DOCTORAL SCHOOL

UNIVERSITY OF MILANO-BICOCCA

Department of
Earth and Environmental Sciences (DiSAT)
PhD program in Environmental Sciences – Cycle XXIX
80 R – Sciences, 80 R - 1

STUDIES OF MIXTURE EFFECTS OF EMERGING CONTAMINANTS IN THE ENVIRONMENT

Candidate: Dr. Valeria Di Nica

Registration number: 78782

Tutor: Prof. Antonio Finizio

Co-tutor: Dr. Sara Villa

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LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. **Di Nica V**., Menaballi L., Azimonti G., Finizio A., 2015. RANKVET: A new ranking method for comparing and prioritizing the environmental risk of veterinary pharmaceuticals. **Ecological Indicators**, 52, 270–276. doi.org/10.1016/j.ecolind.2014.12.021

II. **Di Nica V**., Villa S., Finizio A., 2017. Toxicity of individual pharmaceuticals and their mixtures to *Aliivibrio fischeri*: experimental results for single compounds and considerations of their mechanisms of action and potential acute effects on aquatic organisms. **Environmental Toxicology and Chemistry**, 36, 807–814. doi: 10.1002/etc.3568.

III. **Di Nica V**., Villa S., Finizio A., 2017. Toxicity of individual pharmaceuticals and their mixtures to *Aliivibrio fischeri*. Part II: Evidence of toxicological interactions in binary combinations. **Environmental Toxicology and Chemistry**; 36, 815–822. doi: 10.1002/etc.3686.

IV. **Di Nica V**., Villa S., Finizio A., 2016. Experimental and predicted toxicity of binary combinations of diclofenac sodium, carbamazepine and caffeine to *Aliivibrio fischeri*. **Environmental Engineering Management Journal**, 15, 1971-1980. http://omicron.ch.tuiasi.ro/EEMJ/ IN PRESS.

V. **Di Nica V**., Gallet J., Villa S., Mezzanotte V., 2017. Toxicity of Quaternary Ammonium Compounds (QACs) to aquatic non-target microorganisms: tests with single compounds and mixtures. ACCEPTED in **Ecotoxicology and Environmental Safety.**

[8]Love never fails.

But where there are prophecies, they will cease;

where there are tongues, they will be stilled;

where there is knowledge, it will pass away.

[9]For we know in part and we prophesy in part,

[10]but when completeness comes,

what is in part disappears.

1 Corinthians 13:8-10

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Valeria

TABLE OF CONTENTS

ABSTRACT

Pharmaceuticals and personal care products (PPCPs) constitute a heterogeneous group of emerging environmental contaminants (ECs). In the last years, an increasing number of studies has confirmed the presence of various PPCPs in different environmental compartments, and this raises concerns about the their potential effects to humans and wildlife. This is particularly true if the problem of the presence of mixtures in the environment is considered. In this framework, the present study aimed to contribute to the scientific knowledge about the potential environmental adverse effects of different classes of emerging contaminants. During the PhD, different issues were considered. In a first phase of the study, a prioritization method (RANKVET indicator) was developed and proposed for ranking the environmental risks of veterinary medicinal products (VMPs). As previously stated, the high number of substances that are likely to be found in an environmental compartment enlightens the need for screening tools useful to produce lists of priority substances. Particularly, RANKVET was developed on the basis of the specific requirements of the EU Directives and Regulations for placing VMPs on the market. In a further step, the toxic effects of pharmaceuticals (for veterinary and human use) and quaternary ammonium compounds (QACs) were assessed as single toxicants and as different combinations of binary and multicomponent mixtures by using *Aliivibrio fischeri* as test organism (Microtox® test system). In order to acquire knowledge about their mode of action (MoA), QSAR models, specifically developed for *A. fischeri*, were applied. Results showed a similar MoA for the two separate groups of toxicants: polar narcotic type behaviour for the majority of pharmaceuticals and a-specific reactive MoA for almost all the QACs. Assessed as single chemicals the toxic effects of pharmaceuticals were moderate, whereas a relevant bioluminescent inhibition was obtained for QACs. Single toxicity parameters were used in order to investigate the predictability of mixtures toxicity by using the two widely used eco-toxicological predictive models: Concentration Addition (CA) and Independent Action (IA). Results showed deviations from conceptual expectations in direction of synergistic and antagonistic effects for the majority of tested binary mixture of PhACs. The hypothesis

of interactions among components were confirmed and quantified by the application of the Combination Index method. Synergistic interactions were observed at environmentally relevant concentrations in one case for QACs. From this study some main conclusions were drawn: (i) the knowledge of single toxicity parameters is not always sufficient for a good prediction of mixture effects, especially in the case of pharmaceutical active compounds; (ii) synergistic and antagonistic deviations seem to be confined to mixture of few compounds, (iii) deviations from additivity are mainly dependent of the specific tested combinations of chemicals and on the effect levels under consideration, whereas seem to be independent from the specific mode of action of toxicants towards the organism.

CHAPTER I

Introduction

CHAPTER I

The purpose of this chapter is to define a context for the Ph.D. thesis providing a scientific background about the state of the art of the topics discussed in the next chapters, particularly the issue of mixture toxicity of emerging contaminants that is the main aim of this research activity.

INTRODUCTION

1 Emerging contaminants in aquatic environment

The quality of water resources is of crucial importance for human health and wildlife protection. For many decades, the scientific research in the field of aquatic environment focused on the study of the occurrence and toxicity of xenobiotic substances, such as heavy metals or persistent organic pollutants (POPs), now well recognized as "priority contaminants" and subjected to specific regulations. Thanks to the adoption of appropriate measures, in the industrialized countries, a significant reduction of their emissions and consequently of their occurrence in the environment has been achieved (Barceló and Petrovic, 2008). However, recently, a very wide number of new unregulated substances have been found in aquatic systems thanks to the development of news and more sensitive analytical methods of detection. These new unrecognized contaminants, globally termed as emerging contaminants (ECs), potentially could have adverse effects for human health and for the environment (Barceló and Petrovic, 2008).

A common accepted definition of emerging contaminant is provided by the United States Geological Survey (USGS) as follows: "*any synthetic or naturally occurring chemical or any microorganism that is not commonly monitored in the environment but has the potential to enter the environment and cause known or suspected adverse ecological and/or human health effects. In some cases, release of*

emerging chemical or microbial contaminants to the environment has likely occurred for a long time, but may not have been recognized until new detection methods were developed. In other cases, synthesis of new chemicals or changes in use and disposal of existing chemicals can create new sources of emerging contaminants" (USGS, 2011). Thus, ECs comprise both contaminants that may have existed for a long time (only recently measured in the environment), as well as compounds of new production or new release. For most of these ECs, no regulations for their inclusion in the routinely monitoring programs still exists, but depending on the available data about their measured concentrations and on their potential (eco)toxicological effects, they may be candidates for future legislation interventions (Barcelò and Petrovic, 2008; Mastroianni et al., 2010).

A very high and increasing amount of chemical substances are daily used in industrial, agricultural and domestic fields. For instance about 8,400,000 substances are currently listed in the Chemical Abstract Service website (2013) and more than 100,000 substances are commercially available in European countries (EINECS, Inventory of Commercial Chemical Substances) (Guillen et al., 2012). According to incomplete statistics, more than 40,000 organic chemicals have been identified as ECs (Diamond et al., 2011). This enormous number includes a wide range of different classes of compounds, such as pharmaceuticals of human and veterinary use, drugs of abuse, hormones and endocrine disrupting compounds, active substances contained in personal care products, brominated and organophosphate flame retardants, plasticizers, perfluorinated compounds, surfactants and many others.

On dependence of their volume of use, their chemical-physical properties and their environmental fate, all these chemicals substances along with their transformation products and their metabolites have the potential to enter the aquatic environment, where they could exert unpredictable effects on wildlife. Nevertheless, due to the recent emergence of the issue, data on their environmental occurrence and impact (fate and eco-toxicity) are still very scarce and fragmentary, consequently it is not easy to predict the realistic hazard that they may pose for the environment.

For the specific purpose of this study the author focused on pharmaceuticals for human and veterinary use and on quaternary ammonium compounds as a specific categories of ECs.

In the following sections, a summary of information on their consumption, sources, pathways of releases, occurrence and harmful effects on aquatic systems will be given.

1.1. Pharmaceutical and personal care products as emerging contaminants

In recent years, scientific researches have focused on the study of occurrence and eco-toxicity of active substances (a.s.) contained in pharmaceutical and personal care products (PPCPs) as a class of contaminants of emerging concern. Pharmaceuticals, generally, refer to chemical substances mainly designed to be used for medical diagnosis, prevention, treatment and cure of human and animal diseases, or for animal growth-promoting functions in veterinary field (Daughton and Ternes, 1999). Whereas, personal care products (PCPs) are mainly related to the improvement of the quality of human daily life and include a wide group of products, such as shampoos, deodorants, fragrances, cosmetics, preservatives, insect repellents, sun screens and many others (Boxall et al., 2012).

1.1.1. Human and veterinary pharmaceuticals

1.1.1.1. Consumption

More than 4,000 pharmaceutical active compounds (PhACs) from a wide spectrum of therapeutic classes (analgesics and anti-inflammatories, β-blockers, lipid regulators, antibiotics, psychoactive drugs, anti-neoplastics, antihistamines, etc) are currently marketed worldwide; among these, about 3,000 are authorised in the EU market (Touraud, 2011). The global annual consumption is estimated in

the range of 100,000 - 200,000 tons (KNAPPE, 2008; Tijani et al., 2016) with the EU as the second major consumer accounting for the 24% of the total amount (BIO Intelligence Service, 2013). In 1999, in European countries the percentage between human and veterinary PhACs were 65% and 29% of the total, respectively, whereas the remaining 6% consisted of growth promoters (FEDESA - European Federation of Animal Health, 2001).

The consumption of PhACs among EU countries is greatly heterogeneous (Goossens, 2005; Schuster, 2008; Verbrugh, 2003) and in the majority of cases, because of the high amount of the over-the-counter medicines sold, only an approximate estimation can be made (Dìaz-Cruz and Barcelo, 2004; Stackelberg et al., 2004). In addition, only few information is generally available from public databases and literature. Within Europe, Germany and France account for about two-thirds of the annual EU consumption (BIO Intelligence Service, 2013). As an example, in 2001 in Germany were consumed approximately 38,000 tons of a.s. (Greiner and Rönnefahrt, 2003); anti-inflammatory/analgesic drugs and antibiotics are among the most sold with about 4,000 and 370 tons, respectively, marketed in France (2004) (Monteiro and Boxall, 2010). Conversely, veterinary pharmaceuticals are used in minor amount; e.g. an overall consumption of more than about 8,100 tons of antimicrobials, accounting for about the 90% of the total of veterinary PhACs (Kools et al., 2008) was estimated in 2013 in 26 EU/ European Economic Area countries (EMA, 2015).

1.1.1.2. Sources and pathways of release to the environment

Human and veterinary PhACs, together with metabolites and transformation products, may reach the aquatic environment during all the stages of their life cycle and through different routes of entry (Halling-Sørensen, 1998; Montforts, 1999) as summarized in Fig 1.

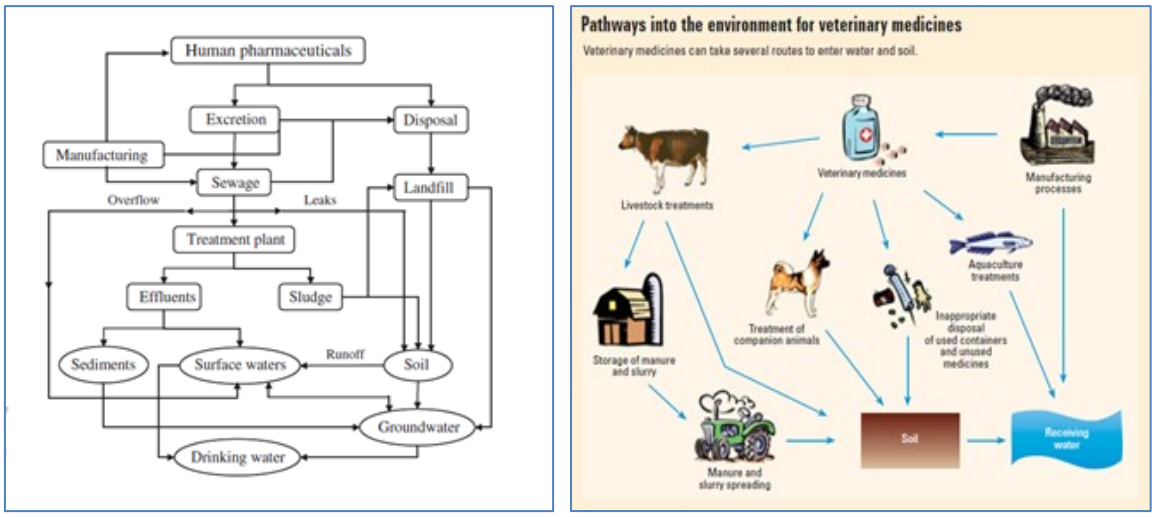

Fig 1. Major pathways of release in the environment for pharmaceuticals of human- (a) and veterinary- (b) use ((a) Monteiro and Boxall, 2010; (b) Boxall et al., 2003)

1.1.2. Human-use pharmaceuticals

1.1.2.1. Emissions during manufacturing

Releases through discharges of manufacturing facility effluents and/or through accidental leakages constitutes a common route for human and veterinary medicinal products. Even though the production of waste in manufacturing facilities is known to be high, in EU countries its direct contribution is considered negligible (EEA, 2010; Williams, 2005) mainly thanks to the application of the Good Manufacturing Practice (GMP) regulations.

1.1.2.2. Discharge from Sewage Treatment Plants

Release into the aquatic environment from treated wastewater effluents is considered the main source of emission for human-use pharmaceuticals (Alder et al., 2001; Daughton and Ternes, 1999). After administration not all the dose of PhACs is metabolised in human body and a variable fraction, ranging from 30 to 90%, remain unchanged before excretion in urine and in faeces (Holtz, 2006; Monteiro and Boxall, 2010; Rang, 1999; Reynolds, 1989). Hence, a mixture of pharmaceutical parental compounds and their metabolites reach, through domestic discharges, the municipal sewerage systems where often are not completely removed (Kümmerer, 2004). Depending on the physical-chemical properties of pharmaceuticals and the specific technology implemented, the percentage of pharmaceutical removal in Sewage Treatment Plants (STPs) can be highly variable, ranging from 0 to 99% (Monteiro and Boxall, 2010). As an example, some a.s. like carbamazepine, diatrizoate and roxithromycin are removed in a percentage lower than 40% after secondary treatments in STPs (Bendz et al., 2005; Vieno et al., 2006). Whereas, a percentage ranged from 20 to 60% of ibuprofen, diclofenac and diazepam is only removed after tertiary treatments (Monteiro and Boxall, 2010). Leakages from sewer drains and STPs may constitute an additional source of pollution for soils, from which active substances may be washed into surface water (Pedersen et al., 2005).

1.1.2.3. Use of sewage sludge and wastewater

In several EU countries, the application of sewage sludge and dirty waters to agricultural soils as fertilizers constitute still a common practice (Topp et al., 2008; Xia et al., 2005). After rainfall events, from contaminated soils, PhAC residues may reach surface and ground water systems through run off and leaching processes (Pedersen et al., 2005; Topp et al., 2008).

1.1.2.4. Disposal of unused drugs

It is estimated that probably, in the EU the percentage of unused medicinal products accounts for about 50% of the total medicines sold (EEA, 2010). Unfortunately, few information on the adequate method of disposal (return to the manufacturer, landfill, waste encapsulation and inertization, incineration) of expired or unused medicines is available (Monteiro and Boxall, 2010). Anyway, some scientific investigations performed in the UK or in Germany have identified the improper disposal of uncollected medicines as a potential source of environmental contamination; e.g. it was estimated that in 2006 in Germany the 23% of unused medicines were discharged in sinks and in toilets accounting for 364 tons per year of a.s. (START, 2008).

1.1.3. Veterinary medicinal products

Veterinary medicinal products (VMPs) are substances designed to protect health and to treat diseases in animals. They include, also, substances incorporated into the feed for the improvement of the animal growth rate. Many therapeutic classes of VMPs share the same area of application of human medicine, such as antimicrobials, anaesthetics, anti-inflammatories, but many others are of exclusive use in veterinary field, like ecto- and endo- parasiticides, antifungals, coccidiostats/antiprotozoals, hormones, etc. Contrarily to human pharmaceuticals that are designed to treat a single species, the target of VMPs is a wide number of species from different taxonomic groups: mammals, birds, fish and invertebrates. Because of this different patterns of use, veterinary pharmaceuticals are subject to more different pathways of entry into the environment (Boxall et al., 2004).

1.1.3.1. Emissions form livestock production and manure

As well as human pharmaceuticals, large amounts of VMPs applied orally, via injections or in feed are excreted unchanged (25-75%) (Elmund et al., 1971) reaching, directly and indirectly, soil and aquatic compartments (Halling-Sørensen et al., 1998). Soils and surface waters may be directly contaminated through faeces and urine of animals intensively reared outdoor and on pasture (Montforts, 1999). Additionally, animals reared intensively indoors annually produce high amounts of manure, slurry and dirty waters. These are directly applied as fertilizers to agricultural soils (Halling-Sørensen et al., 2001; Montforts, 1999) from which, after rainfall events, residues of veterinary medicines may leach to groundwater or reach the nearby surface waterbodies through runoff (Hamscher et al., 2000a,b). Another significant source of contamination for terrestrial and aquatic compartments derive from medicines applied topically. A high amount of VMPs, such as anti-parasiticide products, are used externally in various species (e.g. sheep or poultry) by means of different kind of topical applications (jetters or showers, pour-on formulations; and plunge dipping or sheep dipping). From treated animals, VMPs can be washed onto farmyard, into soil and in surface water.

It becomes clear that, in these cases at least locally, the environmental concentration of veterinary pharmaceutical active substances could be much higher than those of human PhACs as they not undergo any treatment prior the emission (Halling-Sorensen et al., 1998; Sommer et al., 1993; Strong and Wall, 1994).

1.1.3.2. Aquaculture treatments

A significant amount of veterinary PhACs may reach the aquatic environment directly through aquaculture practices. VMPs in pellets of food are usually added to cages and enter the environment via faeces and urine and via food/feed surplus (GACE, 2007). In aquaculture veterinary medicines,

notably antibiotics, are also used for prophylactic purpose to prevent bacterial infections. In this case, drugs are directly added to the water (Lupin, 2003).

1.1.3.3. Domestic animals

VMPs used for companion and domestic animals can also be released into the environment. Anyway, this source is supposed to be less relevant respect to those above mentioned, since pets are kept on a small scale. Actually to date, studies on the environmental fate of medicines used for pets are lacking (Boxall et al., 2003).

1.1.1.3. Occurrence, environmental fate and harmful effects

To date, although a high number of a.s. are used as pharmaceutical ingredients, only a little subset of them has been searched through monitoring surveys (López-Serna et al., 2010). Nevertheless, during the last decades, the ubiquitous occurrence of their residues in aquatic systems has been demonstrated, and at least 150 a.s. have been measured at concentrations ranging from ng L^{-1} to µg L^{-1} in STP effluents, surface water, groundwater and drinking water (Ashton et al., 2004; Calamari et al., 2003; Focazio et al., 2004, Heberer, 2002; Kolpin et al., 2002; Kümmerer, 2004; Metcalfe et al., 2003, 2004; Ternes et al., 2001; Thomas and Hilton, 2003; Zuccato et al., 2004). These figures do not reflect the actual number of PhACs present in aquatic environment, because many of them have not yet been monitored and/or analytical detection techniques have not yet been developed.

Despite pharmaceuticals are, often, considered a single class of contaminants with the principal feature of pharmacological activity, they include a large amount of a.s. belonging to different therapeutic classes. So, respect to other well-known and chemically homogeneous contaminants, such

as dioxins or polycyclic aromatic hydrocarbons, pharmaceuticals represent a group of highly heterogeneous and complex chemicals with different molecular structures, and often multifunctional groups (Cunningham, 2008). PhACs include molecules with distinct functionalities, peculiar physical-chemical and biological properties that enormously influence their behaviour and fate in the environment (Beausse, 2004).

In aquatic environment, sorption to sediments and abiotic and biotic degradation are the predominant fate processes (Andreozzi et al., 2003; Ferrer et al., 2004). Particularly, the first occur for hydrophobic compounds and may have a relevant impact on bioavailability (Kümmerer et al., 2010). Biotic and abiotic reactions, instead, allow a partial or complete transformation and/or degradation of parental compounds (Andreozzi et al., 2003; Ferrer et al., 2004; Kümmerer, 2009). Microorganisms (bacteria and fungi) are able to degrade organic compounds for energy, whereas the main abiotic degradation processes occurring in surface waters are represented by photodegradation and hydrolysis. In general pharmaceuticals are resistant to hydrolysis being designed to be administrated orally, thus photodegradation process predominates. Some intermediate compounds that may present a variable and sometimes increased toxicity and/or stability can be produced (Kümmerer et al., 2010). Some a.s. could show a high environmental persistence, e.g. the pharmaceuticals diazepam, carbamazepine and its metabolite showed a DT_{90} values in water-sediment systems higher than 365 days (Loffler et al., 2005). Anyway, even if PhACs could be easily degraded, in aquatic environment are practically considered persistent pollutants (pseudo-persistence) because of their continuous release from the above mentioned sources (Daughton, 2005).

PhACs are designed to be bioavailable and to have specific biological activity at low doses at least in target organisms (human and animals). For this reason their presence in environment raises particular concern as they might provoke adverse effects also on non-target aquatic species (Brooks et al., 2005; Martinovic et al., 2007; Parrot and Blunt, 2005; Pascoe et al., 2003; Stanley et al., 2007). To date, the

knowledge on eco-toxicological effects of PhACs on wildlife is still scarce and limited to a small set of pharmaceuticals tested on a little number of species in laboratory conditions (Fent, 2008). Despite this, toxicity effects on aquatic species have been demonstrated with regard to different endpoints, such as mortality, growth, reproduction, behaviour (Fent, 2008). For example, some oestrogens, such as 17-α-ethinylestradiol (EE2), have been found being able to affect the reproduction of fish and to provoke a change female-to-male sex ratio already at low concentrations (order of ng L^{-1}) (Lange et al., 2001). Some others pharmaceuticals, such as the lipid-lowering gemfibrozil that has been detected in fish blood at concentration of 113-times greater than water, have the potential to accumulate in biota and in food web (Mimeault et al., 2005) It is also worth mentioning the case of the anti-inflammatory diclofenac accumulated in treated livestock carcasses that caused a severe decline in oriental vulture populations predators (Oaks et al., 2004). Another particular matter of concern is given by the potential spread of antibiotic resistance, not yet fully understood (Kümmerer, 2003). In addition, it should be highlighted that wildlife will be exposed not to single chemicals but to complex mixtures of them, which effects are still largely unknown (Backhaus, 2016).

1.1.4. Quaternary ammonium compounds

Quaternary ammonium compounds (QACs) are a class of organics chemicals widely used as ingredients in a great number of household and personal care products like fabric softeners, detergents, emulsifiers, wetting agents, hair conditioners, disinfectants, biocides and many others (Kaj et al., 2014). Among these, the use of QACs as fabric softeners, accounting for about 66% of the total, dominates over all the other applications (Boethling, 1994).

1.1.4.1. Consumption

QACs are listed among the High Production Volume Chemicals (HPV) of the U.S. Environmental Protection Agency (U.S.EPA) and the Organization for Economic Co-operation and Development (OECD) lists. HPVs are defined as chemicals produced or imported into the U.S.A. for an amount equal to or greater than one million pounds per year; and in OECD countries as chemicals produced for amounts higher than 1,000 tons per producer/importer per year in at least one of the OECD countries. (Tezel, 2009). According the European Committee of Organic Surfactants and their Intermediates (CESIO), in 2004 the worldwide consumption of QACs was estimated at about 500,000 tons (CESIO, 2004), of which in Europe (1998) 98,000 tons were estimated to be used as ingredients in household products and 17,000 tons in industrial products (Madsen et al., 2001).

The large use of this class of compounds is due to their particular physical-chemical properties that allow a wide range of versatile properties, like detergency, antimicrobial properties and surface-activity useful for numerous applications. QACs are characterized by one or more hydrophobic alkyl chain groups and a hydrophilic head positively charged with a central nitrogen atom; other alkyl groups can be presents as short-chain substituents, such as methyl or benzyl groups (Boethling, 1994). This structure give to QACs the capability to modify the surface properties of the water reducing its surface tension, provoking a better distribution and the wetting effects. Additionally during cleansing, chemicals such as those contained in dirt and stain are easily captured in emulsion and removed by rinsing. QACS are also extensively used for their wide spectrum of antibacterial activity over a wide range of pH, due to the attraction of the positively charged group of QACs to the negatively charged substances of bacterial membrane cells, such as structural and enzymatic proteins (Gilbert and Moore, 2005).

1.1.4.2. Sources and pathways of release to the environment

QACs have a high potential to enter the environment. A large amount of QACs used every year reach the wastewater treatment systems (about 75% of the total) whereas, the remaining part reach directly the environment (Tezel, 2009). Similarly to human PhACs, effluent discharges and the spread of sewage sludge represent the main source of release (Martinez-Carballo et al., 2007a,b). Discharges from laundries and from hospitals represent other significant local point sources (Kreuzinger et al., 2007). The mean rate of removal in STPs is, usually, over 90% (Kreuzinger et al., 2007; Martinez-Carballo et al., 2007a) of which biotrasformation account for the major percentage. Anyway, sludge, being in large extent negatively charged, outcompetes biodegradation adsorbing a great amount of QACs (Clara et al., 2007). As an example, a range of 22 - 103 mg/kg of QACs have been found in Austrian STPs sludge samples (Martinez-Carballo et al., 2007b). In sewage sludge QACs may inhibit microorganism activity reducing the removal success of STPs (Ivankovic et al., 2009). Some studies revealed that QACs could be adsorbed prior to be completely biodegraded, thus they can re-enter the environment as such (Clarke and Smith, 2011).

1.1.4.3. Occurrence, environmental fate and harmful effects

Even if the mean rate of removal in STP is high, QACs have been found in effluents from treatment plants, laundries and hospitals (Kreuzinger et al., 2007; Martinez-Carballo et al., 2007) and in surface waters (Ferrer and Furlong, 2001; Kreuzinger et al., 2007; Martinez-Carballo et al., 2007) in the range of μg L^{-1}. In aquatic environment, QACs are mainly degraded by microorganisms under aerobic conditions (Clara et al., 2007; Patrauchan and Oriel, 2003), in addition they have a great affinity for humic materials and sediments where are adsorbed (van Wijk et al., 2009).

Because of their high biological activity, QACs pose particular concern for potential harmful effects on wildlife. Acute toxic effects have been demonstrated on aquatic organisms (García et al., 2001; Kreuzinger et al., 2007; Lavorgna et al., 2016; Sandbacka et al., 2000) at environmentally realistic concentrations, even if in aquatic environment the presence of sorbent materials (sediment, humic acid, clay and suspended matter) could reduce aqueous bioavailability and thus the extent of adverse effects (van Wijk et al., 2009). Anyway, although their widespread use and their high consumption, data on environmental fate and toxicity are greatly limited.

2 Prioritization of chemicals of concern

As seen in the previous sections, the number of PPCPs and in general of chemicals used in the everyday life is enormous. The chemical worldwide annual production is estimated at over 400 million tonnes, with the EU accounting for about 33% of the total (van Leeuwen and Vermeire, 2007). To date, information relating to the environmental fate and the (eco)toxicological effects are available only for some thousands of the over 100,000 chemical substances listed as existing (i.e. placed in the market before 1981) in the EINECS (van Leeuwen and Vermeire, 2007). It is recognized that many of these chemicals can be considered environmental contaminants as they are able to reach the environment. Monitoring or assessing all chemicals potentially detectable in environmental compartments is neither reasonable nor possible, since these processes are very costly and resources available are often very limited. Thus, focus the efforts towards some more relevant compounds, among the many, becomes necessary. There is not a specific method to identify compounds to manage in a priority way. For example, one approach adopted by national and international authorities, beside the creation of chemical inventories (e.g. EINECS) was to identify and list chemicals produced or imported in large volume (e.g. creation of HPVs lists) (Altenburger, 2001).

