view of environmental risk management, the overestimation obtained with the predictive models does not pose a particular concern, as the prediction provided by the additivity concepts was sufficiently protective.

CONCLUSIONS

Our results show that the concentration of PhACs in the treated effluent from the Tonale Pass WTP, in the period of winter tourism peak, do not reach levels that can be harmful to aquatic microorganisms. Microtox results suggest that individual PhACs do not represent a hazard for the local biodiversity. Consistently, Lencioni *et al.* (2020) evaluated the ecological state and water quality of the Vermigliana stream, using the STAR_ICMi (Buffagni and Erba, 2008) and IBE (IRSA-CNR and APAT, 2003) indices, which use macroinvertebrates as biological indicators, and they found a good ecological

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state downstream from the Tonale Pass WTP, even during the winter season. However, some negative effects were found downstream of the WTP on alpha diversity of the macroinvertebrate community and at organism (*e.g.*, mortality) and sub-organism levels (*e.g.*, DNA damage) in local insect populations (Lencioni *et al.*, 2020). Furthermore, Villa *et al.* (2020) assessed an ecological risk downstream of this treatment plant, comparing PhAC concentrations in the Vermigliana stream and the toxicological endpoints for aquatic organisms (algae, *Daphnia*, and fish). So, an ecological risk for the receiving water body cannot be totally excluded, especially for anti-inflammatory compounds, and possibly resulting from the input of nutrients and other organic and civil pollutants (*e.g.*, soaps and oils).

Experimental tests on model species, such as *A. fischeri*, can be useful for preliminary and fast information on the level of risk for freshwater habitats and organisms in a screening phase. However, they should be combined with field studies and experiments on the local population to increase the ecological realism, including the use of other model organisms such as algae, insects, and fish.

Finally, these eco-toxicity data can be potentially used in ecological risk assessment of effluents of this and other WTPs in mountain tourist valleys, where during some periods of the year the receiving water bodies are contaminated by hundreds to thousands of PhACs. Given the importance of alpine waters as drinking water supplies for many Europeans, such studies are also recommended by the Water Framework Directive (European Commission, 2000). The direct toxicity assessment of WTP discharges represents an important tool to maintain or achieve ecological quality objectives in freshwaters.

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CONFLICT OF INTEREST

The authors declare no competing or financial interests.

CONTRIBUTIONS

VDN, investigation, formal analysis, data curation, visualization, original draft preparation; SV, conceptualization, supervision, methodology, investigation, review and editing; VL, conceptualization, supervision, project administration, funding acquisition, review and editing.

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