In the last decades, governmental agencies and research groups have developed a wide number of screening or prioritisation systems with different objectives, e.g. regulatory, ranking or monitoring purposes. For example in the US, a prioritisation tool (CHEMS-1, Chemical Hazard Evaluation for Management Strategies) was developed for the assessment of chemical hazards for the environment and human health (Davis et al., 1994; Swanson et al., 1997). In EU chemicals listed in EINECS have been ranked through the European Union Risk Ranking Method (EURAM) (Hansen et al., 1999). Again, the Italian Environment Protection Agency (ANPA) in 1997-1998 has supported the development of different rating index with the purpose of targeted management of pesticides in different environmental scenarios (Finizio, 1999a; 1999b). Independently of the specific purpose of the prioritization activity, the basic approach involves the identification and the management of the chemical substances posing the greatest concern for human and environmental health, e.g. chemical substances for which eco-toxicological studies or monitoring surveys have to be planned.

In order to select priority substances is, often, necessary to develop a specific methodology. The different prioritization approaches adopted in literature have different degrees of complexity and include a great range of methodologies, from a simple evaluation of chemical hazard characteristics to complex systems based on the assessment of environmental risk (Bu et al., 2013). Anyway, the majority of prioritization schemes is based on the concept of environmental risk assessment, generally defined as the combination of the probability of occurrence of an exposure event and the related adverse effects (Guillen et al., 2012). Whereas, a hazard-based methodology refers in general to the development of a methodology that combine different hazard parameters, such as persistence, potential bioaccumulation on organisms and toxicity, without any consideration on the potential environmental exposure (Bu et al., 2013).

2.2. General approach for the development of prioritization index

A list of priority substances is, often, a result of the application of a specific index; in general, a risk index is developed, following a common scheme with defined steps (Bu et al., 2013; Zaghi et al., 2007):

(i) definition of the chemical universe and the purpose of prioritisation;

(ii) selection of appropriate endpoints or parameters for prioritisation;

(iii) formulation of algorithm for the index calculation;

(iv) final list of priority pollutants.

The starting point is given by the chemicals universe to prioritize. It can include all the chemicals listed in a database (e.g. the EINECS list), or more specifically can consist in a more relatively restricted number of chemicals (e.g. PPCPs used in a given area having the potential to reach a specific environmental compartment). The risk- (or hazard-) index has the advantage to give with a simple numerical value some information on complex phenomena (e.g. the risk posed by the presence of a chemical on a compartment) and, in general, it is developed as a useful tool to drive different kinds of decision-making processes, e.g. regulatory action for high concern chemicals, or for the identification of priority substances, for the setting up of targeted monitoring plans, or the performing of a risk assessment focused only on high-concern substances, or simply to favour the use of more environmentally friendly substances (risk comparison). In relation to the specific environmental scenario (water, soil, air) to which the index is addressed, different parameters of prioritization can be selected. This represent one of the most important step from which depends the level of complexity of the final index. Parameters to be selected, generally, refer to characteristics that make a chemical of concern, i.e. its environmental exposure and effects. Thus, a combination of acute, chronic, sub-chronic toxicity data related to different effect endpoints can be combined for the characterisation of effects. Conversely, in dependence of the level of complexity of the final index, parameters related to intrinsic and extrinsic properties of molecules, such as physical and chemical properties influencing the

fate of a molecule in environment (water solubility, partition coefficients, persistence, etc) and more or less detailed information on usage (e.g. dosage, method of application/administration, frequency of application, etc), can be combined for the characterisation of exposure. This information can be organised in various ways including the use of detailed calculation models (e.g. PEC calculation models). At the end, to obtain the final algorithm of calculation, the selected parameters are combined. The way in which they can be aggregated and organized can vary greatly. In general, after the assignment of some appropriate scores or weights to the set of physical-chemical, ecotoxicological properties or data on usage (or classes of them) they can be combined in an additive way and/or in multiplicative way (Scoring approach) (Bu et al., 2013; Zaghi et al., 2007). The outcome, is a priority list of pollutants ranked on the basis of their environmental risk (or hazard), according to the final numeric value obtained for each substance.

Many approaches, being mainly based on the specific information required by the existing environmental risk assessment guidelines are often built on the basis of a risk quotient concept (PEC/PNEC ratio) (Ratio approach). In the last case, the methodology of prioritization becomes analogous to a quantitative risk assessment methodology, varying in the level of detail in which each term (exposure and effects) is assessed (i.e. conservative approach).

From previous sections, considering the enormous number of chemicals reaching the environment, it becomes evident that in an environmental compartment, chemicals will not be present as single toxicants but rather as mixtures of them. The simultaneous presence in aquatic environment of a great number of potentially harmful chemical substances, including PPCPs, has been demonstrated by several monitoring surveys (Andreozzi et al., 2003; Kostich et al., 2014; Proia et al., 2013; Vulliet and Cren-Olive´, 2011). Consequently, aquatic organisms will be more likely exposed to combinations of chemicals in form of multicomponent and often complex mixtures rather than to isolated toxicants (Backhaus, 2014; Kortenkamp et al., 2009; SCHER, SCCS, SCENIHR, 2012). The exposure to multiple contaminants raises concerns about the potential risk for human health and wildlife since, currently,

the EU procedures for environmental risk assessment focus mainly on the assessment of exposure and toxicity of single chemicals, and no specific mandate exists for the assessment of risk posed by mixtures. This could lead to a severe underestimation of the real ongoing risk in the environment (Kortenkamp et al., 2009; SCHER, SCCS, SCENIHR, 2012). There is large evidences that the toxicity of combined chemicals on aquatic organisms is, usually, larger than the toxicity from individual components even if these are present at concentrations provoking individually negligible or no effects (e.g. NOEC) (Backhaus et al., 2000; Faust et al., 2001; Faust et al., 2003). This leads to the important conclusion that relevant adverse effects could occur even if individual environmental quality thresholds, such as PNEC (Predicted No Effect Concentration), are respected (Backhaus et al., 2000; Backhaus, 2016).

Understanding the combined effects of chemical mixtures and their eco-toxicological role in the environment is an important challenge for scientists and regulators and even if a wide number of scientific publications has been produced, much has yet to be investigated (van Gestel et al., 2011).

3 Chemical mixtures in the environment

The environmental exposure to a multiple combination of chemicals mainly derives from the combination of emission from specific sources and the different distributions or environmental fate that single components undergo. Three main typologies of environmentally relevant mixtures can be identified (European Commission, 2014; Kortenkamp et al., 2009; SCHER, SCCS, SCENIHR, 2012)

- *Intentional mixtures*: technical mixtures designed as such. This may be the case of commercial products and formulations of pharmaceuticals, laundry detergents, cosmetics, etc, composed by two or more active substances (a.s.) and/or other ingredients;

- *Generated mixtures*: mixtures deriving from a specific human activity and often originated from a single source, such as active substances emitted during a production process (e.g. from a pharmaceutical production facility or from an agricultural crop). In these cases, the resulting mixtures could be characterised qualitatively and quantitatively, at least on local scale, even if their composition may vary with time;

- *Random or unintentional mixtures*: mixtures deriving from the co-occurrence of unrelated chemicals in the same environmental compartment (e.g. in a surface water body) due to the presence of different human activities in a given territory. Chemicals are, often, originated from different sources and/or through different pathways. In this case, composition of mixture is greatly unknown.

Once introduced in environment, in dependence of the specific physical-chemical properties, each individual component will undergo a specific environmental fate and distribution process. The identity (composition and concentration) of the resulting mixture components could vary dynamically over time and space, in depending also on the climate, geography and period of the year. This makes clear that the resulting chemical cocktails could be totally different from the original ones, acquiring often an unknown eco-toxicological profile (Boxall, 2008; Boxall and Ericson, 2012; Challis et al., 2014; European Commission, 2014).

In ecosystems, mixtures can act on a multitude of different species characterised by high differences in genetic composition, physiology and biochemistry, as well as in lifecycles. Additionally, the possibility of exerting indirect effects, such as effects on predator–prey relationships, that are substantially unknowns, introduces further levels of complexity (Brodin et al., 2014; SCHER, SCCS, SCENIHR, 2012).

Nevertheless, information on adverse effects caused by contaminants potentially present in the mixture are mainly available only for individual substances, for few limited endpoints (e.g. inhibition of growth, mortality) and for few aquatic species (SCHER, SCCS, SCENIHR, 2012). For all these factors, it

becomes clear that the study of the ecotoxicology of mixtures is still a complex matter that needs some necessary simplifications.

3.2. Approaches used for the assessment of mixture effects

Two main approaches for the assessment of mixture effects are usually adopted: (i) "whole-mixture" assessment and (ii) "component-based" assessment. The first consists in a direct eco-toxicological investigation of a given environmental sample, e.g. toxicity test on a specific effluent, where chemicals are often in a complex undefined mixture (Groten et al., 2001). This method allows a relatively fast and easy application, but is often applicable only for mixtures having constant composition (Kortenkamp et al., 2009). Results cannot be extrapolated to different exposure scenarios and nothing can be said on the contribution of single components; e.g. which chemicals mainly drive mixture toxicity, or if (and which) components interact. This approach can often be used when complex systems have to be tested (e.g. studies on microcosms and mesocosms) (European Commission, 2014).

On the other hand, when information on single mixture components is available (e.g. identity, mode of action, concentration-response relationships) a component-based approach can be applied. This approach allows to link results deriving from mixtures to the individual components. This link allows to understand how mixture effects occur and to generalise the results (van Gestel et al., 2011). Anyway, frequently, information on individual components are lacking.

The linking between single component toxicity data and those resulting from mixtures represents the basis of joint effect predictions. As the assessment of all the mixtures potentially occurring in the environment is very difficult and not feasible, prediction of mixture effects often becomes a necessary tool.

3.3. Principles of mixture toxicology

Historically three basic types of action for chemical mixtures have been defined (Loewe and Muischnek, 1926; Bliss, 1939; Plackett and Hewlett 1948, 1952):

- Similar action (dose/concentration addition)
- Dissimilar action (independent action) and
- Interactions

The first two will be better described in the next paragraphs as they represent the scientific basis of the most widely utilized predictive models of mixture toxicity. Here a brief explanation of interactions phenomena is reported. Some considerations about the risk management of chemical interaction in mixture will be presented later in the text (see paragraph 3.9.).

According to SCHER (2011), interaction describes the combined effect of two or more chemicals as stronger (synergistic, potentiating, supra-additive) or weaker (antagonistic, inhibitive, sub-additive, infra-additive) than would be expected on the basis of dose/concentration addition or response addition. Interactions may therefore vary according to the relative dose levels, the route(s), timing and duration of exposure (including the biological persistence of the mixture components), and the biological target(s). There are different possible interactions among chemicals in mixture (toxicokinetic, metabolic, toxicodynamic). All of them can lead to an enhanced or decreased potency of the mixture. From an environmental risk management point of view, obviously synergy raise higher concern than antagonistic phenomena. However, it must be highlighted, that historically synergism has been utilized commercially to improve the efficacy of plant protection products. For instance, coformulation of pyrethrins with insecticide synergists such as piperonyl butoxide (PBO) has been used for decades as a way to reduce the amount of pyrethrins required to achieve insecticidal potency after application (Gidding et al., 2016). In fact, PBO inhibits breakdown of pyrethrins within the organism so that more parent compound remains available at the site of action (Wickam, 1998).

3.4. Mathematical concepts used for the prediction of mixture toxicity

As previously reported, both concepts, of dose/concentration addition and independent actions have been suggested as default approaches in regulatory risk assessment of chemical mixtures. In reality, however, chemical mixtures are rarely composed of either only similarly or of only dissimilarly acting substances. A brief overview of these two concepts is presented below, and a more detailed description can be found in the review by Kortenkamp et al. (2009).

The component-based approach (see. paragraph 3.2) allows the explanation of the toxicity of a mixture in terms of single component effects. The knowledge of single concentration-response data allows the calculation of the joint effects through the use of two simple pharmacological concepts adopted in ecotoxicology: Concentration Addition (CA) for substances in mixture having the same or similar mode of action (MoA) (Loewe and Muischneck, 1926) and Independent Action (IA) for substances in mixture having different MoA (Bliss, 1939). Both models work making the important assumption of absence of interactions among substances, i.e. mixture components do not influence each other's toxicity by means of chemical or biological interactions (Backhaus, 2014).

Particularly, CA and IA are built on two different ideas about the joint action of components in mixture as described in the following paragraphs.

3.5. Dose/Concentration Addition (CA)

The basic idea of CA model is that if all components in a mixture have the same or similar mode of action (MoA), e.g. act on the same molecular target, it can be assumed that they behave as the same chemical; so each of them could be replaced by an equi-effective concentration (e.g. an EC50) of another, without modifying the final mixture effect (“dilution principle”) (Loewe and Muischneck,

1926). If this assumption works well, the effects of such a mixture can be estimated simply from the sum of the concentrations of the single components, normalized for their potencies (Backhaus, 2014; Kortenkamp et al., 2009; European Commission, 2014). The fractions of equi-effective concentrations of individual components are known as Toxic Units (TUs); CA approach is also called as Toxic Unit Summation. Mathematically, for mixture of *n* components CA concept can be expressed according this formulation (Loewe and Muischneck, 1926):

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

Where *n* is the number of components in the mixture, C_i is the concentration of the single chemical in the mixture that elicits the effect *x*, and EC_{xi} is the concentration of the same chemical that individually provokes the same effect *x*; the ratio C_i/EC_{xi} is the so-called Toxic Unit (TU) (Sprague, 1970). The sum of TUs, in the absence of interactions among components, will be equal 1.

3.6. Independent Action (IA)

IA concept is based on the idea that if all components in the mixture act dissimilarly via different and independent MoA, the overall effect of the mixture can be estimated from the summation of the effects of individual components according to the statistical concept of independent random events (Bliss, 1939). This model is also called Response Addition.

Mathematically, for mixture of *n* components, this concept can be formulated as follows:

$$E(c_{mix}) = 1 - \prod[1 - E(c_i)] \qquad \text{(eq. 2)}$$

with $c_{mix} = c_1 + ... + c_n$

where $E(c_{mix})$ describes the overall effect of the mixture at concentration c_{mix} and $E(c_i)$ is the effect of individual component at the concentration (c_i) at which is present in mixture

According to the expressed mathematical concepts, the application of CA implies that each chemical in mixture contributes to the total mixture effect proportionally to its TU, whatever the concentration in which it is present. On the contrary, IA concept supposes that the overall mixture toxicity is given only by components whose effect is higher than zero at the concentration at which they are present in mixture.

3.7. Quantitative relationship between CA and IA curves

CA and IA predictions can be considered as extremes cases that define the so-called *prediction window*, i.e. the space comprised between the two predicted Concentration-Response Curves (CRCs) with CA curve as the upper bound, since it often predicts a higher toxicity than IA, and the IA curve as the lower bound. Anyway, an environmentally realistic mixture will more likely have an intermediate toxicity falling in the area within the two CRCs. It is very unlikely, in fact, that components in mixture will have all a strictly similar or strictly dissimilar MoA (Walter et al., 2002).

Anyway, it has been experimentally demonstrated that in the majority of cases, CA and IA models estimate a similar toxicity (Backhaus et al., 2002). Several studies have highlighted the dependence of the quantitative relationship between the predicted CRCs on different factors, such as the steepness of the single components' CRCs and the number of components in mixtures. When the number of substances present in the mixture is high (more than about 10) and the individual CRCs are very steep

or very shallow, the differences between prediction from CA and IA become greater than one order of magnitude (Kortenkamp et al., 2009). On the contrary, when single mixture components present a similar slope with an intermediate steepness of their individual CRCs (i.e. neither highly steep nor flat) CA and IA models estimate a similar toxicity (Boedeker et al., 1993; Drescher and Boedeker, 1995).

3.8. Selection of predictive models

In principle, the knowledge of the MoA of mixture components should be the suitable criterion for the selection of the most appropriate predictive model, or for an integrated and stepwise application of both of them. But, besides the fact that it is very hard to find environmentally realistic mixtures whose components have all similar or dissimilar MOA, there are also some different order of difficulties. First of all, there is a substantial lack of knowledge of the components' MoA in non-target organisms, additionally, the MoA of a given substance in a mixture can be different on different taxonomic groups of organisms and finally, if some knowledge is available, this is limited to very few species and/or biological endpoints (Bachkaus et al., 2010) For these reasons, the MoA as a general approach for the selection of the appropriate model to apply is often of difficult use.

When no information on the MoA is available, the most acceptable solution seem to be the *a priori* choice of one of the predictive models. Considering that, as discussed above, large differences between the two predictions are observed only in the case of a high number of components in mixture and in the case of extremely steep or flat individual CRCs and considering that CA is usually slightly more conservative than IA (higher predicted toxicity than IA), for the precautionary principle, CA model can be selected as a pragmatic default approach for regulatory purposes (Backhaus et al., 2010; Kortenkamp et al., 2009; SCHER, SCCS, SCENIHR, 2012). This, also, because the application of IA model is more difficult since, according to its mathematical concept, it requires the knowledge of the entire individual CRCs; conversely, CA concept needs only single ECx values as input data. Considering the

scarce availability of eco-toxicity data often limited to single values of ECx or Lowest Observable Effect Concentrations (LOECs) or No Observable Effect Concentrations (NOECs) CA model is again the preferred approach (Backhaus et al., 2010).

3.9. Deviation from CA and IA predictions

One of the main goal of the component-based approach is to analyse if mixture components meet the additive assumption of predictive models or if some interactions occur. CA and IA models are built under the assumption of simple additivity concepts, i.e. is assumed that compounds in mixtures do not interact neither chemically nor biologically in pharmacokinetic or in pharmacodynamic phases. Nevertheless, in realistic mixtures, interferences and biological interactions might occur at different levels: e.g. a component could enhance the uptake of another component, or could inhibit its excretion, or again could act on enzymes involved in a metabolic pathway (SCHER, SCCS, SCENIHR, 2012). All these interactions can lead to a deviation of the observed toxicity from the conceptual expectations provided by CA or/and IA. Particularly, as a result, the observed mixture toxicity could be higher (more than additive or synergistic effects) or lower than that predicted (less than additive or antagonistic effects) (European Commission, 2014). Thus, the additive response from the application of CA or IA becomes the reference for the qualitative and quantitative identification of synergistic or antagonistic effects.

As previously noted, synergisms or antagonisms cannot be predicted neither quantitatively nor qualitatively. It is not possible to have an anticipation of deviations from additivity expectations on the basis of single mixture component CRCs . Until now, their occurrence is limited to a simple observation of deviations from predictions, even if, from the few available data from literature, the observed synergistic responses seem to be rare and rarely distant more than a factor of 2-3 from CA predictions (Backhaus, 2016). There is a substantial gap in the knowledge of the underlying causes of these

deviations, i.e. the specific chemical or biological interactions among chemicals (Backhaus, 2014; Kortenkamp et al., 2009). This gap prevents a more accurate study of mixture ecotoxicology.

3.10. Combination Index (CI) method

Recently, in eco-toxicological studies, the Combination Index (CI) method was applied to investigate the combined effects of mixtures of environmentally relevant toxicants, e.g. pharmaceuticals, pesticides, perfluorinated surfactants, chlorinated toxicants, heavy metals and many others tested on several aquatic organisms (Gonzalez-Pleiter et al., 2013; Rodea-Palomares et al., 2010; 2012; Rosal et al., 2010). Combination Index is a method historically used in pharmacology to analyse the nature and the extent of interactions among pharmaceutical active compounds (Chou, 2006). Particularly it provides a quantitative determination of synergistic and antagonistic effects at all possible ratio of combination among components and at all levels of effect. Equation underlying CI was built by Chou and Talalay (1983; 1984) on the basis of the median-effect principle of the mass action law (Chou and Talalay, 1983; 1984). An important aspect of this equation is that its application is practically independent from the knowledge and/or any consideration on the MoA of chemicals in mixture (Chou, 2006). CI method share the same underlying concepts of CA model, i.e. the Loewe additivity and the classical concept of isobologram (Berenbaum, 1981; Loewe and Muchnik, 1926), but is mainly oriented on the evaluation of synergistic and antagonistic interactions. For a combination of *n* chemical substances, the general Combination Index equation was developed as follows (Chou and Talalay, 1983; 1984):

$$^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n} \left\{ \frac{[D]_j}{\sum_1^n [D]} \right\}}{(D_m)_j \left\{ \frac{(fa_x)_j}{[1-(fa_x)_j]} \right\}^{1/m_j}} \qquad \text{(eq. 3)}$$

where ${}^{n}(CI)_x$ is the combination index for *n* chemicals at *x%* inhibition, $(D_x)_{1-n}$ is the sum of the dose of *n* chemicals that exerts *x%* inhibition in combination, $\{([D]_j/\sum[D]\}$ is the proportionality of the dose of each of *n* drugs that exerts x% inhibition in combination and $(D_m)_j \{(f_{ax})_j /[1 - (f_{ax})_j]\}^{1/mj}$ is the dose of each chemical alone that provokes *x%* inhibition. D_m is the median-effect dose, f_{ax} is the fractional inhibition at *x%* inhibition and *m* is the slope of the median-effect plot. Plotting the resulting CI values for all the effect levels, a CI curve for each mixture is obtained (*fa-CI* plot). From equation 3, CI<1 indicates that the concentrations of mixture components producing a given mixture effect are lower than concentrations giving theoretical expectation of additivity, this result explains a deviation from additivity in direction of synergistic responses. Correspondingly, CI > 1 implies that the concentrations of chemicals in mixture giving a definite effect are greater than the concentrations expected to give an additive response, this is interpreted as a deviation of mixture response in direction of antagonism. Conversely, CI=1 indicates additive response or absence of interactions. In this way the extent of departure from the additivity at any effect level is quantified.

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CHAPTER II

AIMS

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The present study aims to assess the environmental effects of different classes of emerging contaminants tested as single toxicants and as different combinations of mixtures. The aquatic toxicity of chemicals was tested by using the standardized Microtox® test, which measure the bioluminescence inhibition in *Aliivibrio fischeri* (formerly *Photobacterium phosphoreum*) exposed for 5-30 minutes to toxicants. Microtox® test provides a high validation and standardization of results with a high stability and intra- and inter-laboratory reproducibility. Binary and multicomponent mixtures were assessed in order to evaluate the compliance with conceptual expectations (additivity concepts) by means the application of the two predictive models widely used in ecotoxicology: Concentration Addition (CA) and Independent Action (IA). The presence of toxicological interactions among mixture components was analysed by using the Combination Index method, historically applied in pharmacology to understand the nature of PhAC interactions.

Alongside the main aims of the thesis, which was to contribute to the knowledge of the mixture effects of emerging contaminants in the environment, during the PhD other relevant issues were considered:

(i) how to identify potential and relevant mixture of emerging contaminants. As previously reported, very frequently mixtures derive from the co-occurrence in the same environmental compartment (e.g. in a surface water body) of unrelated chemicals originated from different sources and pathways. In this case, the mixture composition is greatly unknown. Once introduced in the environment, depending on the specific physical-chemical properties, each individual component will undergo a specific environmental fate and distribution process.

(ii) Highlight the mode of action (i.e. narcotic, specific MoA) of the mixture components towards the model organism. This is precious to understand how the mixture behaves on a species. In this study, we used a QSAR (Quantitative Structure Activity Relationship) approach to get these information.

(iii) Identify if CA and IA models were adequate to predict the mixture toxicity or if there were significant deviations.

(iv) Get information about the potential environmental risk posed by single components and mixtures. This was done by comparing the measured environmental concentrations of the selected active substances in surface waters with toxicity data from aquatic organisms representative of the three main trophic levels (algae, *Daphnia* and fish).

To achieve the objectives of the thesis we followed different steps:

I. Development of a new risk-based indicator (RANKVET) specifically implemented for the prioritization of veterinary medicinal products (VMPs) in terms of their potential environmental risk (publication I) and selection of pharmaceuticals to be tested in the next step. RANKVET is presented as useful tool to support risk management strategies. In chapter III the specific methodology followed for the development of RANKVET is presented. The developed methodology is totally based on the specific requirements from Veterinary International Conference on Harmonization (VICH) guidelines for the VMP marketing authorization. For each VMP the characterization of exposure, effects and risk was performed. PEC (Predicted Environmental Concentration) models were applied for each VMP for the estimation of the environmental concentration in surface water and terrestrial compartment and for various kind of husbandry practices according to worst case scenarios. For the characterisation of the effects, the PNEC (Predicted No Effect Concentration) was calculated for each VMP from acute and chronic toxicity data on non-

target aquatic and terrestrial organisms. RANKVET was developed according to a scoring approach, thus some scores were assigned to the obtained ratio PEC/PNEC and to the potential metabolism of the target animals. A priority list of VMPs was obtained by applying RANKVET indicator to the Italian scenario.

II. Assessment of the individual toxicity of ten widely used PhACs on *A. fischeri* using bioluminescence inhibition as toxicity end-point (publication II). This step had the aim to determine and evaluate the main parameters related to the individual concentration-response relationships of the investigated PhACs and to study the mode of action on *A. fischeri.* This basic information is needed for the subsequent application of mixture predictive models (CA and IA) (Component-based approach). Particularly, in chapter IV results from the Microtox® assay and a comparison with a collection of acute toxicity data from other non-target organisms (algae, *Daphnia* and fish) are presented. The MoA was investigated by using the Quantitative Structure–Activity Relationship (QSAR) models specifically developed to predict the effect of narcotic and polar narcotic compounds on the tested organism. Additionally, environmental levels of the selected pharmaceuticals were investigated by means of a detailed analysis on data from literature for the measured environmental concentrations in EU water bodies.

III. Assessment of environmental effects from combination of binary and multicomponent mixtures of veterinary pharmaceuticals tested on *A. fischeri* (publication III). In chapter V, the joint effect of different binary mixtures of pharmaceuticals combined in a ratio corresponding to each individual value of IC_{10} was assessed with the main purpose to evaluate the predictability of CA and IA models and/or assess the potential presence of toxicological interactions. In this step was also evaluated the combined toxicity of a

multicomponent mixture in which each PhAC was combined according to each individual value of PNEC, aiming to assess the reliability of single PNEC values as environmental safety thresholds for the ecosystem also when PhACs are simultaneously present in environment. The hypothesis of toxicological interactions occurring in binary mixtures (in the direction of synergistic or antagonistic effects) was tested by applying the Combination Index (CI) method.

IV. Assessment of inhibition effects on *A. fischeri* exposed to binary combination of 3 human PhACs largely detected in surface water bodies and often used as tracers of human contaminations (publication IV). In chapter VI results from binary mixtures of PhACs combined in a fixed ratio according to their individual values of IC_{50} are described and the hypothesis of additivity of concentrations (CA) and additivity of effects (CI) are tested. Combination method index was also applied in order to confirm results obtained from predictive models. Eco-toxicological studies on combination of pharmaceutical active compounds have the main aim to fill the significant lack of knowledges that still persists about effects from pharmaceutical mixtures.

V. Assessment of individual and combined toxicity to *A. fischeri* of five quaternary ammonium compounds (QACs) widely used as active ingredients in personal care products (publication V). In chapter VII the substantial toxicity of QACs was reported together with the main parameters of the concentration-response curves (i.e. slope, ICx). The MoA was investigated by applying the QSAR models developed for *A. fischeri*, by means of the calculation of the so-called Toxicity Ratio (TR). One multicomponent mixture and ten binary combinations of QACs, all combined in equitoxic ratio corresponding to individual value of IC_{10}, were also assessed and results were compared with predictions from Concentration

Addition (CA) and Independent Action (IA). The accuracy of the predictive approaches was quantified by applying the Model Deviation Ratio (MDR). This work was developed in collaboration with the research group of Dr. Valeria Mezzanotte from the Department of Earth and Environmental Sciences (DiSAT), Milan (Italy).

CHAPTER III

RANKVET: A new ranking method for comparing and prioritizing the environmental risk of veterinary pharmaceuticals

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RANKVET: A new ranking method for comparing and prioritizing the environmental risk of veterinary pharmaceuticals

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ABSTRACT

In recent years veterinary medicinal products (VMPs) have been recognized as emerging contaminants, giving rise to concerns regarding their environmental impact. Due to the high number of utilized VMPs, it is necessary to develop tools (indicators) for ranking these compounds according to their environmental risk relevance. These indicators can be useful, for example, for setting up monitoring programmes, and more in general for risk management purposes. In this paper we propose a new scoring system method (RANKVET) that enables ranking the risk of VMPs for aquatic and terrestrial organisms. The procedure is fully based on the information required by the EU Directives and Regulations for marketing authorization of VMPs and Veterinary International Conference on Harmonization (VICH) guidelines. According to the latter, if the environmental risk assessment of a VMP indicates an unacceptable risk to the environment, i.e., the risk quotient (RQ) consisting of the ratio of Predicted Environmental Concentration (PEC) to Predicted No Effect Concentration (PNEC) is ≥1, then mitigation measures should be proposed by the applicant in order to reduce the risk to an acceptable level. If a risk mitigation measure does not fulfil the criteria mentioned above then the outcome of the risk assessment is that a serious risk for the environment exists. In accordance with Directive 2001/82/EC (as amended) this risk has to be weighed against the favourable aspects of a marketing authorization. The prioritization scheme is based on a quantitative approach and consist of different phases. First, for each VMP, PECs are calculated using simple exposure models and worst case assumptions. PNECs are calculated for non-target organisms representative of the considered ecosystems (soil or surface water). Then numerical scores are given to the calculated PEC/PNEC ratio. Finally, the obtained score is multiplied with a further score which is based on the relevance of the metabolic rate in animals. RANKVET can be applied for surface water and soil systems and for different farming methods (intensive or pasture) and treated species. As an example of its potential use we applied RANKVET to 48 VMPs largely utilized in Italy.

1. Introduction

Veterinary medicinal products (VMPs) are compounds belonging to several chemical classes that are widely used to treat disease and protect the health of terrestrial and aquatic animals (both for food or non-food animals). In recent years VMPs have been recognized as emerging contaminants due to their widespread occurrence in environmental compartments (Brooks et al., 2007). As a consequence, in the last few years, a greater attention has been addressed to such substances (Jørgensen and Halling-Sørensen, 2000; Stuer-Lauridsen et al., 2000; Kümmerer, 2004). Releases of VMPs into the environment can take place at any step in the life cycle of the product. However, it has been recognized that major contamination routes are the direct application in aquaculture, the wash-off from topical treatments, livestock waste treatment plants, runoff from manure-treated farmlands and from excreta of animals on pasture (Boxall et al., 2003a). Many studies report the presence of VMPs in the environment, especially in surface waters (Boxall et al., 2002; Zuccato et al., 2005; Kemper, 2008; Crane et al., 2009; Iglesias et al., 2014). Although some of these compounds are unlikely to be a risk to the environment because of low concentrations combined with low toxicity, others may pose considerable risks. In fact, in some cases it was found that the detected concentrations of VMPS (e.g. ivermectin and doramectin in dung and monensin in soil) exceeded the effect concentrations (Boxall et al., 2003a) and had an adverse effect such as inhibition of reproduction, endocrine disruption and even potential ecosystem-level responses (Kim et al., 2008).

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To prevent the risks to human health and environment, the European Commission issued several Directives which regulates the use of VMPs in EU (EU Directive 90/676/EEC; Directive 2004/28/EC; Regulation EC/726/2004). According to current legislation, before any new VMP can obtain marketing authorization, a stringent analysis has to be carried out by national and/or European authorities to ensure its efficacy, quality and safety to public health as well as to the environment. For the environmental impact assessment of these substances, the VICH phase I and phase II assessments have been implemented in the EU regulatory scheme (VICH, 2000, 2004). Phase I is an assessment of potential environmental exposure not an assessment of risk. It uses a decision tree approach to decide if exposure is low (not extensive): the basic assumption is that since exposure is so low, magnitude of any hazard has no effect in equation and the product is not expected to pose a significant risk for the environment.

In Phase II, the assessment is based on a risk quotient (RQ) approach which is determined for every non-target test species considered as representative of different ecosystems. The RQ is an exposure-toxicity ratio (ETR) and compares a PEC with a PNEC. The first is obtained by using predictive exposure models, whereas PNEC values are derived from the results of ecotoxicological laboratory tests and using appropriate assessment factors (e.g., by applying a factor between 10 and 1000 to the endpoint of each toxicity test performed on non-target organisms). At the end of the Phase II assessment, a VMP may not be expected to cause a significant harm to the environment or, if the RQ remains $\geq$1, a risk to the environment is assumed. In the latter case, risk mitigation measures have to be implemented to allow the authorization of the product (EMA, 2011).

Since a very large amount of VMPs including antibacterials, parasiticides, hormones, antifungals, are marketed yearly, there is a growing need to develop tools useful to support risk management strategies. For instance, monitoring VMPs in surface water and/or ground water is becoming mandatory; however, before implementing a monitoring programme, it is necessary to identify which VMPs have to be included in the analytical protocols. Therefore, the development of tools such as risk indicators or prioritization schemes allowing to rank and compare VMPs according to their environmental significance (in terms of potential risk) could be very useful to define priority lists. In the last years, these tools have been widely used to objectively identify substances of concern and for risk management actions, since they facilitate the effective targeting of resources for subsequent environmental and human health risk assessment (Capleton et al., 2006). For instance, some scoring and ranking systems have been adopted by authorities and regulatory centres mainly as first screening tools to identify the chemicals with greatest potential for adverse effects (Swanson and Jones, 1994; Huijbregts et al., 2000; Senese et al., 2010). In the field of pesticides, risk indicators are largely used as decision support system tool, to assess the potential environmental or economic consequences of pesticide management systems (Finizio et al., 2001; Kovach et al., 1992; HAIR, 2006) or to monitor temporal pesticide environmental risk trends on different scales (Calliera et al., 2006). At present, still very few risk indicators and prioritization schemes have been developed for VMPs. The first method approaching this topic for VMPs has been tackled by Boxall (Boxall et al., 2003b; Kools et al., 2008), who developed a qualitative prioritization system based on the use of VMPs, the degree of metabolism in the animal and degradation during storage of manure prior to land spreading and the toxicity of the substance to terrestrial and aquatic organisms. These data are used in a prioritization scheme (decision tree matrixes) for identifying VMPs having the potential to impact aquatic and terrestrial systems. A different risk indicator more focused on the potential risks to consumers has been proposed by Capleton and coworkers (Capleton et al., 2006). These authors developed a prioritization scheme of VMPs on the basis of their potential for indirect human exposure via the environment and their toxicity profile.

In this paper we propose a new risk indicator (RANKVET) specifically developed for the ranking of VMPs in terms of their potential environmental risk. RANKVET is a scoring system and is fully based on the VICH guidelines used in the VMPs authorization procedures. RANKVET can be applied to different environmental compartments (surface water and soil), different types of farming (intensively reared animals and pasture) and different treated species. To highlight the potential usefulness in risk management actions, the proposed indicator has been applied to 48 VMPs largely utilized in Italy.

2. Materials and methods

The RANKVET indicator is totally based on the information required by VICH guidelines for placing VMPs on the market. The methodological approach followed to develop RANKVET can be divided in different steps:

(1) compilation of a database of active substances present in VMPs, containing the data needed for the application of RANKVET;
(2) risk characterization (based on the PEC/PNEC ratio);
(3) attribution of scores and final ranking. Each step is described in more detail in the following paragraphs.

An EXCEL electronic page for the calculation of the indicator is available upon request to the authors.

2.1. Collection of data

According to the VICH guidelines, the environmental risk assessment of VMPs must be performed for active substances (a.s.). Therefore the complete list of a.s. was retrieved from the authorized VMPs in Italy. The drugs based on natural substances and microorganisms or those used for non-food animals were excluded from further assessment, in agreement with the VICH guidelines that classify these compounds as less harmful for the environment (VICH, 2000). Fish farm medicines were also excluded because the existing PEC calculation models are too complex for a prioritization approach, and since most of them were built for the Scottish aquacultures they cannot be applied to the Italian case. Data on amounts and/or sales of VMPs in Italy were gathered from the European Medicines Agency (EMA, 2013) and from the Italian Ministry of Health (www.salute.gov.it). This process left 48 a.s. which were included in the database. They belong to different chemical classes and different pharmacological types including antibiotics, ecto/endoparasiticides, or anti-inflammatory drugs and progestogens.

For the selected a.s. information on the animal target groups, dosage, formulations, number of treatments and dosing interval were obtained from a database of the Italian Ministry of Health (http://www.salute.gov.it/farmaciVetWeb/). In addition ecotoxicity data on non-target organisms and data on physical-chemical properties (Koc) were gathered from the *Veterinary Substances DataBase* (VSDB) (http://sitem.herts.ac.uk/aeru/vsdb/index.htm) (Tables S1 and S2 of support information). Finally qualitative information on the metabolism of the selected substance were taken from the work of Boxall and coworkers (2002) and from the VSDB database.

Supplementary Tables S1 and S2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecolind.2014.12.021.

Table 1
RANKVET: scores assigned to different categories of risk quotients (RQs) and potential metabolism of VMPs in treated animals (na = not available; L = Low; M = Medium; H = High).

RQ	Score	Metabolism rate	Score
>1	40	n.a.	1
0.1–1	20	L	0.8
0.01–0.1	10	M	0.6
0.001–0.01	5	H	0.4
<0.001	0		

2.2. Exposure characterization

Predicted environmental concentrations in soil and surface water were calculated using the approach suggested by EMA guideline (EMA, 2008). The suggested models were developed for various husbandry scenarios (pasture and intensively reared animals) and are based on the total residue approach (the total amount of the dose applied is excreted from the animal and data on metabolism/excretion are not taken into account). Depending on the considered species, the models provide default information on the weight of treated animals, housing factors, number of animals kept on a place per year, animal turnover rate, fraction of herd treated. Data on representative treatment regimes, (i.e., doses, duration, and frequency of treatments) are also required and must be given by the applicants. PECs in soil and surface water were calculated separately for 7 species (cattle, poultry, horse, rabbit, pig, goat and sheep) using worst case treatment scenarios (where a range of doses was given, the largest was selected; and where a range of treatment durations was possible, the greatest was selected). In cases where different animals were treated, all the PEC results have been considered, leading to different values of RANKVET for the same a.s.

2.3. Effects characterization

PNECs may be defined as the concentration of a given chemical substance that is not expected to produce adverse effects on ecosystems at any exposure time. According to the VICH guidelines, PNECs for VMPs can be deterministically calculated by simply applying an appropriate Assessment Factor (AF) to the experimentally determined effect endpoint on terrestrial and aquatic non-target organisms. The AF is intended to cover uncertainties due to intra- and inter-laboratory and species variation, the need to extrapolate from laboratory study results to the field, and the difference from short term to long term toxicity (acute:chronic ratios). The value varies depending on the type of study conducted. For RANKVET calculation PNEC were calculated for earthworms, arthropods, microorganisms and terrestrial plants for the soil compartment, and algae, *Daphnia* and fish for the aquatic environment.

2.4. RQ characterization and scoring

As described in VICH guidelines GL6 (Phase I) and GL38 (VICH, 2000, 2004) (CVMP/VICH/790/03-FINAL), the risk assessment scheme for VMPs is structured around the risk quotient (RQ) approach, which is the ratio between PEC and PNEC. In RANKVET, the RQs are calculated for all the non-target organisms which are representative of terrestrial and aquatic environments. For compounds that are used on more than one target treatment group, RQs are calculated separately. In addition in RANKVET two different scores are attributed. The first is given to the obtained RQs, according to the different intervals of categories reported in Table 1 (an interval of one order of magnitude was considered and the maximum score is assigned to RQs ≥ 1). The second score is given to the potential metabolism in the target animals (Table 1). Compounds that are administered either orally or by injection (non-topical applications) can be significantly metabolized and this represents the mitigation of the potential exposure.

The RANKVET final score can be obtained by means of the following algorithm:

$$\mathrm{RANKVET_{sp}} = \left(\sum_{i=1}^{n} \mathrm{Score_{RQnto}}\right) \times \mathrm{Score_{met}}$$

where $\mathrm{RANKVET_{sp}}$ = final score reached by an active substance on a particular treated target species; $\Sigma\mathrm{Score_{RQnto}}$ = sum of the scores attributed to the RQs values of non-target terrestrial or aquatic organisms (5 non-target organism for terrestrial and 3 for the aquatic); $\mathrm{Score_{met}}$ = score attributed to the potential metabolism of the substance in the treated target species.

As previously reported, for compounds that are used on more than one target treatment group RANKVET can reach different values.

Since a rather limited set of both acute and chronic toxicity data of VMPs was available, particularly for the terrestrial compartment, the application of RANKVET would have been restricted to just a small number of substances. Therefore, when the ecotoxicological effects on non-target organisms were missing, default scores were attributed as a surrogate for the RQs. The attribution of the score was decided on the basis of the mechanism of action of the active substance and the general sensitivity of the non-target organisms to a particular VMPs class (Table 2).

3. Results and discussion

3.1. General considerations

The lack of data on toxicity was the major obstacle for the proper application of the RANKVET indicator. Particularly, the lack of chronic toxicity data for many compounds was the main hindrance for the calculation of more realistic PNECs. Due to their low, even if constant, occurrence in the environment, VMPs are expected to have, more likely, chronic toxic effects rather than acute. Probably, the lack of chronic toxicity data is due to the recent emergence of the problem of these substances in the environment. From the analysis of the VSDB database, from which data on the effects of VMPs were extrapolated, it was evident that chronic toxicity data were absent for the majority of the selected compounds. As a consequence, in almost all cases, PNECs were calculated using the highest assessment factor (1000). In addition, as reported in Section 2, there were insufficient data for calculating PNECs and RQs, particularly for the terrestrial compartment. In this cases the default score values reported in Table 2 were used. Obviously, all these aspects lead to a high degree of uncertainty in the final scores derived for different VMPs and, as a consequence, the obtained results should be carefully evaluated and considered as a prioritization exercise and as an example of the potential usefulness of the proposed approach for managing the environmental risk of VMPs.

RANKVET generates different outputs each one focussed onto a particular aspect of the environmental profile of VMPs. An overview of the functionality of RANKVET will be given in the next paragraphs.

3.2. Ranking of veterinary medicines for their potential impact in soil and surface water systems (single species)

A first application of RANKVET allows the users to identify those veterinary medicines showing the greatest potential to cause environmental effects in aquatic or soil compartments. The scoring system can be applied on different species and considering different farming methods (intensively reared animals or pasture). As

Table 2
RANKVET: Default score values assignment scheme based on the VMPs categories and non-target organisms.

Drug class	Score						
	Aquatic			Terrestrial			
	Algae	*Daphnia*	Fish	Earthworm	Arthropods	Microorganisms	Terrestrial plants
Antibiotics	40	5	5	5	5	40	5
Parasiticides	5	40	10	10	40	5	5
Others	5	5	5	5	5	5	5

an example, in Fig. 1 the results of the ranking of VMPs used on cattle (intensive herds) are reported. The example is referred to the impact of these substances on aquatic systems and has been selected because:

- cattle is the species treated with the largest number of VMPs included in our study;
- the higher availability of toxicity data on aquatic non-target organisms lower the uncertainty about the reliability of the obtained results.

Taking into account the distribution of chemicals within the range of variability of the indicator four class of priority can be considered (<25 negligible risk; 25–50 medium risk; 50–75 high risk; >75very high risk).

From Fig. 1 the following comments can be made:

(a) RANKVET assigned 4 and 2 substances to the priority compounds class "very high" and "high", respectively. These substances include 4 antibiotics (gentamicin, neomycin, chlortetracycline and streptomycin) and 2 endoparasiticides (ivermectin and fenbendazole).
(b) In almost all cases, at least acute toxicity data were available for these compounds. In some cases (ivermectin, streptomycin and chlortetracycline) also chronic toxicity data were present in the VSDB database. The availability of these data lowered the uncertainty associated to the obtained scores.
(c) Among the antimicrobials gentamicin, neomycin, streptomycin belong to the class of aminoglycoside antibiotics. In addition, other compounds of the same chemical class (paromomycin, kanamycin, dihydrostreptomycin) were assigned in the upper part of the medium risk class. In a comparative study of the effects of the commonly used veterinary antibiotics, 11 compounds (including sulfonamides, tetracyclines, aminoglycosides, fluoroquinolones, and beta-lactams) were evaluated for their acute and chronic aquatic toxicities (Park and Choi, 2008). The authors concluded that neomycin, the only aminoglycoside antibiotic included in their study, was the most toxic compound. A comparison of the available data present in the VSDB indicates that the effects of other aminoglycoside compounds on aquatic organisms range in the same order of magnitude as neomycin, indicating the potential hazard of these class of antibiotics to the aquatic systems.
(d) Ivermectin, chlortetracycline and fenbendazole were also ranked in the highest category of risk. These compounds are known to cause environmental pollution and significant amount of data on their environmental fate, behaviour and ecotoxicity is available (Boxall et al., 2006; Garric et al., 2007; Liebig et al., 2010; Daghrir and Drogui, 2013; Carlsson et al., 2013; Park and Choi, 2008; Yu et al., 2014). Finally, in a study of Oh and coworkers (2006) fenbendazole (among the benzimidazolic anthelmintic compounds) was found to be the most acute toxic compound towards *Daphnia magna*. In the same study, authors highlighted that the survival, reproduction, and growth of *D. magna* were significantly delayed in response to chronic exposure to this substance. In addition, Kolar and Kožuh Eržen, 2006 pointed out that even if there are limited data on the degradation of benzimidazoles, some investigations on

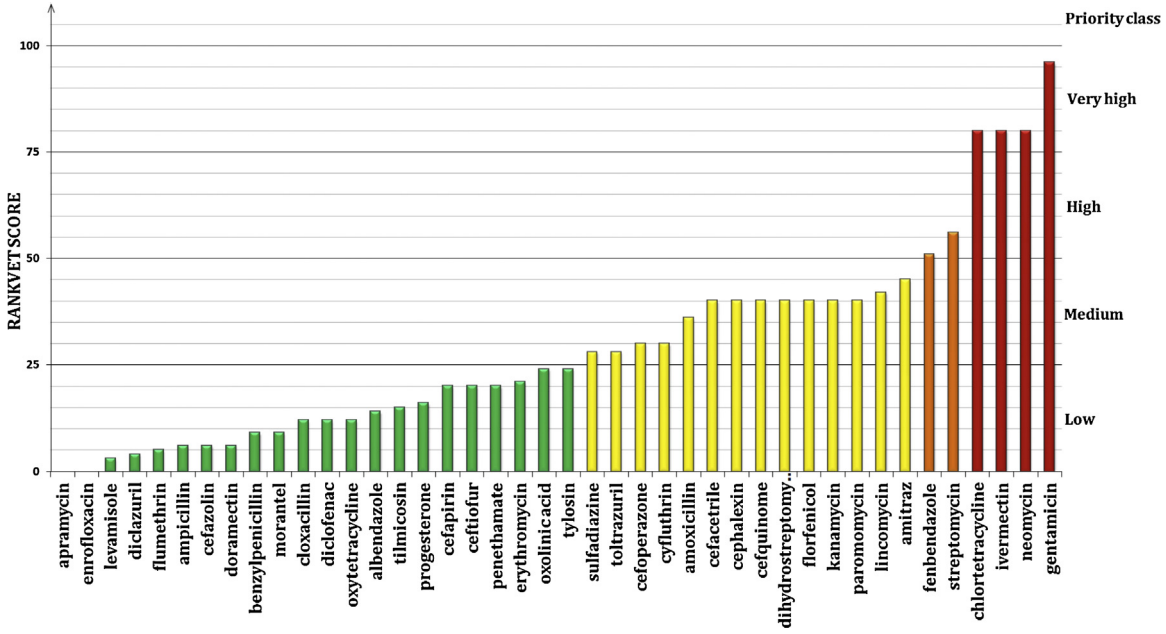

Fig. 1. Risk classification of VMPs for the aquatic compartment. Results are referred to VMPs used in cattle's intensive farming.

the biodegradability of fenbendazole indicate that it degrades slowly indicating probability of exposure for the aquatic environments.

As previously reported, the results in Fig. 1 are referred to risk classification (aquatic systems) for VMPS that are used on intensively reared cattle. However, RANKVET has been also applied for ranking the risk for soil systems and considering different species (farmed intensively or pasture). For the sake of discussion it is not possible to show all the obtained results; however, their analysis suggests that, generally, substances falling in the two highest priority classes are essentially the same independently from the species and the farming practices considered. For this reason, Table 3 summarizes the overall obtained results by grouping all the VMPs included in this study by using their highest class of score. For example, if a compound has a "Medium" class for a species and an "High" class for another one, that substance was considered just in the "High" class. Table 3 reports the VMPs classification both for soil and aquatic systems.

In general, the outcome of RANKVET showed that the top-ranked substances are antibiotics and parasiticides. It is interesting to note that other prioritization studies show a strong analogy with our results. In particular, in the study of Boxall and coworkers (2003b) and Kim and coworkers (2008) VMPs such as apramycin, chlortetracycline, oxytetracycline, diazinon, tylosin, fenbendazole, ivermectin, and toltrazuril were ranked in the highest priority classes. In addition other compounds such as toltrazuril, amitraz and fenbendazole were classified as Group I (highest class of risk) substances.

Some differences appear when the two environmental compartments are taken into account. For instance, the antibiotics apramycin, oxytetracycline and tylosin seem to pose a high risk exclusively to the soil compartment. Apramycin and oxytetracycline belong to the class of aminoglycoside and tetracycline antibiotics. This supports the considerations previously carried out about the environmental risks associated to these class of compounds. The PNECs of apramycin were derived exclusively by no observed effect concentration (NOEC) values present in the VMDB database; therefore, the final score obtained for this substance can be considered reliable, since there was no need to use any default value. On the contrary, due to the lack of ecotoxicity data, the calculation of the scores for oxytetracycline and tylosin required the use of some default values providing, in this way, a risk classification which should be considered with a higher degree of uncertainty.

In general, the number of VMPs present in the lowest class of priority are higher in the aquatic compartment than in the terrestrial one. This mainly depends on the physical chemical properties of the investigated chemicals and particularly from their partition coefficient soil/water (K_d) values. The latter is a measure of how tightly a compound binds or sticks to soil particles (Delle Site, 2001). The greater the K_d value is, the less a chemical will likely leach or contribute to runoff. Most of the VMPs present in this study have high K_d values and great affinity for the soil compartment. Therefore, the calculated PECs for the aquatic compartment are characterized by low levels of exposure for the aquatic non-target organisms.

It is interesting to note that the final scores obtained by the VMPs present in the two top priority classes of risk tend to be higher in the aquatic environment. For instance, the highest score reached by gentamicin in the soil system was 72 (intensively reared cattles) whereas in water the same substance reached a score of 96. However, it is unclear if this is due to a general higher sensitivity of the aquatic organisms to VMPs or to the default values that have been used in the score calculations, particularly for the soil compartment.

3.3. Radar graphs for single drugs on multiple species

The application of VETRANK offers the opportunity to compare the level of risk of single VMPs on different species using radar graphs (for soil and aquatic environments), which can be considered an useful tool to identify appropriate mitigation measures. In

Table 3
RANKVET: risk classification of VMPs in soil and water compartments.

Compartment	Soil				Water			
Priority class	Low	Medium	High	Very high	Low	Medium	High	Very high
Active ingredients	Albendazole Altrenogest Cefapirin Ceftiofur Diclofenac Doramectin Fenbendazole Paromomycin Penethamate Progesterone Tiamulin Trimethoprim	Amoxicillin Amitraz Ampicillin Amprolium Benzylpenicillin Cefacetrile Cefazolin Cefoperazone Cefquinome Cephalexin Cyfluthrin Cloxacillin Diclazuril Dihydrostreptomycin Enrofloxacin Erythromycin Florfenicol Flumethrin Ivermectin Kanamycin Levamisole Lincomycin Morantel Neomycin Oxolinic acid Phoxim Sulfadiazine Tilmicosin	Chlortetracycline Diazinon Gentamicin Oxytetracycline Streptomycin Tylosin Toltrazuril	Apramycin	Albendazole Altrenogest Ampicillin Amprolium Apramycin Benzylpenicillin Cefazolin Ceftiofur Cefapirin Cloxacillin Diclazuril Diclofenac Doramectin Enrofloxacin Flumethrin Levamisole Morantel Oxolinic acid Oxytetracycline Penethamate Progesterone Tiamulin Tilmicosin Tylosin Trimethoprim	Amoxicillin Cefacetrile Cefoperazone Cefquinome Cephalexin Cyfluthrin Diazinon Dihydrostreptomycin Erythromycin Florfenicol Kanamycin Lincomycin Paromomycin Sulfadiazine Toltrazuril	Amitraz Fenbendazole Phoxim	Ivermectin Chlortetracycline Neomycin Gentamicin Streptomycin

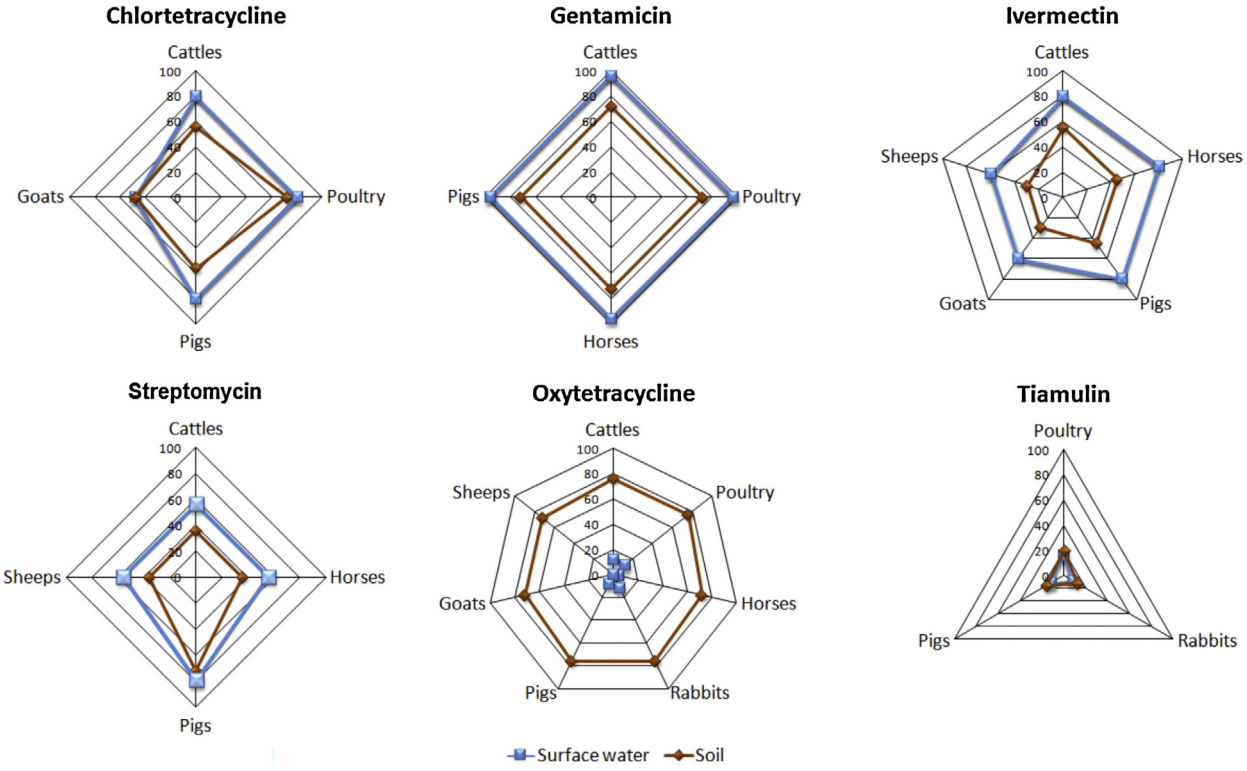

Fig. 2. Radar graphs for single substances and different treated species.

fact, using the graphs such as those reported in Fig. 2 risk managers can easily understand:

(a) the general level of risk of a particular compound and the environmental compartment most at risk. For instance, in Fig. 2 gentamicin seems to be of high concern for both the aquatic and soil environments, whereas tiamulin does not pose any kind of concern. On the contrary, in the case of oxytetracycline the concern is associated exclusively to the soil compartments, whereas for ivermectin the aquatic compartment seems to be most at risk.
(b) the identification of species on which the use of the VMPs compounds gives the higher contribution to their potential environmental impact. For instance, in the case of chlortetracycline reared poultry gives the highest contribution to potential negative impact of this substance in soil systems, whereas in the case of streptomycin the highest contribution is given by pigs.

4. Conclusions

In this work, a new environmental risk indicator (RANKVET) specifically developed for VMPs is proposed. RANKVET is a simple score system fully based on the VICH guidelines which are used for assessing the environmental risk of VMPs in the European authorization process. RANKVET can be applied for aquatic and terrestrial ecosystems and for different types of herds (intensive or pasture) and treated species. Its application could be an useful tool to implement appropriate risk mitigation measures and/or targeted monitoring programmes. To better address its potential use, RANKVET was applied to 48 VMPs largely utilized in Italy. A limitation in the use of RANKVET was due to little or no data availability in the public domain for many VMPs. For this reason, the results obtained in many cases should not be considered real risk classification of VMPs but an example of application, whose validity is related to the data availability. On the other hand, the main objective of this application was to illustrate the methodological approach proposed and the potential usefulness. However, the results of this exercise highlighted aminoglycoside antibiotics (gentamicin, neomycin, streptomycin, apramycin and oxytetracycline) as a high priority class for both the terrestrial and aquatic compartments. Other compounds falling in the highest categories of risk were chlortetracycline and tylosin antibiotics and ivermectin, diazinon and fenbendazole parasiticides. These results show strong similarities with other prioritization schemes present in literature. The application of RANKVET also allows the identification of species for which the use of VMPs generates a greater contribution to the potential environmental impact of these substances. This can contribute to develop more focused risk mitigation measures.

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SUPPORTING INFORMATION

RANKVET: A new ranking method for comparing and prioritizing the environmental risk of veterinary pharmaceuticals

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<u>Supporting information shows:</u> Ecotoxicological data for the aquatic (**Table S1a**) and soil (**Table S1b**) compartments; Soil Organic Carbon-Water Partition Coefficients (Koc) of the studied VMPs (**Table S2**).

Table S1a. Ecotoxicological data for the aquatic compartment (mg L^{-1})

VMPs	EC50 algae	EC50 *Daphnia*	LC50 fish	NOEC algae	NOEC *Daphnia*	NOEC fish
albendazole	-	0.024	-	-	-	-
altrenogest	-	-	-	-	-	-
amitraz	12	0.035	0.74	1	0.002	-
amoxicillin	0.0037	101.6	300	-	-	-
ampicillin	-	1000	1000	1000	-	-
amprolium	160	230	1550	-	-	-
apramycin	100	101.6	300	0.127	-	-
benzylpenicillin	100	-	-	-	-	-
cefacetrile	-	-	-	-	-	-
Cefapirin	-	-	-	-	-	-
Cefazolin	-	-	-	1000	-	-
cefoperazone	-	-	-	-	-	-
cefquinome	-	-	-	-	-	-
ceftiofur	-	-	-	-	-	-
cephalexin	-	-	-	-	-	-
chlortetracycline	3.1	128	0.89	-	-	0.012
cloxacillin	23.4	-	-	-	-	-
cyfluthrin	87.05	0.000025	0.000068	-	0.000015	0.00001
diazinon	15.32	0.01	3.1	1	0.00056	0.7
diclazuril	1.1	0.63	0.58	-	0.16	-
diclofenac	64.8	67	8	0.1	10	0.063
dihydrostreptomycin	0.107	-	-	-	-	-
doramectin	-	0.0001	0.0051	1	0.000025	0.0025
enrofloxacin	4.6	79.9	79.5	-	9.8	18.6
erythromycin	0.02	30.5	349	0.01	0.25	-
fenbendazole	-	0.0165	0.04	-	-	-
florfenicol	-	330	780	2.9	-	10
flumethrin	0.59	0.0027	0.17	-	-	-
gentamicin	-	16.8	0.082	-	-	-
ivermectin	4	0.000025	0.0033	1	3E-10	-
kanamycin	-	-	-	-	-	-
levamisole	-	100	1750	-	-	-
lincomycin	0.07	379.4	980	-	-	-
metaflumizone	0.311	0.331	0.228	-	-	-
morantel	-	40	40	-	-	-
neomycin	-	42.1	80.8	-	0.03	-
oxolinic acid	16	4.6	10	-	0.38	-
oxytetracycline	0.342	102	116	0.183	46.2	2
paromomycin	-	502.8	200	-	-	-
penethamate	-	-	-	-	-	-
phoxim	12.1	0.00081	0.22	0.1	-	-
progesterone	-	-	-	-	0.1	-
streptomycin	0.133	487	180	0.09	32	-
sulphadiazine	7.8	221	103	0.03	8.8	-
tiamulin	0.165	40	5.2	-	5.4	-
tilmicosin	0.35	57.3	851	-	-	-
toltrazuril	4.03	2	0.44	-	-	-
trimethoprim	80.3	123	100	25.5	6	0.157
tylosin	1.38	483	96	0.034	45	-

Table S1b. Ecotoxicological data for soil compartment (mg kg^{-1})

VMPs	LC50 earthworms	LR50 arthropods	LC50 microrg.	EC50 terr. plants	NOEC earthworms	NOEC arthropods	NOEC Microorg.	NOEC terr.plants
albendazole	17.8	-	-	-	-	-	-	-
altrenogest	-	-	-	-	-	-	-	-
amitraz	1000	-	-	-	-	-	-	-
amoxicillin	1000	-	-	-	182	-	-	-
ampicillin	-	-	-	-	-	-	-	-
amprolium	-	-	-	-	-	-	3.06	-
apramycin	-	-	-	-	100	0.77	0.1	160
benzylpenicillin	-	-	-	-	-	-	-	-
cefacetrile	-	-	-	-	-	-	-	-
Cefapirin	-	-	-	-	-	-	-	-
Cefazolin	-	-	-	-	-	-	-	-
cefoperazone	-	-	-	-	-	-	-	-
cefquinome	-	-	-	-	-	-	-	-
ceftiofur	-	-	-	-	-	-	0.25	-
cephalexin	-	-	-	-	-	-	-	-
chlortetracycline	1116	-	-	-	20	-	-	-
cloxacillin	-	-	-	-	-	-	-	-
cyfluthrin	5.5	-	-	-	-	-	-	1.6
diazinon	65	3.03	-	-	0.005	-	-	-
diclazuril	1100	-	-	-	900	-	-	-
diclofenac	-	-	-	-	-	-	-	-
dihydrostreptomycin	-	-	-	-	-	-	-	-
doramectin	1000	12.5	-	-	2	-	40	1.6
enrofloxacin	1000	-	-	125	-	-	-	-
erythromycin	-	-	-	-	-	-	-	-
fenbendazole	1068	-	-	-	56	0.77	1000	36
florfenicol	1000	-	-	-	1.95	-	0.4	-
flumethrin	-	-	-	-	-	-	-	-
gentamicin	-	-	0.066	-	-	0.025	-	-
ivermectin	315	0.036	-	-	2.5	0.3	-	0.56
kanamycin	-	-	-	-	-	-	-	-
levamisole	1000	-	-	-	-	-	-	-
lincomycin	-	-	-	-	1000	-	0.78	-
metaflumizone	500	-	-	-	-	-	0.2	-
morantel	1000	-	-	-	-	-	50	-
neomycin	-	-	-	-	-	-	-	-
oxolinic acid	1000	-	-	-	-	-	-	-
oxytetracycline	1954	5000	-	-	0.1	-	-	-
paromomycin	-	-	-	-	-	-	-	-
penethamate	-	-	-	-	-	-	-	-
phoxim	901.5	-	-	-	-	-	-	-
progesterone	-	-	-	-	-	-	-	-
streptomycin	-	-	-	-	10	10	1000	-
sulphadiazine	984	-	-	28	-	-	-	-
tiamulin	1000	-	-	-	-	-	500	-
tilmicosin	918	-	-	-	-	-	-	-
toltrazuril	52	-	-	-	-	-	-	-
trimethoprim	1017	-	-	-	-	-	-	-
tylosin	4530	5000	-	343	-	-	-	50

Table S2. Soil Organic Carbon-Water Partition Coefficients of the VMPs

VMPs	Koc		VMPs	Koc
albendazole	17650		**fenbendazole**	46
altrenogest	4970		**florfenicol**	38
amitraz	1000		**flumethrin**	4600000
amoxicillin	866		**gentamicin**	10
ampicillin	534.4		**ivermectin**	4000
amprolium	7761		**kanamycin**	10
apramycin	500755		**levamisole**	8652
benzylpenicillin	82.45		**lincomycin**	59
cefacetrile	10		**metaflumizone**	30714
Cefapirin	481.5		**morantel**	5648
Cefazolin	12		**neomycin**	10
cefoperazone	10		**oxolinic acid**	2260
cefquinome	32590		**oxytetracycline**	52875
ceftiofur	3700		**paromomycin**	10
cephalexin	663.4		**penethamate**	3889
chlortetracycline	100.42		**phoxim**	3477
cloxacillin	645.7		**progesterone**	10070
cyfluthrin	64300		**streptomycin**	10
diazinon	609		**sulphadiazine**	81
diclazuril	15370		**tiamulin**	35980
diclofenac	316.23		**tilmicosin**	2800
dihydrostreptomycin	17		**toltrazuril**	17390
doramectin	35900		**trimethoprim**	2835
enrofloxacin	392600		**tylosin**	3744
erythromycin	570			

CHAPTER IV

Toxicity of individual pharmaceuticals and their mixtures to *Aliivibrio fischeri*: Experimental results for single compounds and considerations of their mechanisms of action and potential acute effects on aquatic organisms

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TOXICITY OF INDIVIDUAL PHARMACEUTICALS AND THEIR MIXTURES TO *ALIIVIBRIO FISCHERI*: EXPERIMENTAL RESULTS FOR SINGLE COMPOUNDS AND CONSIDERATIONS OF THEIR MECHANISMS OF ACTION AND POTENTIAL ACUTE EFFECTS ON AQUATIC ORGANISMS

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Abstract: In the first part of a broader study on the effects of individual and multicomponent mixtures of pharmaceutical active compounds, the authors used the Microtox® test system to analyze in detail the effects of 10 widely used human and veterinary pharmaceutical active compounds toward the bioluminescent bacterium *Aliivibrio fischeri*. The experimental results indicated moderate toxicity for the majority of the tested compounds. Comparison between experimental 50% inhibitory concentrations and those predicted from the quantitative structure–activity relationship models indicated that most of the tested pharmaceutical active compounds behave as polar narcotic compounds toward *A. fischeri* (only the antibiotic chlortetracycline seemed to have a specific mechanism of action). A comparison between the experimental results and a collection of acute toxicity data on other nontarget organisms indicated that in general *A. fischeri* has a comparable sensitivity to other aquatic species. However, according to the Globally Harmonized System of Classification and Labelling of Chemicals, the majority of the investigated chemicals can be classified as harmful or nontoxic for aquatic ecosystems. Finally, based on comparisons among the 95th percentile of measured environmental concentrations found in European Union water bodies and acute toxicity data on various aquatic organisms, no risk to aquatic life exists when the tested pharmaceutical active compounds are assessed as individual chemicals. *Environ Toxicol Chem* 2017;36:807–814. © 2016 SETAC

Keywords: *Aliivibrio fischeri* Pharmaceutical Quantitative structure–activity relationship Ecotoxicity Risk

INTRODUCTION

Water contamination by pharmaceutical active compounds has emerged as an environmental issue of concern since the mid-1990s [1]; in fact, in recent decades, more than 100 different drug residues have been detected in effluents from sewage-treatment plants and in groundwater and surface-water systems, with concentrations ranging from nanograms per liter to micrograms per liter [2–4]. The main sources of contamination are related to the use of pharmaceutical active compounds in human and veterinary medicine. Several drugs are excreted unchanged or as metabolites and can enter the environment. For instance, in humans, the quantity remaining unchanged can be up to an average of 70% of the prescribed dose [5]. Consequently, human pharmaceutical active compounds, continuously discharged into domestic wastewater, can reach sewage-treatment plants, where their removal is often incomplete [6–8]. If the drugs and transformation products are not eliminated during sewage treatment, they may enter the aquatic environment and eventually the groundwater [6,9,10]. The same happens with veterinary medicines: Elmund and coworkers [11] reported that 25% to 75% of the prescribed doses of veterinary pharmaceuticals and growth promoters are not metabolized. Direct application in aquaculture, wash-off from topical treatments, livestock waste-treatment plants, and runoff from manure-treated farmlands have been recognized as the most significant entry routes into the environment of these substances [12–15]. Finally, the continuous release of medicines could result in what has been termed "pseudopersistence" in the environment [16].

Regardless of their sources, the occurrence of residues from pharmaceutical active compounds in different environmental compartments raises concern for nontarget organisms because long-term exposure to different active compounds may have potential, often unknown, adverse effects [17,18]. Assessed as single chemicals, most pharmaceutical active compounds seem to pose no or moderate risk to the environment [19] because the effects to nontarget organisms, in general, occur at levels well above the measured environmental concentrations of the chemical. However, notable exceptions are the negative effects on fish reproduction from environmental exposure to estrogenic compounds [20,21], the decline of vulture populations which fed on carcasses of animals recently treated with diclofenac in Pakistan [22], and the inhibition of photosynthesis in algae caused by exposure to β-blockers [23] and fluoxetine [24].

In addition, pharmaceuticals do not occur as single chemicals isolated in environmental compartments. Because a broad number of pharmaceutical active compounds are used simultaneously in human and veterinary medicine, they are present as complex, multicomponent mixtures of compounds [25]. The ecotoxicity of these mixtures can be higher than the effects of the individual components; even in extremely low concentrations that are far below the 10% effective concentration (EC10) of individual chemicals, these mixtures may produce a significant effect [26,27]. The joint effects of these mixtures are basically unknown [28,29]. The present study is part of a broader study that aims to contribute to the knowledge of the effects of the presence of veterinary and human pharmaceutical residues in aquatic environments. Particularly, in the present study, we report the toxicity, which was determined using the Microtox® test system, of 10

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different pharmaceutical active compounds. The effects of their binary and multicomponent mixtures are described in Di Nica et al. [30] in this issue. The Microtox test system is based on bioluminescence inhibition of the marine bacterium *Aliivibrio fischeri* (formerly *Vibrio fischeri* or *Photobacterium phosphoreum*). This test offers ease of application, reproducibility, sensitivity, low cost, and standardization [31]; furthermore, several studies have shown significant correlations among toxicity data from tests of *A. fischeri* and those obtained for other species, including fish, crustaceans, and algae [32,33].

To reach a deeper insight on the mechanism of action of these compounds to *A. fischeri*, in the present study we also calculated the so-called toxicity ratio [34,35]. The toxicity ratio is the ratio between the estimated baseline toxicity (also called "nonpolar narcosis") and the corresponding experimental concentration causing 50% inhibition (IC50). For all compounds, the baseline toxicities were obtained using 2 quantitative structure–activity relationship (QSAR) models specifically developed to predict the toxicity of nonpolar and polar narcotic compounds to *A. fischeri* [36]. For organic pollutants, 4 main mechanisms of action can be distinguished based on the primary processes that cause toxicity [34]. The first, nonpolar narcosis, results from rather inert chemicals, which act via a nonspecific mode of action and mainly depend on the hydrophobicity of the compound. The second main mechanism is polar narcosis. These chemicals also act by narcosis, but they are slightly more toxic than nonpolar narcotics. The third mechanism is aspecific reactivity. Chemicals belonging to this class may exhibit their toxicity through different modes of action and show a pronouncedly enhanced toxicity compared with narcosis. The fourth and final mechanism is specific reactivity. This class consists of toxicants with a specific cellular receptor as a target. For this reason they are extremely toxic. Through the calculation of the toxicity ratio, it was possible to classify the studied pharmaceutical active compounds.

Finally, in the present study, a detailed analysis was performed on data from the literature that report measured levels (measured environmental concentrations) of the investigated pharmaceutical active compounds in aquatic systems of the European Union. The calculated 95th percentile of measured environmental concentrations were then compared with the acute toxicity data to obtain some general indications about the realistic risk related to the presence of residues of the studied compounds in European Union water bodies.

MATERIALS AND METHODS

Chemicals

Test compounds were chosen to represent different classes of widely used veterinary and human pharmaceutical active compounds (Table 1). Chemicals were purchased in the highest available purity from Sigma-Aldrich Laboratories. In Table 1, the Chemical Abstracts Service number together with some relevant physical–chemical properties are reported for each chemical.

The solutions of the tested chemicals were prepared according to the method reported by Vighi and coworkers [36]. Briefly, stock solutions of amoxicillin, chlortetracycline, diclofenac sodium, acetylsalicylic acid, and caffeine were prepared directly in saline water solutions (2% sodium chloride [NaCl]). Because the other investigated pharmaceutical active compounds exhibit low water solubility (Table 1), stock solutions were prepared in dimethyl sulfoxide (DMSO; sulfamethizole, furosemide, carbamazepine, bezafibrate, ibuprofen). However, in the final test samples, the DMSO was always below 1% (v/v). Dong and coworkers [37] highlighted that in an *A. fischeri* toxicity test, at this level of concentration, the influence of DMSO for low–water solubility compounds is negligible.

Final solutions were buffered with 0.1 M sodium phosphate buffer, and pH was adjusted with the aid of a pH meter (model 250; Denver Instrument) in the range of 6 to 8.50 (DIN EN ISO 11348-3). For all tests, we refer to the nominal (= initial) concentrations of pharmaceutical active compounds.

All the tested pharmaceutical active compounds are ionizable and can dissociate to various extents as a function of pH and their dissociation constants (pK_a). The parent and ionic forms can differ in biological effects and physical chemical properties quite a bit. The hydrophobicity of uncharged compounds is generally expressed in terms of *n*-octanol–water partition coefficient ($\log K_{OW}$) values. However, for ionizing chemicals, the term $\log D_{OW}$ (for distribution) is preferred because the distribution between the 2 phases is strongly dependent on the experimental pH conditions used in the determination of the partition coefficient. Note that the $\log K_{OW}$ is a ratio at the equilibrium of the concentrations of the non-ionized compound between 2 different phases, whereas the $\log D_{OW}$ is the ratio of the sum of the concentrations for both forms of the compound (ionized and non-ionized). For un-ionizable compounds, $\log K_{OW}$ is equal to $\log D_{OW}$. In the present study, only experimental $\log D_{OW}$ values determined at pH approximately 7.0 to 7.5 (in parentheses in Table 1) were considered.

Table 1. Relevant physical chemical properties of the tested compounds

Pharmaceutical groups	Compounds	CAS No.	Water solubility ($mg\,L^{-1}$)	pK_a	Log D_{OW} (pH)
Antibiotic	Amoxicillin	26787-78-0	3430 [68]	2.4, 7.4, 9.6 [69]	–1.17 (7.4) [70]
	Chlortetracycline	64-72-2	630 [71]	3.3, 7.4, 9.3 [72]	–0.80 (7.0) [70]
	Sulfamethizole	144-82-1	1050 [71]	2.1, 5.3 [73]	–1.11 (7.5) [70]
Anti-inflammatory	Acetylsalycilic acid	50-78-2	4600 [71]	3.49 [74]	1.07 (7.4) [70]
	Diclofenac sodium	15-307-79-6	2425 [45]	4.15 [75]	1.3 (7.4) [76]
	Ibuprofen	15687-27-1	21 [71]	4.42 [77]	1.44 (7.4) [76]
Diuretic	Furosemide	54-31-9	73.1 [71]	3.8 [78]	–0.24 (7.4) [76]
Anticonvulsant	Carbamazepine	298-46-4	112 [79]	13.9 [58]	2.45 (7.4) [80]
Hypolipidemic	Bezafibrate	41859-67-0	0.355 [79]	3.6 [81]	–0.11 (7.4) [83]
Central nervous system stimulant	Caffeine	58-08-2	21600 [71]	14 [82]	–0.10 (7.4) [70]

CAS = Chemical Abstracts Service; D_{OW} = octanol–water distribution constant; pK_a = dissociation constant.

Toxicity test for single chemicals

Toxicity tests were carried out in duplicate on a control and 9 different concentrations of stock solutions from a serial dilution in 2% NaCl solution (20 °C). Each test was performed at least 3 times.

The total concentrations varied systematically with an adequate factor of dilution for describing a complete concentration–response relation.

Testing procedure

In accordance with the test conditions and operating protocol of the Microtox system operating manual [38], toxicity tests were carried out using bioluminescent bacterium *A. fischeri* after short-term exposure to toxic compounds. Luminescence inhibition was measured using a Microtox model 500 analyzer in the acute mode.

The freeze-dried bacterium and the reconstituent solution were purchased from Ecotox LDS. Bacteria were exposed to the compounds for 5 min and 15 min. The temperature during the exposure was 15 °C according to the Microtox standard procedure. For the final analysis, only the data from the 15-min exposure were reported because of the negligible difference in toxicity between results from different durations of exposure. The decrease of bacterial light production with respect to the control reflects the toxic effect (IC*x*) of the chemicals.

Data analysis

The observed single-compound concentration–response data were fitted to a nonlinear regression Weibull model (Equation 1) using R software [39,40]

$$I = (c, \beta) = 1 - \exp\{-\exp\{\ln(\beta_2(\ln(c) - \ln(\beta_1))\}\} \quad (1)$$

where I is the response in terms of inhibition of luminescence, β_1 and β_2 are the parameters of the Weibull model, and c is the experimental concentration of the single chemical.

Measured environmental concentrations in European Union water bodies

An extensive literature search (limited to the last decade) was carried out to characterize the occurrence of the investigated pharmaceutical active compounds in European water bodies. The 95th percentile of the values of the measured environmental concentrations was then calculated (Supplemental Data, Table S1).

RESULTS AND DISCUSSION

Toxicity of individual compounds to A. fischeri

Table 2 shows the individual effect concentrations (IC10 and IC50 values) and the 95% confidence limits of the investigated pharmaceutical active compounds in the *A. fischeri* bioluminescence inhibition test (15 min). In Table 2, the substances are reported in decreasing order of toxicity (IC50). In the same table (Table 2), the slopes of the concentration–response curves (Weibull function) are also summarized. The corresponding concentration–response functions with regard to the curve, shape, and position are visualized in Figure 1.

The IC50 values span approximately 3 orders of magnitude, ranging from 12.1 mg L^{-1} for chlortetracycline to >1702 mg L^{-1} for amoxicillin. For amoxicillin, application of the Weibull function resulted in an estimated IC50 of 2845 mg L^{-1}. However, because this value is above the maximum tested concentration, in Table 2 an IC50 >1702 mg L^{-1} (the maximum tested concentration) is reported.

Among antibiotics, chlortetracycline showed the highest toxicity to *A. fischeri*, followed by sulfamethizole and amoxicillin. The results are in agreement with previously reported data. For chlortetracycline and amoxicillin, Park and Choi [41] reported an IC50 of 13 mg L^{-1} and 3597 mg L^{-1}, respectively (for the latter the reported value is very close to that predicted by our Weibull model). For sulfamethizole, Białk-Bielinska and coworkers [42] did not reach the IC50 up to the tested concentration (100 mg L^{-1}). Except for acetylsalicylic acid, the anti-inflammatory drugs seem to exert a similar degree of toxicity to *A. fischeri* (toxicity range 13.77–15.9 mg L^{-1}). The present findings are largely in agreement with data from the literature. In a bioluminescence inhibition test using *A. fischeri* to assess the toxicity of ibuprofen, Farrè and coworkers [43] reported an IC50 of 19.1 mg L^{-1}, whereas Ra and coworkers [44] found a value of 37.5 mg L^{-1}. For diclofenac, Ferrari and coworkers [45] reported an IC50 of 11.45 mg L^{-1}, whereas Farrè and coworkers [43] and Ra and coworkers [44] reported IC50s of 13.7 mg L^{-1} and 9.7 mg L^{-1}, respectively.

Finally, for acetylsalicylic acid, the present findings are on the same order of magnitude of those reported by de García and coworkers (133.59 mg L^{-1}) [46], whereas Calleja and coworkers reported a value of 26.12 mg L^{-1} [47]. However, in the latter study, the authors did not buffer the solution used for the Microtox test, and consequently their results are not directly comparable.

The other tested pharmaceutical active compounds can be classified as moderately toxic (carbamazepine and furosemide) or nontoxic (bezafibrate and caffeine) to *A. fischeri*. In the vast majority of cases, data from the literature are in agreement with the present results. Jos and coworkers [48] and Kim and coworkers [49] reported IC50 values of 78.44 mg L^{-1} and 52.5 mg L^{-1} for carbamazepine; in addition, an IC50 of 672 mg L^{-1} was reported for caffeine [46], whereas for bezafibrate Rosal and coworkers [50] and Rodea-Palomares and coworkers [51] reported IC50 values of 179 mg L^{-1} and

Table 2. Toxicity data for the tested chemicals with *Aliivibrio fischeri* (milligrams per liter, 15 min)

Compounds	IC10	IC50	Slope (IC80/IC20)
Chlortetracycline	2.0 (1.49–2.61)	12.1 (10.94–13.18)	6.4
Diclofenac sodium	5.9 (5.03–6.74)	15.9 (14.59–17.26)	5.1
Ibuprofen	1.4 (0.99–1.72)	18.3 (15.96–20.58)	15.3
Furosemide	7.5 (4.78–10.18)	33.2 (23.23–43.18)	11.6
Carbamazepine	4.2 (2.85–5.47)	94.0 (82.03–105.91)	26.3
Bezafibrate	15.2 (12.3–18.1)	150.7 (135.0–166.4)	11.1
Acetylsalycilic acid	45.8 (40.70–50.8)	267.6 (255.2–279.9)	6.3
Sulfamethizole	90.4 (73.2–107.5)	581.4 (530.4–632.5)	7.0
Caffeine	24.5(17.9–31.1)	632.0 (569.1–694.7)	30.2
Amoxicillin	717.6 (537.5–897.7)	>1702	7.3

C*x* = concentration causing *x*% inhibition.

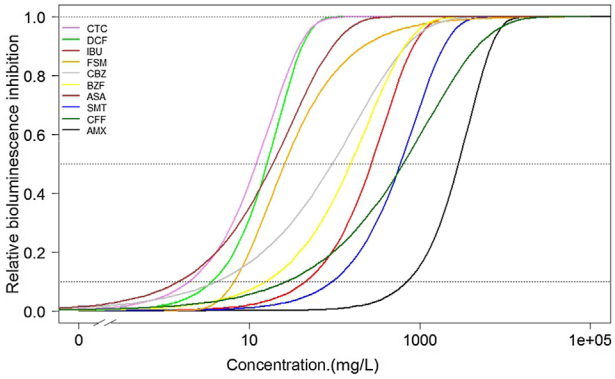

Figure 1. Concentration–response curves (Weibull) for the investigated pharmaceutical active compounds. Identity of individual compounds and the corresponding slopes are given in Table 2 in order of increasing 50% inhibitory concentration values. CTC = chlortetracycline; DCF = diclofenac sodium; IBU = ibuprofen; FSM = furosemide; CBZ = carbamazepine; BZF = bezafibrate; ASA = acetylsalycilic acid; SMT = sulfamethizole; CFF = caffeine; AMX = amoxicillin.

150 mg L^{-1}, respectively. The present results, however, are quite different from those reported in the literature for furosemide. For this substance, Isidori and coworkers [52] reported an IC50 of >200 mg L^{-1}; however, also in this case the authors did not buffer the test solution, and thus, their results are not comparable.

The concentration–response curves obtained for all the tested substances are reported in Supplemental Data, Figure S1. An overview of the obtained results is shown in Figure 1. For almost all chemicals, the shapes of the concentration–response curves (Figure 1) appear rather similar, but they are not strictly parallel. This can also be observed in the slopes (ratio EC80/EC20) reported in Table 2. In some cases, the difference in slopes led to an intersection between the curves. For instance, at lower doses (IC10), ibuprofen, carbamazepine, and caffeine become more toxic than other compounds. According to Backhaus and coworkers [53], the differences in shape and slope may be interpreted in terms of the different toxicokinetic properties, but they may also indicate different additional binding to nonspecific sites.

Mode of action toward A. fischeri

Recently, Vighi et al. [36] developed simple QSAR equations to predict the effect of narcotic and polar narcotic compounds on *A. fischeri*

$$\mathrm{Log}1/C_{\mathrm{nar\ cotic}}\ (\mathrm{mmol\ L^{-1}}) = 0.94 \log K_{\mathrm{OW}} - 2.61 \quad (n = 23,\ R^2 = 0.92) \qquad (2)$$

$$\mathrm{Log}1/C_{\mathrm{polarnar\ cotic}}\ (\mathrm{mmol\ L^{-1}}) = 0.502\ \log K_{\mathrm{OW}} + 0.294\ (n = 23,\ R^2 = 0.81) \qquad (3)$$

Both equations are based on the hydrophobicity of the compounds (expressed as log K_{OW}). As described in *Materials and Methods*, the partitioning behavior between *n*-octanol and water for ionisable compounds is better described by log D_{OW}. Because the majority of the selected pharmaceutical active compounds were ionizable, we considered the values of log D_{OW} measured at pH as close as possible to the pH of the test solutions used to perform the Microtox tests (Table 1). This made it possible to directly compare the experimental obtained IC50 values and those predicted by the use of QSAR models.

The application of Equation 2 to the studied compounds allowed us to calculate the so-called baseline toxicity, which is the minimal toxic effect exerted by a chemical (also named "nonpolar narcosis") and the toxicity ratio ($TR = IC50_{narcotic}/IC50_{experimental}$) [34,35,54]. Because of the absence of specific mechanisms of toxicity, the toxic potency of a narcotic chemical is strongly related to its hydrophobicity; and this forms the basis of several QSAR models, such as those proposed by Vighi and coworkers [36]. If the obtained toxicity ratio approximates 1, then the compound can be considered to show no specific mode of action (inert chemicals). Analogously, the use of Equation 3 and the calculation of a different toxicity ratio ($TR_1 = IC50_{polarnarcotic}/IC50_{experimental}$) allows the classification of those compounds showing polar narcotic behavior. Polar narcotic compounds, also called "less inert" chemicals, are slightly more toxic than chemicals with baseline toxicity. The results obtained using Equations 2 and 3 and the calculated toxicity ratios are reported in Table 3.

Based on the results, almost all the studied pharmaceutical active compounds are polar narcotic compounds to *A. fischeri*. In fact, in 6 cases (acetylsalicylic acid, ibuprofen, diclofenac sodium, sulfamethizole, bezafibrate, and furosemide) the TR_1 was very close to the ideal ratio of 1. In addition, in another 2 cases (caffeine and amoxicillin) the TR_1 was slightly less than 1. On the contrary, compared with its baseline toxicity, the antibiotic chlortetracycline seemed to show an enhanced toxicity to *A. fischeri*, whereas the toxicity ratio of 5.1 obtained for carbamazepine indicated a narcotic-type behavior for this compound.

Our results are in agreement with previous studies on other aquatic organisms. For instance, Sanderson and Thomsen [55] concluded that for the vast majority of pharmaceutical active compounds the acute mode of action was nonspecific narcosis. According to these authors, 85% of the 59 pharmaceutical active compounds examined had a toxicity ratio of <7, and the majority of the log EC50–log K_{OW} regression slopes (–0.49 to –0.86) for the pharmaceutical active compounds were within the range of the universal narcosis slopes. In addition, Cleuvers [28,29], by comparing the experimental toxicity data obtained in *Daphnia* and algae with those predicted by the application of 2 QSAR models for narcosis, demonstrated that carbamazepine, diclofenac sodium, ibuprofen, and acetylsalicylic acid act as narcotic-type compounds to both organisms. Finally, Halling-Sørensen [56] found that chlortetracycline was highly toxic to *Microcystis aeruginosa* and, in general, to

Table 3. Quantitative structure–activity relationship prediction of the IC50 to *Aliivibrio fischeri* using Equations 2 and 3 and the relative toxicity ratios (TR and TR_1)[a]

Compounds	$IC50_{narcotic}$ (Equation 2) (mg L^{-1})	TR	$IC50_{polarnarcotic}$ (Equation 3) (mg L^{-1})	TR_1
Carbamazepine	479	5.1	7.1	0.08
Caffeine	98 226	155	111	0.18
Ibuprofen	3722	270	19.8	1.44
Diclofenac sodium	7773	488	36	2.26
Bezafibrate	187 022	1241	209	1.39
Amoxicillin	3 995 585	1404	1076	0.38
Sulfamethizole	1 217 010	2093	496	0.85
Acetylsalycilic acid	884 420	3306	346	1.29
Furosemide	226 516	6821	222	6.7
Chlortetracycline	1 186 025	98 344	660	54.7

[a]Compounds are reported in increasing order of toxicity ratio.
IC50 = concentration causing 50% inhibition.

cyanobacteria through the inhibition of protein synthesis in prokaryotes, indicating a specific mechanism of action.

Potential harmful effects on aquatic organisms

The potential risk of a substance to the environment is often characterized by calculating the so-called risk quotient, which is the ratio between the predicted environmental concentration and the predicted no-effect concentration (PNEC). Predicted environmental concentrations are estimated using calculation methods that allow the generation of likely concentrations in surface waters or in other environmental compartments [57–60]. Alternatively, when monitoring data are available, measured environmental concentrations instead of predicted environmental concentrations can be utilized to calculate the risk quotient [61]. Predicted no-effect concentrations are derived from the acute and chronic toxicity data by using an appropriate assessment factor [62]. For instance, according to the European Medicines Agency directives for pharmaceutical active compounds [63,64], the assessment factor to be applied to acute toxicity data is 1000; this is considered a conservative and protective approach and is applied in the presence of limited available information about the effect on nontarget organisms.

An overview of the acute toxicity data on algae (or cyanobacteria), invertebrates, and fish found in the literature is summarized in Supplemental Data, Table S1. In the same table, values for measured environmental concentrations corresponding to the 95th percentile of the concentrations measured in European surface water bodies are also reported.

The data reported in Supplemental Data, Table S1, were also plotted in Figure 2 (log scale). Using a worst-case assumption, only the lowest available toxicity data were considered (highest effect). Figure 2 also includes the IC50 obtained in the present study for *A. fischeri* (Table 2) and the Globally Harmonized System of Classification and Labelling of Chemicals [65]. The latter is an internationally agreed-on system, created by the United Nations (2013), to address classification of chemicals by types of hazard and to propose harmonized hazard communication elements, including labels and safety data sheets. According to this scheme, compounds can be classified as highly toxic (acute category I: 50% lethal/effective concentration [L(E)C50] $\leq 1\,mg\,L^{-1}$), toxic (acute category II: $1\,mg\,L^{-1} < L(E)C50 \leq 10\,mg\,L^{-1}$), or harmful (acute category III: $10\,mg\,L^{-1} < L(E)C50 \leq 100\,mg\,L^{-1}$) to aquatic organisms. Some regulatory systems also consider a further category (not toxic) for compounds showing an L(E)C50 $>100\,mg\,L^{-1}$.

From Figure 2 the following 2 situations should be considered. For the first situation, according to the Globally Harmonized System of Classification and Labelling of Chemicals scheme [65], in almost all cases, the investigated chemicals can be classified as harmful or nontoxic for aquatic ecosystems. Only sulfamethizole and chlortetracycline fall in the categories of toxic and highly toxic compounds, respectively. However, it should be taken into consideration that the EC50 ($5\,mg\,L^{-1}$) reported for sulfamethizole in *Daphnia* was estimated using a QSAR model (ECOSAR software). Consequently, the ecotoxicity of sulfamethizole toward this organism could be overestimated. In contrast, as previously reported, chlortetracycline has a specific mode of action on algae and particularly on cyanobacteria and, consequently, is highly toxic to these organisms. For the second situation, the relatively low toxicity and the small differences in sensitivity among the species seem to indicate that these substances act as narcotic-type compounds toward aquatic organisms. In addition, the comparability of toxicity data between the IC50 values obtained using the luminescent bacteria test and the other aquatic bioassays confirms those previously reported by Kaiser [32], who investigated the usefulness of this test to obtain preliminary information on compounds for which little is known about their toxicity on aquatic organisms. In addition, this test may be performed with relative ease and speed and at a limited cost, which is an important consideration. However, in the present study, we provide evidence that this approach works exclusively with narcotic-type compounds or when no specific mechanisms of action are present.

From the comparison of the few available acute toxicity data (even the IC10 for *A. fischeri* reported in Table 2) with the corresponding measured environmental concentration values (95th percentile), it appears evident that the latter are 3 to 6 orders of magnitude lower, giving an indication that their realistic concentrations should not pose unacceptable acute risks to aquatic organisms (in European Union water bodies). In fact, the highest measured environmental concentration/PNEC ratio (using an assessment factor of 1000 to calculate the PNECs) was obtained for chlortetracycline (risk quotient = 0.2). However,

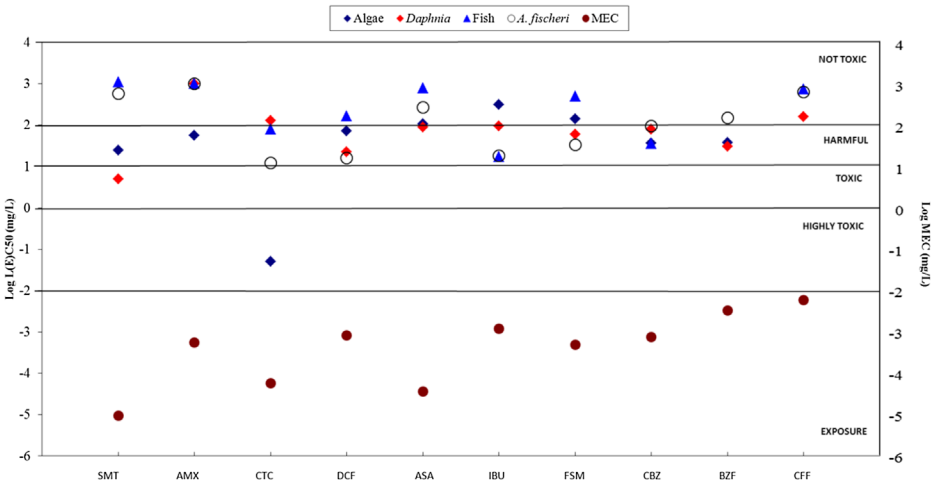

Figure 2. Acute ecotoxicity data, relative Globally Harmonized System of Classification and Labelling of Chemicals [65] classification, and measured environmental concentrations (95th percentile) in European Union water bodies of the considered pharmaceutical active compounds. L(E)C50 = 50% lethal and effective concentrations; MEC = measured environmental concentration.

our results should be considered according to the context; in fact, at the local scale, the situation could be different. For instance, Bouissou-Schurtz and coworkers [66] found a measured environmental concentration/PNEC ratio >1 for ibuprofen. In their study, the measured environmental concentrations referred to concentrations of ibuprofen measured in a national survey in French water bodies, and the calculated PNEC was considerably lower than that calculated in the present study. However, the measured environmental concentrations utilized in the present study are based on the few available monitoring data present in the literature. Consequently, these examples may not be representative of the general conditions of European Union water bodies. The lack of measured environmental data can hinder understanding of the real ecological consequences of the presence of residues of pharmaceutical active compounds in the environment. Therefore, to estimate the potential ecological risk of pharmaceutical active compounds, further monitoring studies are needed. Finally, even if environmental concentrations are much lower than the known L(E)C50, there is evidence that important adverse effects can occur under certain circumstances, as observed in the case of the collapse of vulture populations on the Indian subcontinent. A lack of knowledge exists about the potential long-term ecotoxicological effects for many pharmaceuticals, particularly with respect to potential disturbances in hormonal homeostasis (endocrine disruption), immunological status, or gene activation and silencing during long-term exposure [17]. Finally, it is important to note that pharmaceuticals do not occur as pure isolated substances in aquatic environments. Because a broad range of different substances is used simultaneously in humans and other animals, in any given area these substances are present as multicomponent combinations (mixtures). Thus, ignoring the combined effects of mixtures of pharmaceutical active compounds and other contaminants, potentially present in the environment, could severely underestimate the real environmental risk posed by them because this may be more than a factor of 1000 higher than the risk quotient of a single random pharmaceutical [67]. In addition, mixtures of substances could produce a stronger ecotoxicological effect, including synergistic ones.

CONCLUSION

The toxicity toward *A. fischeri* of 10 pharmaceutical active compounds was determined, and the obtained IC50 values extended approximately 3 orders of magnitude. Comparison of the experimental results with those obtained from 2 QSAR equations, specifically developed for *A. fischeri*, indicated that almost all of the selected compounds act as polar narcotics. Only chlortetracycline seems to show an enhanced toxicity compared with its baseline toxicity, whereas for carbamazepine the results indicated a narcotic-type behavior to this organism.

The present experimental results, together with a collection of acute toxicity data on other nontarget organisms, allowed us to identify that *A. fischeri* has a comparable sensitivity to other aquatic species at least for short-term exposure conditions and for compounds that act as narcotics. In addition, the majority of the studied compounds are at least harmful to aquatic organisms according to the Globally Harmonized System of Classification and Labelling of Chemicals [65] (chlortetracycline was classified as highly toxic).

Finally, the measured environmental concentration/PNEC ratio gave an indication that the residue levels found in European Union water bodies are sufficiently low that no acute risk to aquatic organisms is suspected to occur. However, because pharmaceutical active compounds are normally present as mixtures in aquatic environments, further studies are needed to evaluate their potential joint effects. The results of the mixture effects assessment of the different combinations of the tested pharmaceutical active compounds to *A. fischeri* are presented in Di Nica et al. [30] in this issue.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3568.

Data availability—Data, associated metadata, and calculation tools are available from the corresponding author (v.dinica@campus.unimib.it).

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SUPPORTING INFORMATION

Toxicity of individual pharmaceuticals and their mixtures to *Aliivibrio fischeri*. Part I: experimental results for single compounds, considerations of their mechanisms of action and potential acute effects on aquatic organisms

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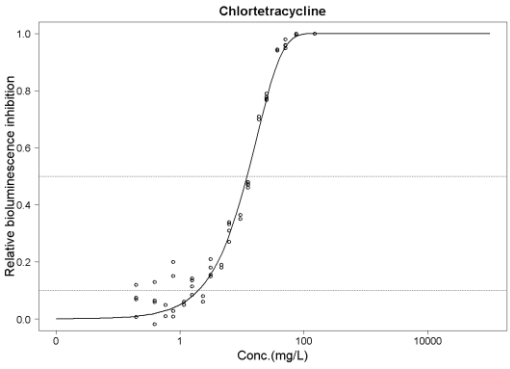
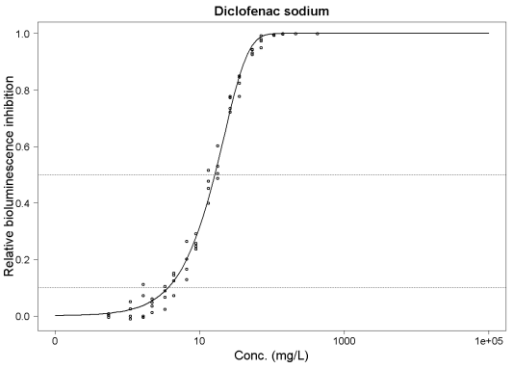
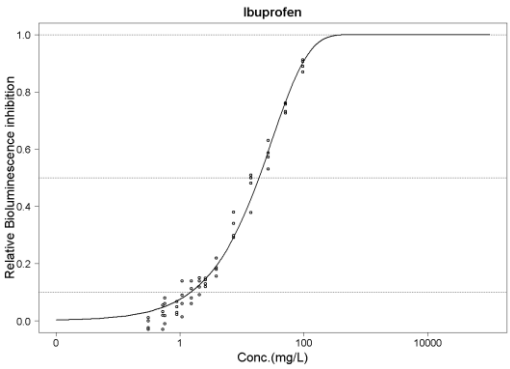
Contents

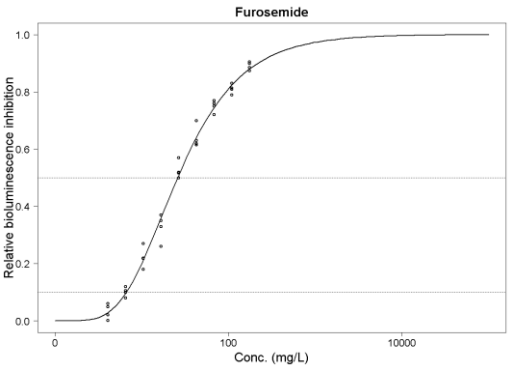
Supporting Information shows: Concentration-response curves (Weibull) for the investigated pharmaceutical active compounds (PhACs) with the experimental points. **(Figure S1)**; available data for the acute toxicity of the investigated substances to different trophic levels in aquatic systems and for the MECs (95^{th} percentile) in the EU water bodies (**Table S1)**.

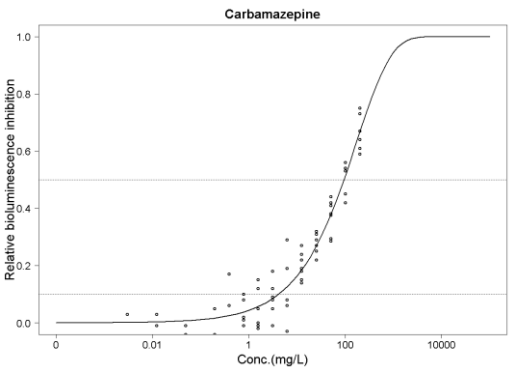
Abbreviations: AMX, amoxicillin; ASA, acetylsalycilic acid; BZF, bezafibrate; CBZ, carbamazepine; CFF, caffeine; CTC, chlortetracycline; DCF, diclofenac sodium; FSM, furosemide; IBU, ibuprofen; SMT, sulfamethizole;

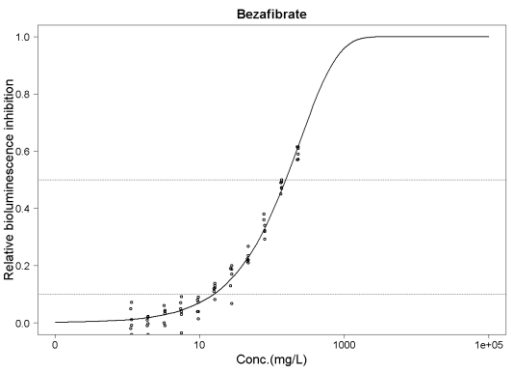

Chlortetracycline
Relative bioluminescence inhibition
Conc.(mg/L)

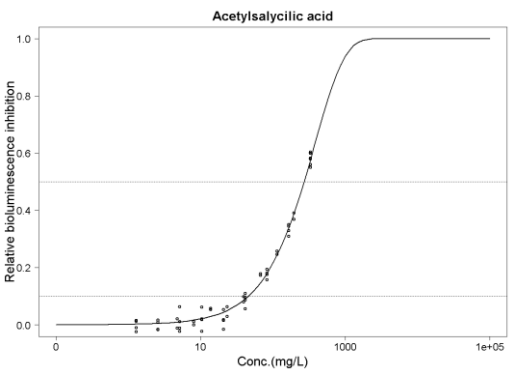

Diclofenac sodium
Relative bioluminescence inhibition
Conc. (mg/L)

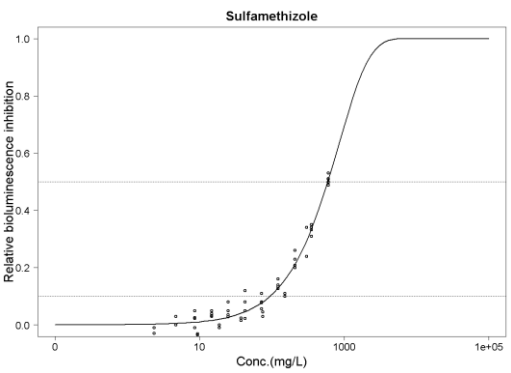

Ibuprofen
Relative Bioluminescence inhibition
Conc.(mg/L)


Furosemide
Relative bioluminescence inhibition
Conc. (mg/L)


Carbamazepine
Relative bioluminescence inhibition
Conc.(mg/L)


Bezafibrate
Relative bioluminescence inhibition
Conc.(mg/L)


Acetylsalycilic acid
Relative bioluminescence inhibition
Conc.(mg/L)


Sulfamethizole
Relative bioluminescence inhibition
Conc.(mg/L)

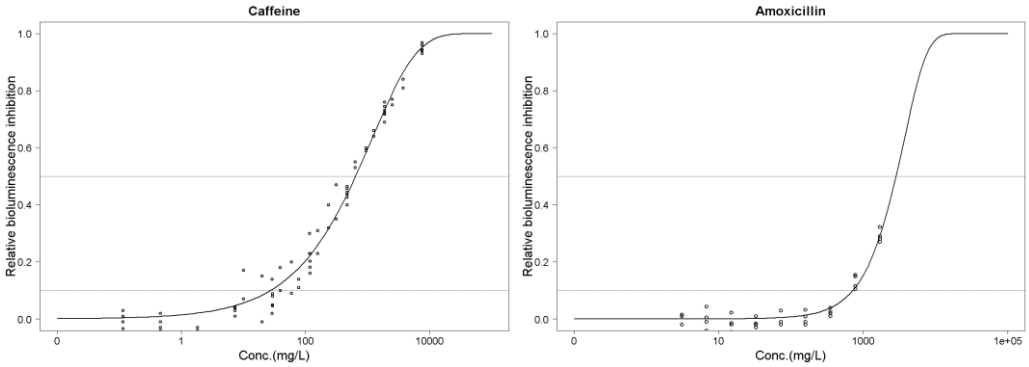


Figure S1. Experimental concentration-response curves (solid line: Weibull fit to the data) for the investigated PhACs.

Table S1. Summary of the acute toxicity of the investigated substances to different trophic levels in aquatic systems (algae or cyanobacteria, *Daphnia*, fish) and MECs (95th percentile) in the EU water bodies. (* = predicted toxicity values by QSAR studies; ** = data referred to cyanobacteria)

Compounds	**EC_{50} Algae (mg L^{-1})**	**EC_{50} *Daphnia* (mg L^{-1})**	**LC_{50} Fish (mg L^{-1})**	**MEC (mg L-1)**	**Monitoring sites**	**Ref. MEC values**
SMT	24.94[1], 60[2-3]*	5.0[2-3]*	1113[2-3]*	9.5E^{-06}	Spain	[4-8]
AMX	56.3[9]**, >1500[9]	>1000[10]	>1000[10]	5.65E^{-04}	Italy, UK, France, Poland	[11-17]
CTC	0.050**[18], 3.1[18], 1.8[19]	127.43[20], 153.04[20], 380[10], 225 [10]	78.9[10]	5.7E^{-05}	France, Spain	[17, 21-23]
DCF	72[24], 71.9[25], 68[25]	22.43[26], 68[24, 25], 60.7[27], 39.9[28], 44.7[28], 22.70[26], 142.6[27]	166.6[29]	8.29E^{-04}	UK, Spain, Sweden, Greece, France, Italy, Finland	[8, 11, 12, 21-23, 30-46]
ASA	106.7[25]	88.1[25], 1293.05[47], 647.31[47], 167.91[48]	796.00[2-3]*	3.7E^{-05}	UK, France, Romanie	[11, 12, 35, 41, 49]
IBU	315[24], 324[25]	108[24], 132.6[50], 94.9[25]	17.1[51]	1.2E^{-03}	Italy, UK, Spain, Sweden, Greece, Romanie, France	[11-13, 15, 21-23, 31-33, 35, 36, 38-42, 44-46, 49, 52]
FSM	142[53]	84.09[54], 60.62[54]	166[2-3]*, 497 [53]	4.94E^{-04}	Italy, UK, Spain	[11, 12, 15, 21-23, 34, 36, 37
CBZ	36.62[55], 110.93[55], 74[24]	77.7[26, 56], 112.22[55]	86.5[57], 35.4[54]	7.6E^{-04}	Italy, UK, Poland; Sweden, Romanie, France, Greece, Finland	12, 13, 16, 21-23, 31-33, 35-41, 43, 45, 46, 49, 58]
BZF	37.28[59]	100.08[54];30.3[60];240.40[59]; 75.79[54]	5.30[2-3]*	3.3E^{-03}	Italy; UK; Spain; France; Finland	[12, 15, 21, 22, 31, 33, 35-38, 40, 45, 46]
CFF	46.00[2-3]*	159.62[51]	735.98[61] 87[62]	5.9E^{-03}	Spain; Romanie; Greece; Sweden; France	[32, 40, 41, 43, 49, 63]

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CHAPTER V

Toxicity of individual pharmaceuticals and their mixtures to *Aliivibrio fischeri*: Evidence of toxicological interactions in binary combinations

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TOXICITY OF INDIVIDUAL PHARMACEUTICALS AND THEIR MIXTURES TO *ALIIVIBRIO FISCHERI*: EVIDENCE OF TOXICOLOGICAL INTERACTIONS IN BINARY COMBINATIONS

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Abstract: The combined toxicities of binary mixtures of veterinary pharmaceutical active compounds were examined using the bioluminescent bacterium *Aliivibrio fischeri* as a test organism (Microtox® test). Mixtures were prepared at an equitoxic ratio that corresponded to the inhibitory concentration, 10% (IC10) of individual pharmaceutical active compounds. In addition, the toxicity was determined of a multicomponent mixture that contained all of the investigated pharmaceutical active compounds mixed at a ratio corresponding to their individual predicted no-effect concentration (PNEC) values. The experimental results were successively compared with those obtained by applying the 2 most widely used models for predicting mixture toxicity, the concentration addition (CA) and independent action (IA) models. Although the toxicity of the multicomponent mixture tested was well predicted by the CA and IA models, deviations from the model predictions were found for almost all of the binary mixtures. The deviations from the CA and IA models were greater at lower concentrations, particularly when diclofenac sodium and amoxicillin were present in the mixture. Based on these results, another hypothesis was tested, that of toxicological interactions occurring in binary mixtures (in the direction of synergistic or antagonistic effects), by applying the combination index method, which allowed for computerized quantification of synergism, the additive effect and antagonism. The application of this method confirmed, for at least half of the binary combinations, the clear presence of synergistic deviations at the lowest tested concentrations, with a tendency toward antagonism at the higher ones. In 1 case, a relevant antagonistic interaction was observed. *Environ Toxicol Chem* 2017;36:815–822. © 2016 SETAC

Keywords: *Aliivibrio fischeri* Mixture toxicity Veterinary pharmaceuticals Combination index Synergism

INTRODUCTION

Organisms are typically exposed to a cocktail of chemicals rather than 1 individual substance. This is particularly true in surface water systems, where monitoring studies have generally detected a multitude of heterogeneous and potentially toxic substances [1–4]. Hence, the risk posed by the presence of mixtures in environmental compartments should be evaluated. During the last decade, the European Commission became aware of the problem of chemical mixtures in the environment [5] and defined the challenges that require scientific support in its communication on the combination effects of chemicals [6]. However, presently, environmental risk assessment procedures are generally developed for single substances, which may lead to a severe underestimation of the real risk facing the environment [7,8].

Our understanding of the ecotoxicology of mixtures is in the first stages, because of the complexity of the variables that need to be taken into consideration [9]. The experimental assessment of the ecotoxicity of every potentially occurring mixture is not feasible and is still limited because of the wide temporal and spatial variability of compounds in a given area. In addition, obtaining a full prediction of mixture effects by using models is not easy. Two simple component-based predictive models for assessing the responses of mixtures are commonly used: the concentration addition (CA) or dose addition [10] and independent action (IA) [11] models. The CA approach is thought to be applicable to mixtures of chemicals that share a similar or common mode of action. Conversely IA, which is also known as response addition, is widely held to be appropriate for mixtures of agents that show a dissimilar mode of action. These concepts are both based on knowledge of the concentration–response relationships of the single components. In many cases mixtures in which components had similar or dissimilar modes of action were correctly predicted with the concepts of CA and IA, respectively [12–14]. The results from the IA model usually lead to slightly lower responses than those predicted by CA, and thus CA is often used as the acceptable worst case. However, in some cases, the so-called prediction window [15,16] is very small, which indicates that both models are able to predict the response of mixtures independently based on the mode of action of single components [7,17,18].

By definition, the CA and IA concepts ignore synergistic and antagonistic effects, and both models assume that the components of a mixture do not interact chemically or in toxicokinetic/toxicodynamic phases [9]. For the quantification of both phenomena, Chou and Talalay introduced the combination index method [19]. This approach was historically applied in pharmacology to understand the nature of the interactions among pharmaceutical active compounds [20], and it was more recently applied in the environmental field to study the interactions among chemical contaminants present in environmental mixtures [21–23]. In the first part of our study, the toxicity of 10 different pharmaceutical active compounds (for both veterinary and human use) to *Aliivibrio fischeri* (formerly *Vibrio fischeri* or *Photobacterium phosphoreum*) was determined using the Microtox® test system [24]. In this second part, we focused on pharmaceutical active compounds registered for veterinary use. In particular, we investigated the effects of a series of their binary or multicomponent mixtures on *A. fischeri*. In the previous study [24], we demonstrated that almost all the selected pharmaceutical active compounds act as polar-narcotic compounds toward *A. fischeri*. The only exception was the antibiotic chlortetracycline,which had a specific mode of action. Based on these findings, the mixture

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responses containing narcotic-type pharmaceutical active compounds should be well predicted by the CA model. In contrast, the IA approach should be more suitable for the prediction of mixture toxicities containing chlortetracycline. The binary mixtures were prepared at an equitoxic ratio that corresponded to the individual inhibitory concentration, 10% (IC10) obtained in our previous work [24]. The multicomponent mixture was prepared at a ratio corresponding to the individual predicted no-effect concentration (PNEC) value. The experimental results were compared with those obtained by the application of the CA and IA models, to assess the additive behavior of chemicals in a mixture or the existence of deviations from conceptual expectations. Finally, the nature of the pharmaceutical active compound interactions was also investigated by applying the combination index method to verify the existence of possible synergistic and/or antagonistic effects.

MATERIALS AND METHODS

Chemicals and testing procedures

The tested veterinary pharmaceutical active compound compounds were the same as those reported by Di Nica et al. [24]: amoxicillin, chlortetracycline, sulfamethizole, diclofenac sodium, and acetylsalycilic acid. The CAS numbers, main physicochemical properties, and individual acute toxicity data (IC50 and IC10) for *A. fischeri* have been reported by Di Nica et al. [24]. The stock solutions were prepared using the method described in Vighi et al. [25]. Acute toxicity (15 min) to the bioluminescent bacterium *A. fischeri* (purchased from Ecotox) was measured using the Microtox model 500 analyzer (Ecotox). The testing procedure was described by Di Nica et al. [24]. For binary mixtures, the tested concentrations (from 9 to 22 experimental concentrations) ranged from 0.02 to 35 toxic units, where the toxic units of the mixture correspond to the sum of the ratio between the actual concentration of the chemicals present in the mixture and their relative IC10. Solutions were buffered with 0.1 M sodium phosphate and pH was adjusted to a range of 6 to 8.50 (DIN EN ISO 11358-3) using a pH meter model 250 (Denver Instruments). All tests are referred to nominal (= initial) concentrations of pharmaceutical active compounds. Tests were performed in 4 replicates. Stock solutions of sulfamethizole, amoxicillin, and acetylsalycilic acid were prepared by using dimethyl sulfoxide (DMSO). For mixtures containing these pharmaceutical active compounds, the final test samples contained a concentration of DMSO that was always below 1.5% (v/v). As reported in our previous study [24], at this level of concentration, the influence of DMSO for low-water-solubility compounds is negligible.

Experimental design for selected mixtures

Eleven mixtures were prepared by mixing individual chemicals at equitoxic concentrations in the saline solution (2% NaCl) that was used for the toxicity test. This concentration ratio was kept constant, and, using a serial dilution, different concentrations of stock solutions were tested (fixed ratio design).

The tested mixtures were as follows. First, 10 combinations of binary mixtures of compounds mixed in a ratio corresponding to their individual IC10 value (acetylsalycilic acid–amoxicillin, acetylsalycilic acid–chlortetracycline, acetylsalycilic acid–diclofenac sodium, acetylsalycilic acid–sulfamethizole, chlortetracycline–amoxicillin, chlortetracycline–diclofenac sodium, chlortetracycline–sulfamethizole, diclofenac sodium–amoxicillin, diclofenac sodium–sulfamethizole, sulfamethizole–amoxicillin, with individual IC10 values of the selected pharmaceutical active compounds shown in the Supplemental Data, Table S1). Second, 1 multicomponent mixture of the 5 compounds mixed in a ratio corresponding to their individual PNEC value (mix–PNEC: chlortetracycline–diclofenac sodium–acetylsalycilic acid–sulfamethizole–amoxicillin).

In line with the current guidelines on environmental risk assessment of pharmaceuticals [26,27], PNECs were extrapolated by applying an appropriate assessment factor to the lowest relevant observed value within the toxicity dataset available in the literature. The calculated PNEC values are reported in the Supplemental Data, Table S2.

Concentration–response curve fitting

To quantitatively describe the concentration–response curves, the observed concentration–effect data were fitted to the nonlinear regression Weibull model (Equation 1):

$$I = (c, \beta) = 1 - \exp\{-\exp\{\ln(\beta_2(\ln(c) - \ln(\beta_1))\}\} \quad (1)$$

where I is the fractional response ($0 \leq I \leq 1$) in terms of the inhibition of luminescence, c is the chemicals concentration and β_1 and β_2 are the parameters of the model. The I_x ($100 \times I_x$%) and the confidence intervals were obtained using the R® software package [28,29].

Prediction of mixture toxicities by CA and IA models

Based on the fixed ratio of the mixture components, it was possible to express the concentration of single chemicals as a fraction of the total concentration. Therefore, it was possible to apply the CA model using Equation 2 [15]:

$$ECx_{\text{mix}} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{x_i}} \right)^{-1} \quad (2)$$

where ECx_{mix} is the total concentration of the mixture that produces an x% of the effect, p_i (C_i/C_{mix}) is the fraction of the component i in the mixture, and $EC_{x,i}$ is the concentration of the i^{th} component when present individually and elicits the same effect (x%) as the mixture. It was possible to derive the predicted concentration-response curve using Equation 2.

The alternative IA model is commonly used to predict the mixture toxicity of substances that have different or dissimilar mechanisms of action. It may be applied using Equation 3:

$$\begin{aligned} E(C_{\text{mix}}) &= 1 - \Pi_{1=1}^{n}(1 - E(C_i)) \text{ with } C_{\text{mix}} \\ &= c_1 .. \ \ldots \ \ldots + c_n \end{aligned} \quad (3)$$

where C_i denotes the concentrations of the i^{th} mixture component, $E(c_i)$ is its corresponding effect, and $E(C_{\text{mix}})$ is the overall effect as a result of the mixture [11,15].

Application of the combination index-isobologram equation

To highlight possible synergistic or antagonistic effects, a further analysis of the response of *A. fischeri* to pharmaceutical active compound mixtures was carried out using the combination index approach [20,30,31] (Equation 4):

$${}^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n} \left\{ \frac{[D]_j}{\sum_1^n [D]} \right\}}{(D_m)_j \left\{ \frac{(fa_x)_j}{[1-(fa_x)_j]} \right\}^{1/m_j}} \quad (4)$$

where ${}^{n}(CI)_x$ is the combination index for n chemicals at x% inhibition, $(D_x)_{1-n}$ is the sum of the dose of n chemicals that

exerts $x\%$ inhibition in combination, $\{([D]_j/\sum[D]\}$ is the proportionality of the dose of each of n drugs that exerts $x\%$ inhibition in combination, and $(D_m)_j\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the dose of each drug alone that exerts $x\%$ inhibition. D_m is the median-effect dose, f_{ax} is the fractional inhibition at $x\%$ inhibition, and m is the slope of the median-effect plot.

From Equation 4, combination index < 1, combination index $= 1$, and combination index > 1 indicate synergism, the additive effect, and antagonism, respectively.

The computer program CompuSyn was used to calculate the combination index values for the different mixtures at different ranges of effect levels (10%, 25%, 50%, 75%, and 90% of inhibition of bioluminescence) [32].

RESULTS AND DISCUSSION

Experimental mixture toxicities

Binary mixtures of pharmaceutical active compounds. Values of IC10 and IC50 were derived from the concentration–response curves of the tested binary pharmaceutical active compound combinations (Table 1). As shown in Table 1, the IC50 values obtained spanned over 2 orders of magnitude.

In particular, the binary mixture chlortetracycline–diclofenac sodium showed the highest toxicity (IC10 = 0.68 mg L^{-1} and IC50 = 10.1 mg L^{-1}), followed by the diclofenac sodium–acetylsalycilic acid combination (IC10 = 18.2 mg L^{-1} and IC50 = 111.6 mg L^{-1}). In contrast, the binary combinations containing amoxicillin showed the lowest IC50.

These results were not surprising considering that, in our previous study [24], chlortetracycline and diclofenac sodium tested singly were the most toxic pharmaceutical active compounds to *A. fischeri* (IC10 = 2.0 mg L^{-1} and IC50 = 12.1 mg L^{-1} for chlortetracycline; IC10 = 5.9 mg L^{-1} and IC50 = 15.9 mg L^{-1} for diclofenac sodium) whereas the amoxicillin was the least toxic 1 (IC50 = >1702 mg L^{-1}).

For the majority of the tested binary mixtures, a complete concentration–response relationship was obtained (Figure 1). In 4 cases the maximum percentage of inhibition reached was near 50% (acetylsalycilic acid–amoxicillin and sulfamethizole–amoxicillin 43%; acetylsalycilic acid–sulfamethizole and chlortetracycline–amoxicillin 48%).

Multicomponent mixtures of pharmaceutical active compounds. A complete concentration–response curve was obtained for the tested multicomponent mixture of veterinary pharmaceutical active compounds (mixed at their PNEC ratio). The resulting IC10 and IC50 values were 25.0 mg L^{-1} and 220.7 mg L^{-1}, respectively. The results obtained showed that at concentrations corresponding to the sum of individual PNECs (0.16 mg L^{-1}), a very negligible effect was measured on *A. fischeri*; in addition, the IC10 was 2 orders of magnitude greater than the sum of the single-compound safety threshold for aquatic organisms, and at least 4 orders of magnitude above their joint measured environmental concentrations in European water bodies that are in the range of ng L^{-1} [24].

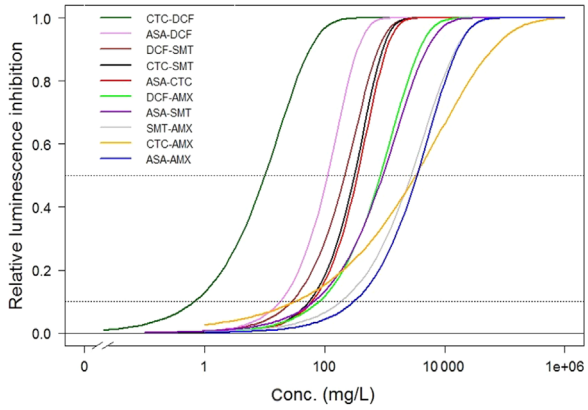

Figure 1. Concentration response curves (Weibull model) of the tested binary mixtures of veterinary pharmaceutical active compounds (dashed lines = intersections at values of individual acute toxicity data [IC50 and IC10, respectively]). AMX = amoxicillin; ASA = acetylsalycilic acid; CTC = chlortetracycline; DCF = diclofenac sodium; SMT = sulfamethizole.

These results are not surprising considering that PNECs have been calculated by applying an assessment factor to acute or chronic toxicity data for the most sensitive species and that *A. fischeri* is not the most sensitive for all the tested compounds, as demonstrated in our previous study [24].

Predicted mixture toxicity by CA and IA models and comparison with experimental results

The experimental concentration–response curves obtained were compared with the classical models of CA and IA. Some of the results are depicted in Figure 2, with the confidence intervals also reported for the experimental concentration–responses curves. A more complete picture of the results can

Table 1. Inhibitory concentration (IC10 and IC50; 15-min acute Microtox test; mg L^{-1}) values with the corresponding 95% confidence intervals (in parentheses) for the 10 tested binary combinations of pharmaceutical active compounds

		CTC	DCF	ASA	SMT	AMX
	mg L^{-1}					
CTC	IC10	—				
	IC50	—				
DCF	IC10	0.68 (0.41–0.96)	—			
	IC50	10.1 (8.72–11.5)	—			
ASA	IC10	56.0 (50.3–61.8)	18.2 (15.6–20.8)	—		
	IC50	378.6 (340.7–406.4)	111.6 (105.9–117.2)	—		
SMT	IC10	55.6 (46.8–64.3)	28.3 (25.8–30.9)	66.1 (49.1–83.2)	—	
	IC50	311.1 (294.6–327.6)	215.4 (208.4–222.3)	927.6 (774.8–1080.5)	—	
AMX	IC10	32.7 (20.9–45.2)	80.7 (63.3–98.0)	308.7 (187.5–430.0)	187.4 (150.5–224.3)	—
	IC50	3270.4[a] (2471.9–4068.9)	846.2 (783.8–908.6)	3549.2[a] (2397.0–4501.4)	2738.2[a] (2352.2–3135.2)	—

[a]Values estimated with the Weibull model.
AMX = amoxicillin; ASA = acetylsalycilic acid; CTC = chlortetracycline; DCF = diclofenac sodium; SMT = sulfamethizole.

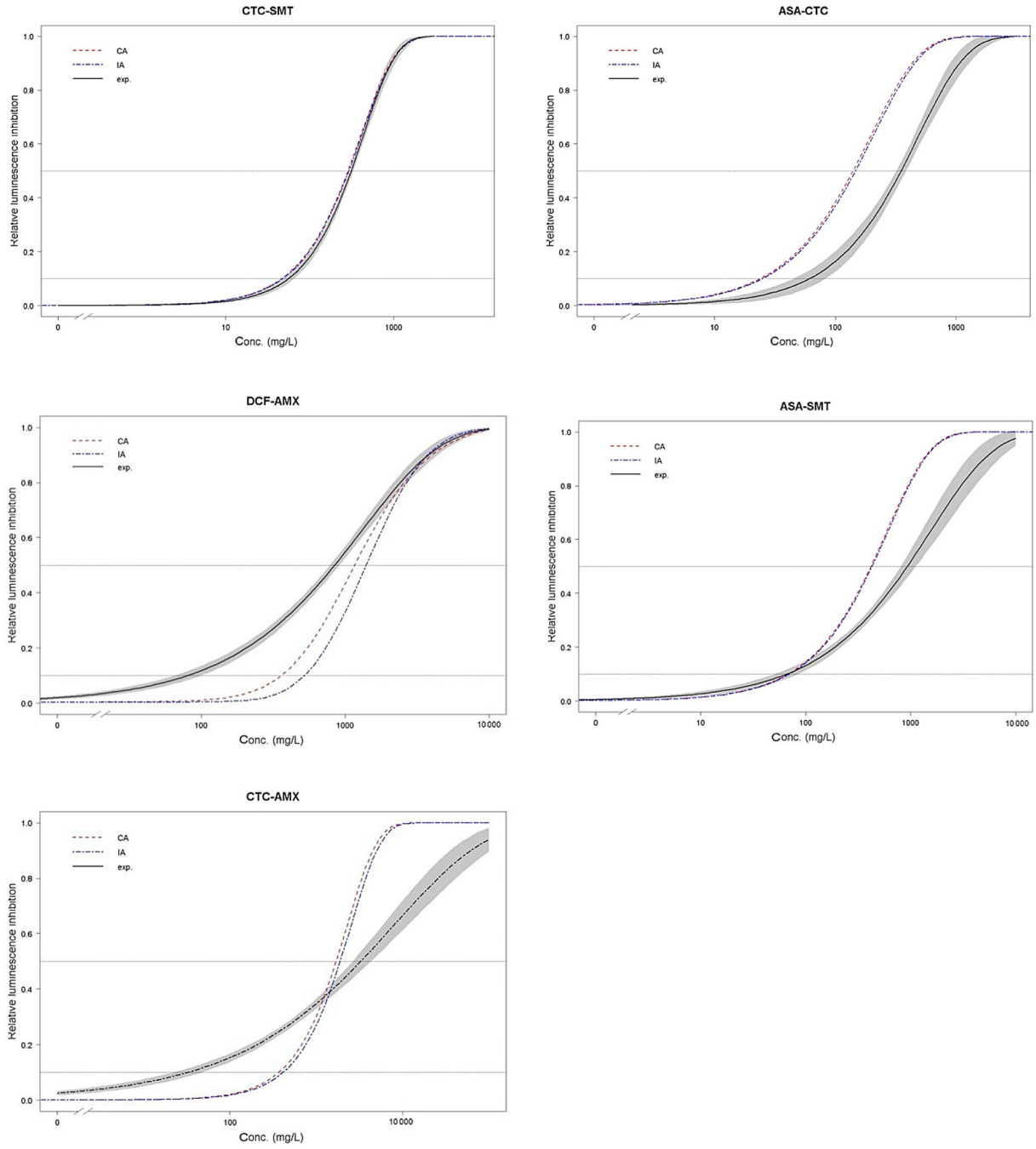

Figure 2. Predicted (concentration addition = red line; independent action = blue line) and observed toxicity values (black line) of binary mixtures (at inhibition concentration [IC10] concentration ratio) of veterinary pharmaceutical active compounds (gray shading = 95% confidence intervals). AMX = amoxicillin; ASA = acetylsalycilic acid; CTC = chlortetracycline; DCF = diclofenac sodium; SMT = sulfamethizole.

be found in the Supplemental Data, Figure S1 and Table S3 (IC50 and IC10 predicted by CA and IA). The observed concentration responses are not always sufficiently described by the CA and IA models. In fact, with the exception of 3 binary combinations (chlortetracycline–sulfamethizole, diclofenac sodium–sulfamethizole, and acetylsalycilic acid–diclofenac sodium) and of the multicomponent mixture, the experimental toxicity data deviate from the conceptual expectations of the CA and IA. In 1 case (the binary mixture acetylsalycilic acid–chlortetracycline), both models clearly overestimate the bioluminescence inhibition response at all concentrations tested. In all the other cases, the extent of the deviation is mainly dependent on the mixture components and the effect level considered. In particular, for binary combinations showing greater observed toxicities than those predicted by models (e.g., chlortetracycline–diclofenac sodium, diclofenac sodium–amoxicillin), the deviations are considerably higher at lower concentrations. Recently, Marx et al. [33], using literature data, analyzed the presence of synergistic and antagonistic effects in antibiotic mixtures and found a potential increase in synergistic effects with decreasing concentrations. However, the authors concluded that because of the low number of investigations into the concentration dependency of mixture interactions and because

of the dependency of this influence on the targeted organism and on the specific combination of chemicals, no general statement can be made at present.

In contrast, for acetylsalycilic acid–sulfamethizole (Figure 2) and acetylsalycilic acid–amoxicillin (Supplemental Data, Figure S1) combinations, the magnitude of deviation increased with an increase in concentration. However, in both cases, the deviations could be an artifact resulting from the lack of experimental data. (As reported above in the section *Binary mixtures of pharmaceutical active compounds*, the maximum percentage of inhibition reached was near 50%.) Finally, both models seem to underestimate the effects of chlortetracycline–amoxicillin (Figure 2) and sulfamethizole–amoxicillin (Supplemental Data, Figure S1) mixtures at lower tested concentrations.

Toxicological interactions of the tested pharmaceutical active compounds in mixtures

The results reported in the section *Predicted mixture toxicity by CA and IA models and comparison with experimental results* suggested possible interactions among the mixture components. The presence of synergism or antagonism in the mixtures of pharmaceutical active compounds (particularly in binary mixtures) or other pollutants has been reported in previous studies [14,21,23,34–37] and in the recent review by Backhaus [9]. Cleuvers [14] tested combinations of various pharmaceutical active compounds on algae and *Daphnia* and demonstrated that the evaluated compounds behaved as nonpolar narcotic compounds toward both organisms. In the algal test, the combination effect of a binary mixture of ibuprofen and diclofenac (2 nonsteroidal anti-inflammatory drugs [NSAIDs]) was well predicted by the CA model (as expected for substances with similar modes of action). However, in the *Daphnia* test, the effect of the mixture was stronger than that predicted by the CA model. In a further study, Cleuvers [34] evaluated a 4-component mixture of NSAIDs and confirmed that, in the algal test, the effect of increasing the number of NSAID components in the mixture was well predicted by the CA. In the *Daphnia* test, the results were quite different: at low doses (up to EC20/4), the mixture showed practically no effect (much lower than that predicted by the CA model), whereas at the higher concentrations, the effect was much higher than that predicted by the model. Rodea-Palomares et al. [21] found contrasting results when assessing the toxicological interactions of binary combinations of fibrates in *V. fischeri* and *Anabaena.* In fact, these authors reported that in the *Vibrio* test, the binary mixtures showed antagonism at low effect levels that turned into an additive effect or synergism at higher effect levels. However, in the *Anabaena* test, they found a strong synergism at the lowest effect levels that turned into a very strong antagonism at high effect levels. Gonzalez-Pleiter et al. [36] studied the toxicity of binary and multicomponent mixtures of 5 antibiotics to the green alga *Pseudokirchneriella subcapitata* and the cyanobacterium *Anabaena* CPB4337. The study found a clear predominance of synergistic behavior for most of the antibiotic interactions in these organisms. In particular, the amoxicillin–tetracycline combination tested on cyanobacterium was strongly synergistic. Conversely, the same authors [36] found a dual behavior with rapid changes of interactions (from strong synergism to strong antagonism) when tetracycline in combination with erythromycin was tested in *Anabaena*. Brezovsek et al. [37] tested the toxicity of 3 binary mixtures of antineoplastic drugs to the green alga *P. subcapitata* and the cyanobacterium *Synechococcus leopolinensis* and compared their results with predictive models (CA and IA). The binary combination of 5-fluorouracil and imatinib had a synergistic effect for *P. subcapitata* and an antagonistic effect on *S. leopolinensis*. Based on these results, the authors noted the importance of species-dependent interactions between the pharmaceuticals in a mixture.

Backhaus [9] highlighted that, for pharmaceuticals, the synergisms and antagonisms were specific for the tested mixture and the toxicity test. In particular, synergistic mixtures seemed to be largely confined to mixtures of only a few compounds, usually not more than 2 or 3. In contrast, synergistic or antagonistic mixture effects were rarely observed in toxicity tests for multicomponent mixtures. Backhaus [9] argued that this phenomenon might be explained by the presence of a sort of buffering against synergistic or antagonistic interactions when the contributions of the latter are not predominant.

Warne and Hawker [38], in their work on the funnel hypothesis, explained the variation in the toxicity of multicomponent mixtures of nonspecific toxicants (narcotics) and concluded that mixtures of this type of toxicants tended to approach additivity (becoming less synergistic or antagonistic) as the number of components increased.

In the present study, to evaluate the presence and eventually quantify the nature of such interactions (e.g., synergism, antagonism) at any effect level, the combination index method was applied. Figure 3 shows a plot of the combination index values obtained as a function of effect levels (f_a) for all of the tested mixtures. The corresponding combination index values at the main representative f_a levels (effect level of fraction inhibited with respect to the control) are reported in the Supplemental Data, Table S3. The f_a-combination index plots allowed us to observe, at any f_a level, the trend of the nature of the interactions among pharmaceutical active compounds in mixtures. In Figure 3, combination index values up to the effect level (f_a) of 0.5 are reported, for those binary mixtures for which the entire concentration–response curves were not obtained.

From the analysis of Figure 3 and Supplemental Data, Table S4, the following inferences may be made. First, for almost all the tested mixtures, f_a-combination index plots and combination index values seems to confirm the presence of interactions among the components. The only exceptions are the chlortetracycline–sulfamethizole, diclofenac sodium–sulfamethizole, and acetylsalycilic acid–diclofenac sodium binary combinations, which are fairly additive (combination index $\sim$1) at all the effect levels considered. Second, synergism or antagonism seem to be independent of the mode of action toward the tested organism. As highlighted in our previous study [24], we demonstrated that, with the exception of chlortetracycline, all of the selected pharmaceutical active compounds act as polar narcotic compounds to *A. fischeri*. In agreement with the previously reported results of Cleuvers [14] on *Daphnia*, even if these substances share (broadly speaking) the same mechanism of action, we found that the effect of the binary mixtures deviates from CA predictions. In addition, binary combinations composed of substances with different mechanisms of action (i.e., those including chlortetracycline) show very different and unpredictable behaviors. Third, clear synergistic effects are present for the chlortetracycline–diclofenac sodium and diclofenac sodium–amoxicillin combinations up to the effect level (f_a) of 0.65 to 0.70. The results obtained for chlortetracycline–diclofenac sodium seem to be in line with those of previous studies. Olajuyigbe and Afolayan [39] tested combinations of tetracycline and amoxicillin in 8 different kinds of antibiotic-resistant bacteria (either gram-negative or gram-positive bacteria) and found that the combinations often had a synergistic effect. Interestingly the

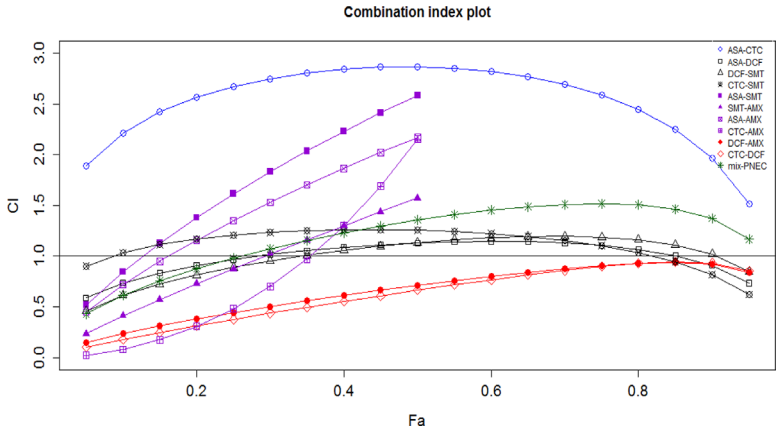

Figure 3. Combination index plot (f_a-combination index plot) for binary and multicomponent mixtures of tested pharmaceutical active compounds toward *Aliivibrio fischeri*. The combination index values are plotted as a function of the fractional inhibition of bioluminescence (f_a) by computer simulation (CompuSyn). Combination index $<1, =1$, and >1 indicate synergism, the additive effect, and antagonism, respectively [32]. AMX = amoxicillin; ASA = acetylsalycilic acid; CTC = chlortetracycline; DCF = diclofenac sodium; SMT = sulfamethizole.

same authors [39] also found that the synergistic effects were concentration dependent between the 2 antibiotics. In contrast, acetylsalycilic acid–chlortetracycline clearly interacted in an antagonistic way over the whole range of effect levels. Fourth, for other binary combinations (chlortetracycline–amoxicillin, sulfamethizole–amoxicillin acetylsalycilic acid–sulfamethizole, acetylsalycilic acid–amoxicillin), a somewhat heterogeneous pattern is observed, with interactions changing from synergism at low f_a (0.1–0.3) to a clear antagonism within a narrow range of concentrations. The presence of synergism at lower concentrations and the tendency toward additivity, or antagonism, at the higher levels is not easy to explain, because the nature of the interactions are complex and mostly unknown. In any case, as discussed previously, similar results are found in the literature [21,35,36]. For instance, Magdaleno et al. [40] tested the effects on the growth of *P. subcapitata* of combinations of several antibiotics and found that at low concentrations ($<10\ mg\ L^{-1}$), all binary combinations showed synergism, whereas in almost all cases, at the higher concentrations tested, the response became close to the predictions.

In some cases, the nature of the interactions seems to be related to the presence of certain compounds. For instance, at the lower tested concentrations, the presence of the anti-inflammatory diclofenac sodium seems to enhance the effects of the antibiotics amoxicillin, chlortetracycline, and (to a lesser extent) sulfamethizole). Several studies have noted that the efficiency of some antibiotics (streptomycin, gentamicin, tetracycline, and ampicillin) toward bacteria can be synergistically enhanced by the presence of diclofenac sodium [41–45]. These studies found that diclofenac sodium has the pability to damage the bacterial membrane [46], and to favor alteration in the permeability of the microorganism to antibiotics [45,47,48]. In contrast, in the present study, the presence of the anti-inflammatory acetylsalycilic acid seems to weaken their toxicity.

Finally, the multicomponent mixture showed no strong synergism or antagonism. In fact, the combination index values ranged from 0.61 (IC10) to 1.37 (IC90) until the tested effect level. The results we obtained in the toxicity test for the multicomponent mixtures seem to support the buffering hypothesis proposed by Backaus [9].

As reported by Kortenkamp et al. [49], synergistic cases are highly specific for specific compounds, their concentrations, and their mixture ratios. In the present study, the concentrations of the tested mixtures giving synergistic responses are above the environmentally relevant concentrations and in addition, for our experimental purpose, substances were combined in equal ratios according to their individual IC10 value, which could be quite different from the real ratio at which toxicants might be found in the environment. Thus responses from naturally occurring combinations could be different.

CONCLUSIONS

The toxicity of binary and multicomponent mixtures of widely used veterinary pharmaceuticals was evaluated using the Microtox test system. For the multicomponent mixtures tested, the results indicated that at concentrations corresponding to the sum of individual PNECs, no effects on *A. fischeri* were present. This finding suggested that PNEC values were sufficiently protective when the investigated pharmaceutical active compounds were contemporaneously present in aquatic environments, at least for microorganisms and for short-term exposure. By comparing the experimental toxicity curves with those predicted by the CA and IA models, it was possible to hypothesize the presence, in binary mixtures, of toxicological interactions between the components, which, in several cases, leads to synergistic effects at low concentrations (particularly when either diclofenac sodium or amoxicillin was 1 of the components). The subsequent application of the combination index method confirmed the presence of synergistic and antagonistic interactions. Finally, synergism or antagonism seemed to be independent of the mode of action of the toxicants tested. This seems to confirm that for combinations of pharmaceutical active compounds, use of the predictive models may have some limitations in terms of assessment of the real mixture toxicity.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3686.

Data Availability—Data, associated metadata, and calculation tools are available from the corresponding author (v.dinica@campus.unimib.it).

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SUPPORTING INFORMATIONS

Toxicity of individual pharmaceuticals and their mixtures to *Aliivibrio fischeri*. Part II: Evidence of toxicological interactions in binary combinations

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Contents

Supporting Information shows: PNECs values used for mixing pharmaceuticals in the multicomponent mixture (Table S1); Predicted (CA and IA) and observed concentration response curves for binary e multicomponent mixtures of veterinary PhACs (**Figure S1**); IC_{10} and IC_{50} values predicted by Concentration Addition (CA) and Independent Action (IA) models for the tested mixtures (**Table S2**); Combination Index (CI) values for IC_{10}, IC_{25}; IC_{50}; IC_{75}; IC_{90} effect levels for the tested mixtures (**Table S3**).

Abbreviations: ASA: acetylsalycilic acid; AMX: amoxicillin; CTC: chlortetracycline; DCF: diclofenac sodium; SMT: sulfamethizole; mix-PNEC: CTC-DCF-ASA-SMT-AMX;

Table S1. PNECs calculation from the most sensitive aquatic species and the Assessment Factors (AF) selected for mix-PNEC components (mg L^{-1})

Most Sensitive Species			**References**		
				AF*	**PNEC (mg L^{-1})**
Compound	**Species**	**Ecotox data** (mg L^{-1})			
CTC	*M. aeruginosa*	0.05 (EC_{50})	Halling-Sorensen, 2000	1000	0.00005
ASA	*D. magna*	88.1 (EC_{50})	Cleuvers, 2004	1000	0.0881
SMT	*L. minor*	2.54 (EC_{50})	Bialk-Bielinska et al., 2011	1000	0.00254
AMX	*Anabaena*	56.3 (EC_{50})	Gonzales-Pleiter et al., 2013	1000	0.0563
DCF	*C. dubia.*	10 (NOEC)	Carlsson et al., 2006	10	0.01

*The AF were chosen on the basis of the availability of toxicity data

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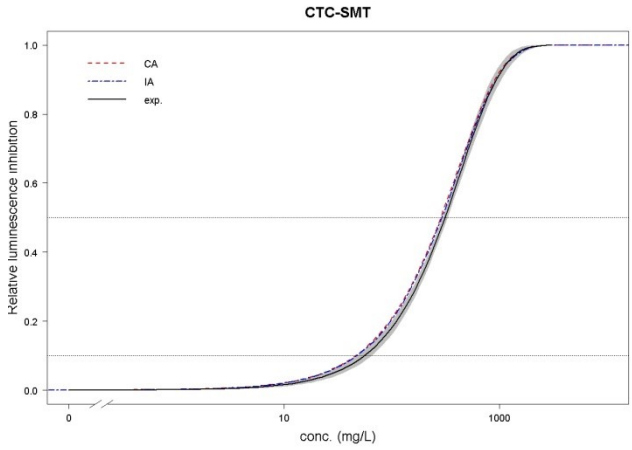
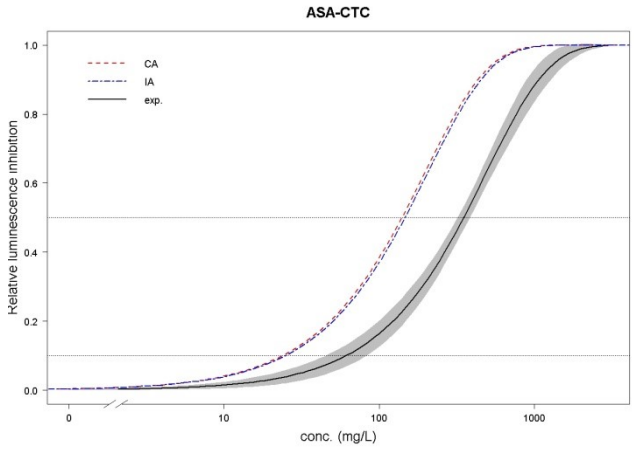

CTC-SMT
CA
IA
exp.
Relative luminescence inhibition
conc. (mg/L)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exp.
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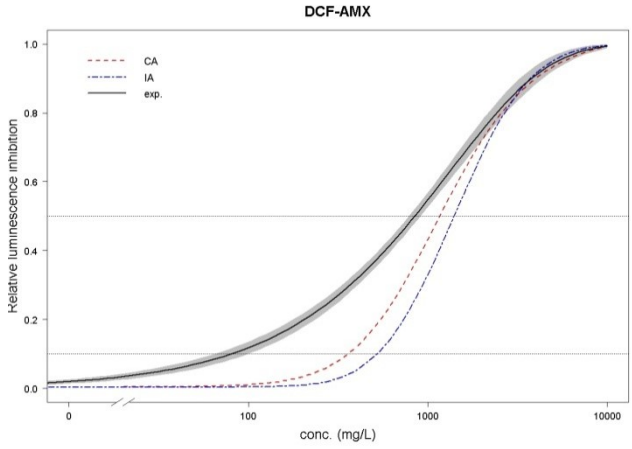
conc. (mg/L)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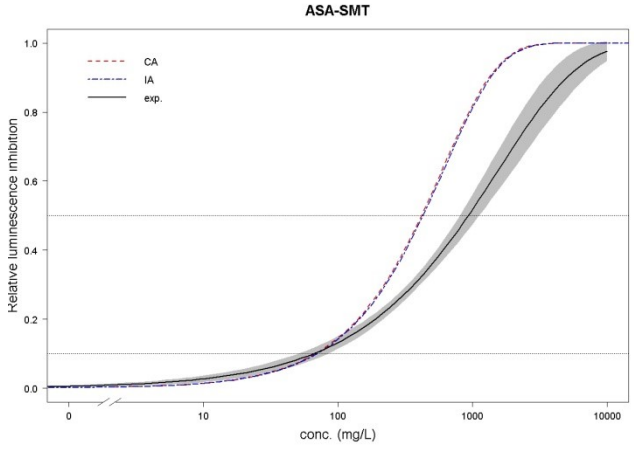
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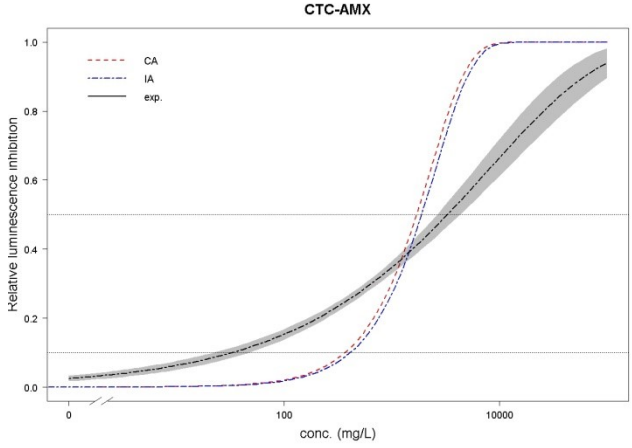
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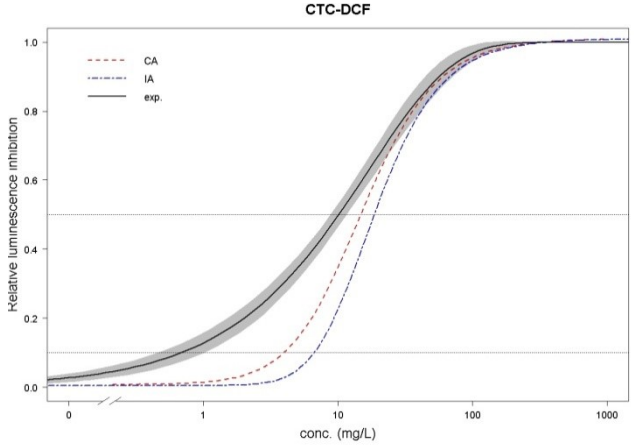
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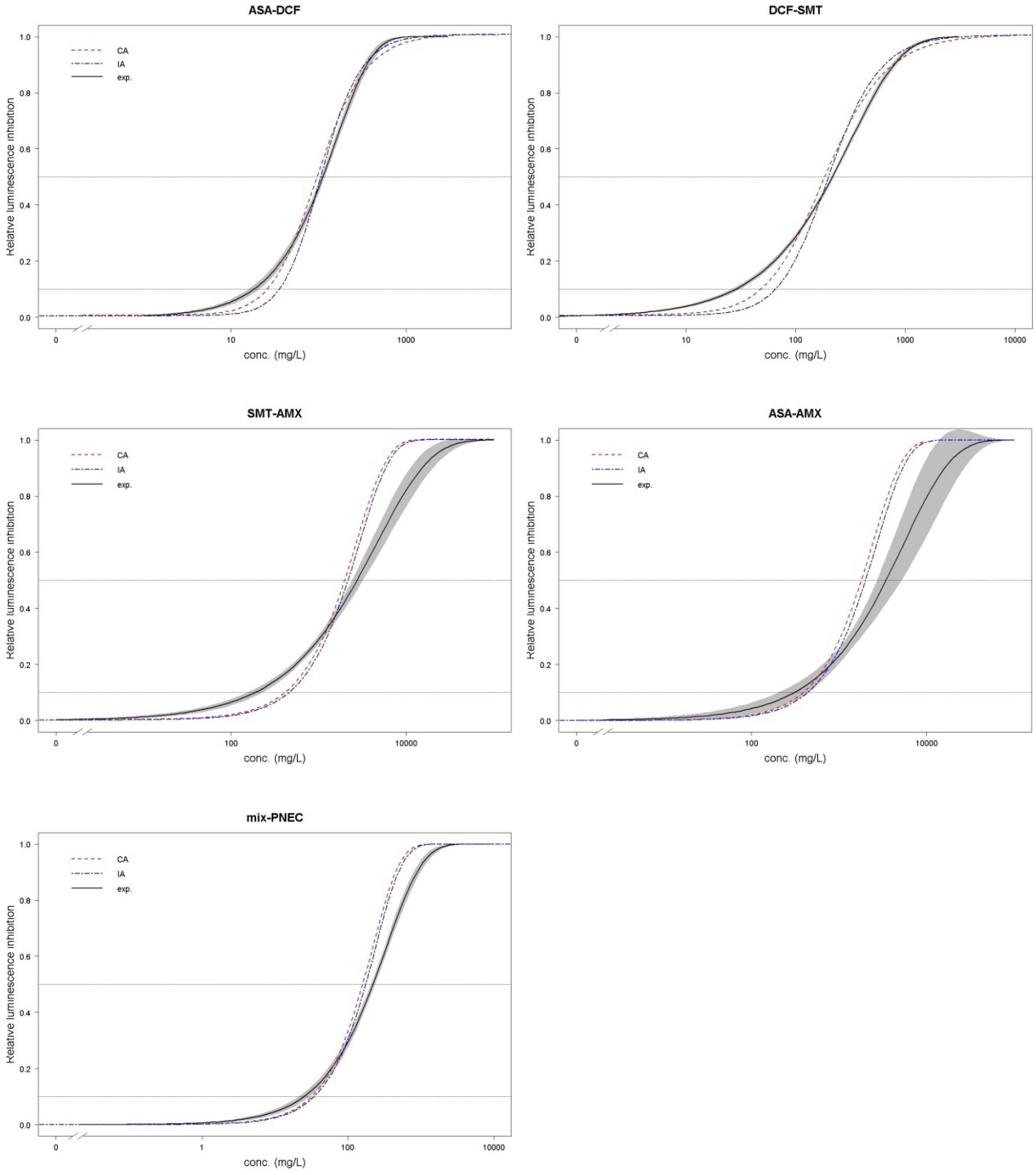

Figure S1. Predicted (CA = red line; IA = blue line) and observed toxicity values (black line) of binary (at IC_{10} concentration ratio) and multicomponent mixtures (at individual PNECs concentration ratio) of veterinary PhACs (in shadow are the confidence intervals at 95 percent)

Table S2. IC_{10} and IC_{50} values for the tested mixtures of PhACs as predicted by CA and IA models

Mixture		CA	IA	Mixture		CA	IA
CTC-DCF	IC_{10}	4.15	6.8	**ASA-DCF**	IC_{10}	27.0	36.5
	IC_{50}	15.1	18.8		IC_{50}	97.9	107.0
DCF-SMT	IC_{10}	50.6	67.5	**CTC-SMT-**	IC_{10}	46.3	47.4
	IC_{50}	187.2	201.4		IC_{50}	284.0	291.0
ASA-CTC	IC_{10}	23.9	24.9	**DCF-AMX**	IC_{10}	372.5	530.0
	IC_{50}	140.2	146.3		IC_{50}	1207.3	1411.0
ASA-SMT	IC_{10}	68.1	69.9	**SMT-AMX**	IC_{10}	421.1	462.6
	IC_{50}	416.9	427.7		IC_{50}	1992.1	2166.4
CTC-AMX	IC_{10}	367.0	410.9	**ASA-AMX**	IC_{10}	388.4	436.0
	IC_{50}	1701.7	1892.0		IC_{50}	1799.5	2004.7
mix-PNEC	IC_{10}	31.7	34.7				
	IC_{50}	159.9	175.1				

Table S3. Combination Index (CI) values with the intensity of the interactions

Mixt.		CI	CI graded symbols	Description	Mixt.		CI	CI graded symbols	Description
CTC-DCF	IC_{10}	0.18	+ + + +	Strong synergism	ASA-DCF	IC_{10}	0.73	+ +	Moderate synergism
	IC_{25}	0.38	+ + +	Synergism		IC_{25}	0.97	±	Nearly additive
	IC_{50}	0.66	+ + +	Synergism		IC_{50}	1.13	-	Slight antagonism
	IC_{75}	0.90	±	Nearly additive		IC_{75}	1.11	-	Slight antagonism
	IC_{90}	0.93	±	Nearly additive		IC_{90}	0.91	±	Nearly additive
DCF-SMT	IC_{10}	0.61	+ + +	Synergism	CTC-SMT	IC_{10}	1.03	±	Nearly additive
	IC_{25}	0.89	+	Slight synergism		IC_{25}	1.21	- -	Moderate antagonism
	IC_{50}	1.13	-	Slight antagonism		IC_{50}	1.26	- -	Moderate antagonism
	IC_{75}	1.19	-	Slight antagonism		IC_{75}	1.10	±	Nearly additive
	IC_{90}	1.02	±	Nearly additive		IC_{90}	0.82	+ +	Moderate synergism
ASA-CTC	IC_{10}	2.21	- - -	Antagonism	DCF-AMX	IC_{10}	0.23	+ + + +	Strong synergism
	IC_{25}	2.67	- - -	Antagonism		IC_{25}	0.44	+ + +	Synergism
	IC_{50}	3.01	- - -	Antagonism		IC_{50}	0.71	+ +	Moderate synergism
	IC_{75}	2.87	- - -	Antagonism		IC_{75}	0.91	±	Nearly additive
	IC_{90}	1.97	- - -	Antagonism		IC_{90}	*	*	No data
ASA-SMT	IC_{10}	0.85	+	Slight synergism	SMT-AMX	IC_{10}	0.41	+ + +	Synergism
	IC_{25}	1.61	- - -	Antagonism		IC_{25}	0.87	+	Slight synergism
	IC_{50}	2.58	- - -	Antagonism		IC_{50}	1.57	- - -	Antagonism
	IC_{75}	*	*	No data		IC_{75}	*	*	No data
	IC_{90}	*	*	No data		IC_{90}	*	*	No data
CTC-AMX	IC_{10}	0.08	+ + + + +	Very strong synergism	ASA-AMX	IC_{10}	0.72	+ + +	Moderate synergism
	IC_{25}	0.48	+ + +	Synergism		IC_{25}	1.35	±	Moderate antagonism
	IC_{50}	2.16	- - -	Antagonism		IC_{50}	2.17 *	- - - *	Antagonism
	IC_{75}	*	*	No data		IC_{75}	*	*	No data
	IC_{90}	*	*	No data		IC_{90}	*	*	No data
mix-PNEC	IC_{10}	0.61	+ + +	Synergism					
	IC_{25}	0.98	±	Nearly additive					
	IC_{50}	1.36	- -	Moderate antagonism					

IC_{75}	1.51	- - -	Antagonism
IC_{90}	1.37	- -	Moderate antagonism

CHAPTER VI

Experimental and predicted toxicity of binary combinations of diclofenac sodium, carbamazepine and caffeine to *Aliivibrio fischeri*.

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EXPERIMENTAL AND PREDICTED TOXICITY OF BINARY COMBINATIONS OF DICLOFENAC SODIUM, CARBAMAZEPINE AND CAFFEINE TO *Aliivibrio fischeri*

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Abstract

In this study, the toxic effects of binary mixtures of Pharmaceutical Active Compounds (PhACs) that are observed in the effluents from a wide range sewage treatment plants and surface water bodies were investigated using the bioluminescent bacterium *Aliivibrio fischeri* (Microtox® test). The selected chemicals were the nonsteroidal anti-inflammatory drug (NSAID) diclofenac sodium [DCF] and the anti-epileptic carbamazepine [CBZ]. In addition, caffeine [CFF], a psychoactive stimulant of the central nervous system, was also included in the study. Binary combinations were prepared at a predefined ratio that corresponded to the individual IC_{50} values of the investigated compounds (equitoxic ratio). The experimental results were compared with those obtained using the two most frequently used predictive models in aquatic toxicology: the Concentration Addition (CA) and Independent Action (IA) models. The results indicated that both models predict the observed mixture toxicity of the DCF-CBZ and DCF-CFF mixtures quite well. However, in the case of CFF-CBZ, both models slightly overestimated the experimental results, suggesting the presence of a potential antagonistic effect. The application of the Combination Index (CI) method, which allows us to identify and quantify the nature of the interactions between the chemicals present in a mixture (synergistic, additive or antagonistic effects), confirmed the additive behaviour of the DCF-CBZ and DCF-CFF combinations and the slightly antagonistic effect observed for the binary mixture of CFF-CBZ.

Key words: Aliivibrio fischeri, combination index, concentration addition, human pharmaceuticals, mixture toxicity

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1. Introduction

For both humans and for organisms living in the environment, chemical exposure rarely consists of single chemicals, but in many cases, it consists of mixtures of chemicals, often of fluctuating compositions and concentrations (van Gestel et al., 2011). There is accepted evidence demonstrating that mixture toxicities may be higher than the toxicity observed for their individual components (Cleuvers, 2003; Di Nica et al., 2016b; Gonzalez-Pleiter et al., 2013; Villa et al., 2012). Monitoring studies demonstrated the presence of residues of Pharmaceutical Active Compounds (PhACs) and their metabolites in water bodies in concentrations ranging from ng L^{-1} to µg L^{-1} (Daughton and Ternes, 1999; Halling-Sørensen et al., 1998; Heberer, 2002; Hilton and Thomas 2003; Kümmerer, 2001; López-Serna et al., 2010; Monteiro and Boxall, 2010). According to Voulvoulis et al. (2016), approximately one hundred pharmaceuticals from many classes of drugs and some of their metabolites were identified in treated sewage, rivers and creeks, seawater, groundwater and drinking water all around the world. Hence, in the last few years, the evaluation of the effects of mixtures of PhACs has become an emergent topic in ecotoxicological studies (Backhaus, 2014). Due to their large consumption,

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carbamazepine (CBZ), diclofenac sodium (DCF) and caffeine (CFF) are among the most commonly observed drug residues in aquatic systems. In fact, Zhang et al. (2008) estimated that the global consumption volumes of CBZ and DCF were 1014 tons and 940 tons per year, respectively. For CFF, Gokulakrishnan et al. (2005) estimated an average global consumption of 80-400 mg per person per day.

CBZ is typically used for the treatment of epilepsy and neuropathic pain. Together with its degradation products, 10,11-dihydrocarbamazepine and 10,11-dihydroxy-10,11-dihydrocarbamazepine (Hummel et al. 2006), this compound is regularly detected in sewage treatment plant (STP) effluents, freshwater (rivers and lakes) and even in seawater (Buser et al., 1998; Weigel et al., 2004). For this reason, CBZ has been proposed as an anthropogenic marker in water bodies (Clara et al., 2004). Thaker (2005) reported the presence of carbamazepine residues in forty-four rivers across the USA, with an average level of 60 ng L^{-1} in water and 4.2 ng mg^{-1} in sediments. In the Elbe River and its tributaries, Wiegel et al. (2004) found levels of CBZ up to 1.2 µg L^{-1}, whereas Monteiro and Boxall (2010) reviewed the presence of this compound and observed concentrations of up to 7.1 µg L^{-1} in Germany. In Italy, Zuccato et al. (2005) reported median concentrations of 291 ng L^{-1} and 175 ng L^{-1} in STPs and the Lambro River, respectively. Further evidence of the presence of CBZ in STP effluents, surface waters, drinking waters and groundwater in Europe, the United States and Canada are shown in the reports of Benotti and Brownawell (2007), Focazio et al. (2008), Hao et al. (2006), Loos et al. (2008) and Zhang et al. (2008).

DCF, a nonsteroidal anti-inflammatory drug (NSAID), is perhaps the most widely used analgesic (Cleuvers et al., 2004). In a long-term monitoring investigation of sewage and surface water samples, DCF was identified as one of the most relevant PhACs residues (Heberer, 2002). In the UK, DCF has been found in STP effluents (median concentration of 424 ng L^{-1}) (Ashton et al., 2004). Furthermore, it has also been measured in groundwater (Heberer et al., 2001). More recently, Iglesias et al. (2014) reported a mean concentration of 13.6 ng·L^{-1} in surface waters collected from rural areas in Northwestern Spain. More information about the presence of DCF residues in water bodies can be found in a recent review of Cherik et al. (2015). CFF is popularly consumed as a stimulant of the central nervous system (Ferreira, 2005). The presence of caffeine residues in water bodies is largely attributed to discharges of domestic wastewater (Martín et al., 2012; Metcalfe et al., 2003; Seiler et al., 1999; Wu et al., 2010), particularly from the disposal of unconsumed coffee, tea or soft drinks down household drains. Due to its high solubility in water, low octanol-water partition coefficient and low volatility, CFF fits the characteristics for a good chemical marker of pollution that is directly related to anthropogenic influences, with no potential biogenic sources (Siegener and Chen, 2002). The amount of CFF residues in water bodies is highly variable. For instance, in surface waters around Madrid (Spain), concentration ranges varying from 675 to 13167 ng L^{-1} were measured (Valcárcel et al., 2011). Based on this evidence, it is not surprising that residues of these compounds are present contemporaneously in surface waters. For instance, Heberer and Feldmann (2005) calculated that in total, 2.0 kg of carbamazepine per week (105 kg per year) and 4.4 kg of diclofenac sodium per week (226 per year) were discharged into Berlin's surface water. In a recent study, Al-Qaima et al. (2014) demonstrated the presence of the three PhACs, CFF, CBZ and DCF, in the Langat and Muar Rivers (Malaysia) at concentrations of 410, 15 and 39 ng L^{-1}, respectively. Consequently, they can potentially exert a joint effect on water organisms.

Assessed as individual compounds, the selected PhACs exert a moderate acute toxicity for aquatic organisms, at levels well above those measured in the aquatic compartment ($L(E)C_{50}$ ranging from tens to hundreds mg L^{-1}). For instance, the reported toxicity to aquatic organisms of DCF ranges from 22 mg L^{-1} (*D. magna*) to 167 mg L^{-1} (fish) (Cleuvers, 2003, 2004; Ferrari et al., 2003; Praskova et al., 2011). The same levels of toxicity are reported for CBZ and CFF (Cleuvers, 2003; Jos et al., 2003; Pounds et al., 2008; Selderslaghs et al., 2012; Van den Brandhof and Montforts, 2010). Anyway, the study of the joint effects and the potential interactions (e.g. synergism) of chemicals in mixture is of more concern for scientists and regulators. Currently, according to the EU legislation for the environmental risk assessment (ERA) of the human medicine products, no specific mandate for the assessment of mixtures exists. However, in 2011, the SCHER (Scientific Committee on Health and Environmental Risks; opinion on Toxicity and Assessment of Chemical Mixtures, 2011) specifically indicated, the need to take into account the existing scientific information on potential effects of combination of chemicals (including pharmaceuticals) in the environment. In this context, the aim of this study is to contribute to the improvement of the current knowledge in the field of the joint effects of human pharmaceuticals. In fact, the study of binary mixtures can be a suitable screening for the individuation of combinations of particular concern (Backhaus, 2014; Deneer, 2000).

To the best of our knowledge, there are very few data available in literature on the ecotoxicological effects of the mixtures composed of a combination of these compounds (Nieto et al., 2013; Stancova et al., 2014). In recognition of the lack of information concerning the joint effects of these compounds, in this study, we investigated the toxicity of their binary mixtures to *A. fischeri*. The binary mixtures were prepared at an equitoxic ratio corresponding to the individual IC_{50} values obtained in a previous study (Di Nica et al., 2016a). The combined experimental results were compared with

those obtained by the application of two predictive models commonly used in ecotoxicology: Concentration Addition (CA) and Independent Action (IA) (Bliss, 1939; Greco et al., 1992; Loewe and Muischnek, 1926). Finally, the nature of the interactions between the PhACs was also investigated by applying the Combination Index (CI) method (Chou, 2006) to verify the existence of possible synergistic or antagonistic effects. The CI method, commonly applied in pharmacology, has been recently applied in ecotoxicological studies (Boltes et al., 2012; Di Nica et al., 2016b; Rodea-Palomares et al., 2010; Rosal et al., 2010).

2. Materials and methods

2.1. Chemicals

Diclofenac sodium [DCF] (CAS 15-307-79-6), carbamazepine [CBZ] (CAS 298-46-4), and caffeine [CFF] (CAS 58-08-2) were purchased at the highest available purity from Sigma-Aldrich (Milan, Italy). Information about their main physical-chemical properties are reported in Table 1. The stock solutions were prepared according to the method reported in Vighi et al. (2009).

2.2. Tested mixtures

Three binary mixtures (DCF-CFF, DCF-CBZ, and CFF-CBZ) were prepared by mixing the individual chemicals at equitoxic concentrations. The chemicals were mixed in a ratio corresponding to their individual IC_{50} values. The stock solutions of DCF-CFF mixture were directly prepared in the same saline solution used for the toxicity test (2% NaCl). On the contrary, mixtures stock solutions containing CBZ were prepared using the saline solution (2% NaCl) plus DMSO (2% v/v). DMSO was used to increase the solubility in water of CBZ. In the final test samples, the concentration of DMSO solvent did not produce any effect on bacteria. Tests were performed just after preparation of fresh solutions.

A fixed ratio design was used to determine the mixture toxicities (Backhaus et al., 2000). According to this approach, the mixture of interest is analyzed at a constant concentration ratio, while the total concentration of the mixture is systematically varied.

2.3. Testing procedure

Toxicity tests were performed according to the test conditions and operating protocol of the Microtox-system operating manual (Azur Environmental, 1998) using the luminescent marine bacterium *A. fischeri*. The reagents (the freeze-dried bioluminescent bacterium *A. fischeri*) and the other required test solutions were purchased from Ecotox LDS S.r.l. (Milan, Italy). The test was based on the reduction in luminescence after a short-term exposure to the pharmaceuticals (15 minutes). The reduction in luminescence reflected the acute toxic effect of the mixture (ICx) and was measured using a Microtox Model 500 analyzer. The described testing protocol was performed in duplicate using a control and nine different concentrations of the mixtures obtained by serial dilution from a stock solution (diluent = 2% NaCl solution at 20°C). The tested concentrations ranged from 0.39 mg L^{-1} to 100 mg L^{-1} for DCF-CBZ, from 1.42 mg L^{-1} to 726 mg L^{-1} for CFF-CBZ and from 0.035 mg L^{-1} to 2280 mg L^{-1} for DCF –CFF. Prior the tests no adjustment of pH was needed as the pH was in the range of 6–8 according to the EN ISO 11348–3:1999. The tests were repeated twice for DCF-CBZ and CFF-CBZ and three times for DCF-CFF.

2.4. Concentration-response curve fitting

The observed concentration-response data were fitted to a non-linear regression Weibull model (Eq. 1) to quantitatively describe the Concentration Response Curves (CRC) (R Core Team, 2015; drc package, Ritz and Streibig, 2005).

$$1 = (c, \beta) = 1 - exp\{-exp\{ln(\beta_2(ln(c) - ln(\beta)))\}\} \quad (1)$$

where: β_1 and β_2 were the parameters of the model, c was the concentration of the chemicals and I was the fractional response ($0 \leq E \leq 1$) in terms of the inhibition of the luminescence.

2.5. Statistical analysis

The analysis of the statistical parameters of the estimated regression coefficients was performed in order to check the goodness-of-fit of the selected model. The comparison of different fitting models gave negligible differences among models (application of the log likelihood functions and the Akaike Information Criterion). The high significance (*p-value*<0.001) of the statistic parameters ($\beta 1$ and $\beta 2$) clearly indicated the capability of the Weibull model to provide a very good estimation of bioluminescence inhibition (I). The null hypothesis of normality was not rejected (Kolmogorov-Smirnov test: *p-value* is >0.05). IC_x (IC_{50} and IC_{10}) values together with the corresponding confidence intervals (95%) were derived using the R software package" (R Core Team, 2015; drc package; Ritz and Streibig, 2005).

Table 1. Relevant physical chemical properties of the tested compounds

Pharmaceutical groups	***Compounds***	***CAS Nr.***	***Water Sol. (mg L^{-1})***	***pKa***
Anti-inflammatory	Diclofenac sodium [DCF]	15-307-79-6	2425 [a]	4.15 [b]
Anti-epileptic	Carbamazepine [CBZ]	298-46-4	112 [c]	13.9 [d]
SNC stimulant	Caffeine [CFF]	58-08-2	21600 [e]	14 [f]

[a] *Ferrari et al., 2003;* ***[b]*** *Sangster, 1994;* ***[c]*** *Claessens et al., 2013;* ***[d]*** *Jones et al., 2002;* ***[e]*** *Yalkowsky and Dannenfelser, 1992;* ***[f]*** *Martin et al., 1969.*

2.6. Theoretical calculation of the mixture response

The CA and IA models were applied to predict the effect of a mixture (ECx_{mix}). The first one is commonly used for chemicals with similar mode of action (Faust et al., 2003) (Eq. 2).

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{x_i}} \right)^{-1} \quad (2)$$

The mixture components are present in a fixed ratio; thus, it is possible to express the concentration of single chemical as a fraction of the total concentration (p_i). ECx_{mix} is the concentration of the mixture that causes *x%* of the effect, p_i is the fraction (C_i/C_{mix}; C_i = concentration of i^{th} component in the mixture and C_{mix} = total concentration of the mixture) of the i^{th} component in the mixture, ECx_i is the individual concentration of component *i* alone that provokes the same effect (*x%*) as the mixture.

The alternative IA model (Bliss, 1939; Faust et al., 2003) predicts the effect of concentration for those mixtures of chemicals showing different or dissimilar mechanism of action. According to this model, the mixture effect can be calculated using Eq. (3).

$$E(C_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(C_i)) \quad (3)$$

in which $c_{mix}=\sum c_i$; $E(c_{mix})$ is the predicted joint concentration-response relationship provoked by the total concentration of the mixture, and $E(c_i)$ is the effect of the individual i^{th} component when applied individually at concentration c_i .

2.7. Application of Combination Index (CI) equations

The Combination index (CI) equations (Chou, 1976; 2006; Chou and Talalay, 1984) were used to investigate the nature of the possible interactions between chemicals that can lead to possible synergistic or antagonistic effects in *A. fischeri* in response to exposure to the binary mixtures. For *n* chemicals, these equations can be expressed as follows (Eq. 4).

$${}^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n} \left\{ \frac{[D]_j}{\sum_{1}^{n}[D]} \right\}}{(D_m)_j \left\{ \frac{(f_{a_x})_j}{[1-(f_{a_x})_j]} \right\}^{1/m_j}} \quad (4)$$

where: ${}^{n}(CI)_x$ is the combination index for *n* chemicals at *x%* inhibition, $(D_x)_{1-n}$ is the sum of the dose of *n* chemicals that exerts *x%* inhibition in combination, $\{([D]_j/\sum[D]\}$ is the proportionality of the dose of each of *n* drugs that exerts x% inhibition in combination, and $(D_m)_j \{(f_{ax})_j /[1 - (f_{ax})_j]\}^{1/mj}$ is the dose of each drug alone that exerts *x%* inhibition. D_m is the median-effect dose, f_{ax} is the fractional inhibition at *x%* inhibition, and *m* is the slope of the median-effect plot.

The synergistic, additive, and antagonistic effects are indicated by CI < 1, = 1, and > 1, respectively, obtained from Eq. (4). CI represents a special case of the CA model (Backhaus, 2014), where a deviation of CI values from 1 indicates a deviation from additive effects (CI is the ratio between the observed and predicted mixture effects using CA model). The computer program CompuSyn (Chou and Martin, 2005). (Compusyn Inc., USA) was used to calculate the CI values at different effect levels of the tested mixtures (from 0.05 to 0.95 of f_a). The F_a-CI plot (plot of CI values at different concentrations of mixtures versus the fraction affected, f_a) was also obtained.

3. Results and discussion

3.1. Toxicity of the tested mixture

Table 2 reports the individual effects of the concentrations (IC_{10} and IC_{50} values) of the investigated PhACs using the Microtox® test system (duration 15 min). In the study of Di Nica et al. (2016a), the authors indicated that these PhACs show a narcotic mechanism of action towards this organism.

Table 2. Toxicity data of chemicals tested with *A. fischeri* (mg L^{-1}; mean ± 95% confidence intervals in brackets) (Di Nica et al., 2016a)

Chemical	IC_{10}	IC_{50}
CBZ	4.2 (±1.3)	94.0 (±12)
CFF	24.5 (±6.6)	632.0 (±62.7)
DCF	5.9 (±0.8)	15.9 (±1.3)

The experimental bioluminescence response was inhibited by the binary mixtures of PhACs to *A. fischeri* (Weibull function) and is shown in Fig. 1, together with the curves predicted by the CA and IA models. The IC_{50} and IC_{10} (both experimental and predicted values) are reported in Table 3. All of the data were obtained by fitting the experimental results using the Weibull mathematical model, which gave us a good estimate of the effective concentrations obtained (*p-value*<0.05). For DCF-CBZ and CFF-CBZ combinations, the maximum percentage of inhibition was 65% and 57%, respectively. Nevertheless, the entire CRC was obtained with a good approximation with the regression model used here.

The obtained IC_{10} and IC_{50} values ranged between the highest and lowest toxicity of each mixture component (Tables 2 and 3). However, for the CFF-CBZ mixture, the toxicity at lower concentrations (e.g., IC_{10}) was lower than the values observed for the single components. Based on the experimental results, it seems that the tested combinations exert low toxicity towards *A. fischeri*,

with DCF-CBZ > DCF-CFF > CFF-CBZ. These results are not surprising considering that both substances show a low level of toxicity to *A. fischeri* (Table 2). In addition, these substances act as narcotic-type compounds towards this organism (Di Nica et al., 2016a). It is well known that narcotics show an additive-type behaviour when are present in mixtures (Hermens, 1989).

For the DCF-CBZ and DCF-CFF combinations, the predictions of the CA and IA models are very close to the obtained experimental results. In addition, the prediction window (the ratio between IA/CA predictions) is <1, indicating that the two reference models predicted the toxicity of both mixtures equally well. In fact, the variation is very small (0.62 < IA/CA < 0.99) for all of the concentration-response relationships; this led to very small differences in the predictions of the IC_{10} and IC_{50} values between the two models (Table 3). As previously reported, Di Nica et al. (2016a) demonstrated that these compounds can be classified as exhibiting non-polar (CBZ) and polar (DCF) narcotic-type actions on *A. fischeri*. The narcotic-type behaviour of these chemicals could be equated to a similar mode of action towards *A. fischeri*. Könemann (1981) and Hermens et al. (1985) have shown that multicomponent mixtures of non-specifically acting organic substances (narcotics) induce combined effects in aquatic organisms and that the median effective concentrations of their combinations could be predicted fairly well using CA, even for multicomponent mixtures of chemicals. Consequently, this allows us to explain the ability of the CA model to predict the toxicity of binary combinations of DCF-CBZ and DCF-CFF.

On the other hand, in the literature, there are a number of studies reporting that the IA model can predict the toxicity of mixtures containing chemicals with the same or similar mechanisms of action (Backhaus et al., 2004; Syberg et al., 2008). In addition, it has been noted that the predictions of the CA and IA models will be more similar when there are fewer mixture constituents (Drescher and Boedeker, 1995; Villa et al., 2014).

Table 3. Experimental toxicity data (IC_{10} and IC_{50}; mg L^{-1}) for the binary mixtures, together with the 95% confidence intervals (in brackets) and predicted values from CA and IA models

Mixtures	*IC_{10} (Exp)*	*IC_{10} (CA)*	*IC_{10} (IA)*	*IC_{50} (Exp)*	*IC_{50} (CA)*	*IC_{50} (IA)*
DCF-CBZ	5.1 (±1.2)	4.0	4.0	59.2 (±5.4)	55.0	49.8
DCF-CFF	13.8 (±3.0)	14.3	13.3	275.5 (±20.7)	276.3	238.5
CFF-CBZ	47.0 (±10.2)	15.0	9.3	528.3 (±70.3)	362.9	224.2

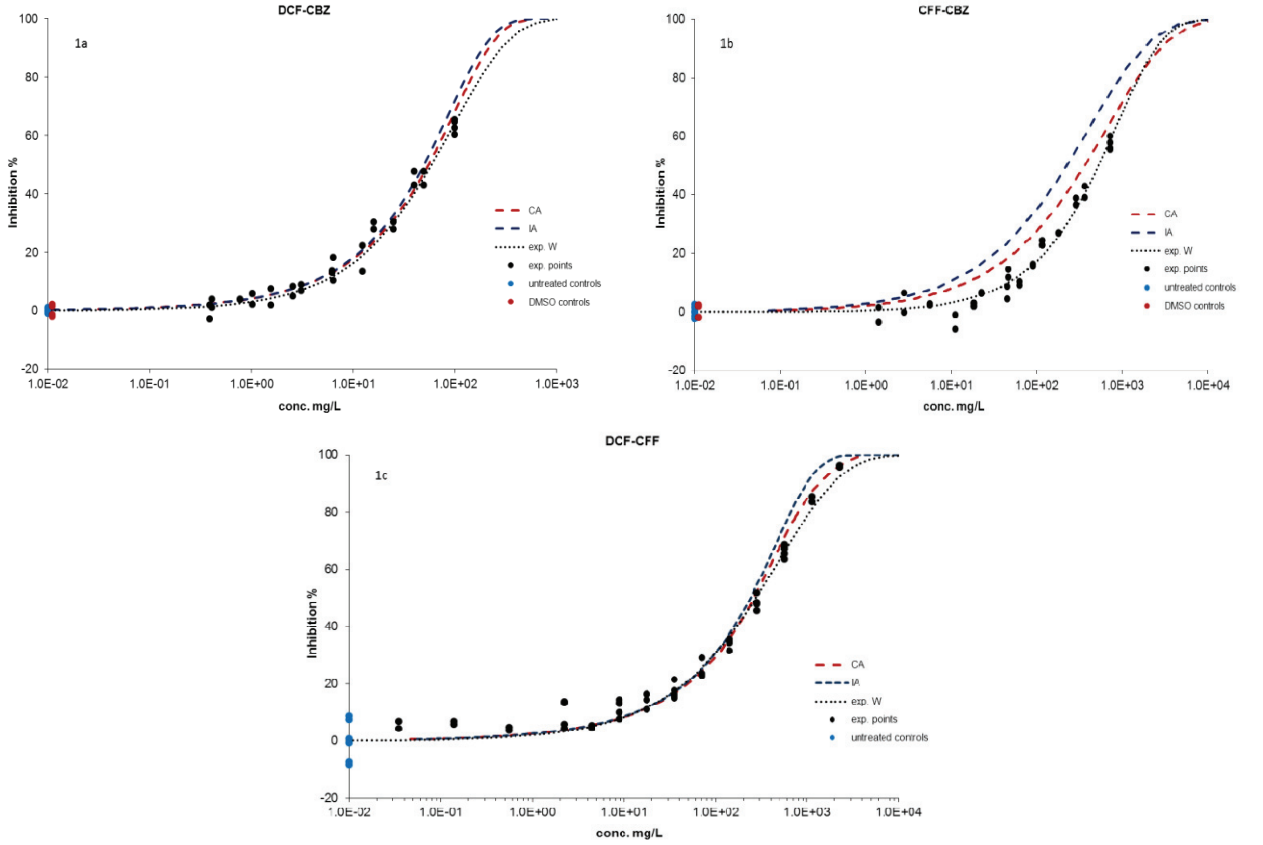

Fig. 1. Predicted values (CA in red lines and IA in blue lines) and observed toxicity values (black dashed line) of the tested binary mixtures of PhACs. (Each test was performed in duplicate and was repeated twice for DCF-CBZ (a) and CFF-CBZ (b) and three times for DCF-CFF (c))

Our results seem to be in contrast with those obtained in the study of Nieto et al. (2013). These authors tested a binary combination of CBZ and DCF on the freshwater shrimp *Atyaephyra desmarestii* and found that the experimental values did not fit with CA and IA models at lower concentrations. In particular, the measured effects were higher than the toxicity predicted by both models; on the contrary, at higher exposure concentrations, the tendency was better approximated by the IA model curve. The different results in the two studies suggest that mixtures can have different behaviours, depending on the tested organism. In some cases, they show dose-response variability: from synergy (at lower concentrations) to less than additive (at higher concentrations) in the case of *A. desmarestii*; in other cases, there is constancy in the dose-response relationship (additivity in the case of *A. fischeri*). For the CFF-CBZ combination, the IC_{10} and IC_{50} experimental values indicated a toxicity of the mixture that was lower than that predicted by both models, suggesting potential interactions between the two components that led to slight antagonistic effects. In the literature, there are a number of studies reporting observed mixture toxicities of PhCAs that significantly deviated from the conceptual expectation of the CA and/or IA models (synergism or antagonism) (Boltes et al., 2012; Brezovsěk et al., 2014; Cleuvers et al., 2003, 2004; Di Nica et al., 2016b; Gonzalez-Pleiter et al., 2013; Rodea-Palomares et al., 2010; Rosal et al., 2010; Shakya, 2011).

The effects resulting from interactions between chemicals (synergistic or antagonistic effects) seem to be independent of the mode of action of the chemicals. For instance, Cleuvers (2003) tested various PhACs that act as non-polar narcotic compounds on algae and *Daphnia*. The author found that the ibuprofen-diclofenac sodium combination produced an additive effect (well predicted by the CA model) on algae, while the effect of the same mixture on *Daphnia* was much higher than that predicted by the CA model (synergism). The same author (Cleuvers, 2004) obtained similar results with a combination of four anti-inflammatory compounds (all non-polar narcotic substances). In the study of Di Nica et al. (2016b), some synergistic and antagonistic effects on *A. fischeri* were observed using different binary combinations of polar and non-polar narcotic compounds (pharmaceuticals of veterinary use).

In an algal growth test using *Synechococcus*, Brezovsěk et al. (2014) observed antagonism with a binary combination of anti-neoplastic pharmaceuticals (5-fluoruracil and imatinib). In their study on the joint effects of fibrates towards *A. fischeri* and *Anabaena* CPB4337, Rodea-Palomares et al. (2010) highlighted a strong antagonistic effect of fenofibric acid and bezafibrate on *Anabaena*. The results obtained by Shakya (2011) indicated that the interaction between high concentrations of a binary mixture of metformin and metoprolol is antagonistic towards *Daphnia magna*. Di Nica et al. (2016b) in their studies on the bioluminescent bacteria *A. fischeri* observed a clearly antagonistic response with a binary combination of acetylsalicylic acid and chlortetracycline, and a dose-dependent antagonism behaviour in other cases, e.g. the binary combination of acetylsalicylic acid and sulfamethizole. For the last combination, the authors found an additive behaviour at lower doses, which became clearly antagonistic at the higher ones.

Based on this evidence, Backhaus (2014) concluded that the synergism and antagonism phenomena are rather specific for the tested mixture and bioassay; in addition, these phenomena are largely confined to mixtures of only a few compounds (two or three compounds). Backhaus argued that these phenomena might be explained by a presence of a sort of buffering effect in multicomponent mixtures, leading to a reduction of the impact of a few synergistic or antagonistic interactions.

The Combination Index (CI) method was applied to verify the nature of the interactions in the tested binary mixtures. Fig. 2 shows the so-called f_a-*CI plots* for the three tested mixtures, and the corresponding CI values at the main representative f_a levels (fractional inhibition with respect to the control) are reported in Table 4. The f_a-*CI plots* allow us to observe the trend of the nature of interactions present in the mixtures at any f_a levels (Chou, 2006).

The analysis of CI values and the f_a-*CI* plot allowed us to confirm the observations of the CA and IA models. In fact, the effects of the DCF-CBZ and DCF-CFF combinations were additive ($0.9<CI<1.10$: absence of interactions between the individual components). The slight synergism observed at low concentrations ($CI<1$) of DCF-CFF is likely due to the higher variability of the experimental data at lower concentrations, as fitted by the applied non-linear regression model. On the contrary, the application of the CI method to the CFF-CBZ combination confirmed the presence of interactions leading an antagonistic effect at low concentrations that becomes near additive behaviour at higher f_a levels. The CI graded symbols refer to the CI ranking that quantifies the magnitude of the synergistic or antagonistic effect, as refined by Chou (2006).

Table 4. Predicted data from the Concentration Addition (CA) and Independent Action (IA) models, as well as the Combination Index (CI) values and the corresponding graded symbols for 10% and the 50% inhibition concentration levels

Mixtures	*ICx*	*CA*	*IA*	*CI*	*CI graded symbols*
DCF-CBZ	IC10	4.03	3.98	0.97	±
	IC50	55.01	49.81	1.10	±
DCF-CFF	IC10	14.25	13.32	0.76	+ +
	IC50	276.28	238.50	1.09	±
CFF-CBZ	IC10	14.97	9.29	2.46	- - -
	IC50	362.94	224.92	1.89	- - -

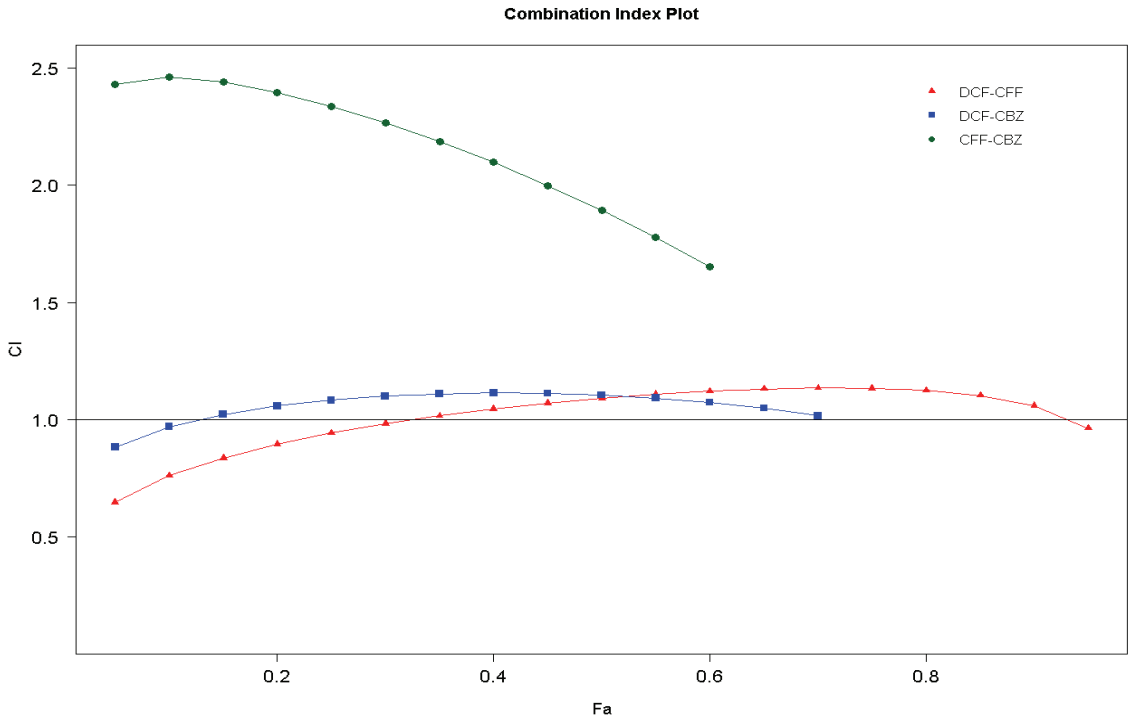

Fig. 2. CI plot (f_a-*CI plot*) for the effect of the binary mixtures of the tested PhACs on *A. fischeri*. The CI values are plotted as a function of the fractional inhibition of bioluminescence (*fa*) using a computer simulation (CompuSyn). CI < 1, = 1 and > 1 indicate the synergistic, additive and antagonistic effects, respectively (Chou and Martin, 2005)

4. Conclusions

In this study, binary combinations of DCF, CBZ and CFF (equitoxic ratio) were tested on the bioluminescent bacterium *Aliivibrio fischeri*. The resulting experimental values (IC_{50} and IC_{10}) indicated a low toxicity of the three tested mixtures (DCF-CBZ > DCF-CFF > CFF-CBZ).

Both the CA and IA models predicted the joint toxicities of DCF-CBZ and DCF-CFF equally well. These results are in accord with the narcotic behaviour of these compounds, which can be assimilated to exert a similar mechanism of action towards *A. fischeri*.

On the other hand, the CA and IA models overestimated the joint toxicity of CFF-CBZ, leading to the hypothesis of a potential interaction between the two components. The use of the Combination Index (CI) method allowed us to confirm both the additive behaviour (no interactions) for the DCF-CFF and DCF-CBZ mixtures (CI values near 1) and the antagonistic behaviour (stronger at low concentrations) for the CFF-CBZ combination.

However, from the obtained results, it can be concluded that there is no particular risk towards this organism with regard to the presence of these substances, at least in the form of their binary mixtures and when present at equitoxic concentrations. In fact, the concentrations of the individual compounds that produce the EC_{50} of the mixtures are at least 4 orders of magnitude higher than the median measured environmental concentrations in European surface waters.

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CHAPTER VII

Toxicity of Quaternary Ammonium Compounds (QACs) to aquatic non-target microorganisms: Tests with single compounds and mixtures

Valeria Di Nica, Julie Gallet, Sara Villa, Valeria Mezzanotte. ACCEPTED in **Ecotoxicology and Environmental Safety**

Toxicity of Quaternary Ammonium Compounds (QACs) as single compounds and mixtures to aquatic non-target microorganisms: experimental data and predictive models

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Abstract

The toxic effects of five quaternary ammonium compounds (QACs) that are widely used as active ingredients in personal care products were assessed using the bioluminescent bacterium *Aliivibrio fischeri* (formerly *Vibrio fischeri*) (Microtox® test system). The experimental results showed a relevant toxicity for almost all of the single QACs, with IC_{50} values lower than 1 mg L^{-1}. Analysis of the mode of action through the application of the Quantitative Structure-Activity Relationship (QSAR) models indicated an a-specific reactivity for most of the QACs toward *A. fischeri*. Only hexadecyl trimethyl ammonium chloride (ATMAC-16) behaved as a polar-narcotic, with a low reactivity toward the bacterial cell membrane. The concentration response curves of the different binary and multicomponent mixtures of QACs were also evaluated with respect to the predictions from the Concentration Addition (CA) and Independent Action (IA) models. For almost all of the binary and multicomponent mixtures (7 out of 11 mixtures tested), an agreement between the experimental and predicted IC_x was observed and confirmed via application of the Model Deviation Ratio (MDR). In four cases, some deviations from the expected behaviour were observed (potential antagonistic and synergistic interactions) at concentrations on the order of hundreds of µg L^{-1}, which could be of environmental concern, especially in the case of synergistic effects. The analysis of aquatic ecotoxicity data and the few available values of the measured environmental concentrations (MECs) from the literature for wastewaters and receiving waterbodies suggest that a potential risk toward aquatic life cannot be excluded.

Keywords: *Aliivibrio fischeri*; cationic surfactants; mixture toxicity; Concentration Addition; BAC-12; risk.

Abbreviations

ATMAC-16: hexadecyl trimethyl ammonium chloride

BAC-12: benzyl dimethyl dodecyl ammonium chloride

CA: Concentration Addition

C10TAB: decyl trimethyl ammonium bromide

C14TAB: tetradecyl trimethyl ammonium bromide

DADMAC-10: didecyl dimethyl ammonium chloride

HWW: hospital wastewater

IA: Independent Action

MEC: measured environmental concentration

MDR: Model Deviation Ratio

MoA: Mode of Action

QAC: Quaternary Ammonium Compound

QSAR: Quantitative Structure-Activity Relationship

SAA: Surface Active Agent

WWTP wastewater treatment plant

T_{con}: total connectivity index

TU: Toxic Unit

1. Introduction

Surfactants (Surface Active Agents - SAAs) are the main ingredients in detergents and disinfectants and comprise a broad group of chemical compounds (mainly anionic, non-ionic and cationic compounds) that are used in many areas of human activity because of their specific physicochemical properties. Among the cationic types, Quaternary Ammonium Compounds (QACs) are most widely used for their strong cationic surface activity in products such as fabric softeners, anti-statics, disinfectants, biocides, detergents, phase transfer agents and personal care products (Tsai and Ding, 2004; Kreuzinger et al., 2007; Lara-Martín et al., 2010). In 2013, in EU countries, the total production of SAAs was 2,985 kt (European Committee of Surfactants and their Organic Intermediates - CESIO: Statistics 2013), 8% of which (229 kt) was cationic surfactants.

Benzyl alkyl dimethyl ethyl ammonium compounds (BACs) with alkyl chain lengths from C12 to C18, alkyl trimethyl ammonium compounds (CTMACs) (C12–C18) and dialkyl dimethyl ammonium compounds (DADMACs) (C8-C18) are the most widely used QACs and are consequently found in natural environments (Zhang et al., 2015).

All SAAs show marked biological activities, but among these, QACs are the most toxic (Ivanković and Hrenović, 2010; Qv and Jiang, 2013; Ying, 2006). The biocidal mechanism of QACs typically consists of the disruption of cell membranes by their long alkyl chain (Xia and Onyuksel, 2000), causing a leakage of the intracellular constituents (Denyer, 1995; Ioannou et al., 2007; Salton, 1968; Sutterlin et al., 2008). Several authors studied the acute toxic effects of these compounds on different aquatic organisms (Chen et al., 2014; García et al., 2001; Ge et al., 2010; Kreuzinger et al., 2007; Sandbacka et al., 2000; Zhu et al., 2010); nevertheless the available toxicity data on QACs are still fragmentary, especially regarding their possible interactions with other similar chemicals. There is accepted evidence that the toxicities of mixtures may be higher than the toxicities of the individual compounds, even if all the compounds are present at low concentrations, even below

their individual EC_{10} values (Backhaus, 2014; Cedergreen, 2014; Gonzalez-Pleiter et al., 2013; Villa et al., 2012).

As a very high number of mixtures can be potentially generated in a given environmental compartment (e.g. surface water body), the experimental toxicity assessment of all of them is not feasible. The use of some predictive approaches becomes necessary. Two basic ecotoxicological models are accepted by the scientific community: Concentration Addition (CA) (Loewe and Muischnek, 1926) and Independent Action (IA) (Bliss, 1939). These concepts enable a conceptual link between the toxic effects of single chemicals and their mixture through the knowledge of the main parameters of single components such as toxicity, concentration-response relationships and mode of action (MoA) (component-based approach). The choice of the most suitable model is mainly based on the knowledge of the MoA as CA is basically applied when chemicals in mixture behave similarly towards a biological endpoint and IA can be more appropriately applied when they act dissimilarly. Anyway, both models are built on the assumption of absence of chemical and/or biological interactions among substances (Backhaus, 2014).

The presence of disinfectants and detergents from households and, especially, hospitals may contribute to the overall ecotoxicity of treated effluents (Boillot and Perrodin, 2008; Emmanuel et.al., 2004). The concentration of disinfectants in hospital wastewater (HWW) ranges between 2 and 200 mg L^{-1} and is dependent on the size of the hospital (Hartemann et al., 2005). Hospital wastewaters are more intrinsically toxic, by at least 5–15 times, than municipal wastewater (Leprat, 1998). Hartemann et al. (2005) concluded that a hospital with 1,000 beds and an internal laundry can generate a toxic impact on the aquatic environment comparable to the impact from a town with a population of 10,000 inhabitants . Despite their specific nature, HWW is often directly discharged without any prior treatment into the municipal wastewater network, where it is mixed with urban sewage and collectively sent to municipal wastewater treatment plants (WWTPs) to be co-treated (Brown et al., 2006; Emmanuel et al., 2004; Kümmerer, 2001; Langford and Thomas, 2009;

Verlicchi et al., 2010). This common practice of co-treatment is not considered adequate by many authors because it generates a diluted mixture of different types of emerging contaminants and toxic substances (Pauwels and Verstraete, 2006; Verlicchi et al., 2010; Vieno et al., 2007) that are not sufficiently removed by conventional wastewater treatment methods (Heberer 2002; Joss et al. 2005; Ternes, 1998). In this context, a potential risk to aquatic organisms in the receiving waterbody could be related to exposure to the effluent discharge (Emmanuel et al., 2005; Orias and Perrodin, 2013).

The aim of this study is to contribute to the knowledge of the toxic effects of five common QACs on the bioluminescent bacterium *A. fischeri* using the Microtox® test system.

Microtox® test is a standardized method accepted by organizations like the International Organization for Standardization (ISO), the Organization for Economic Co-operation and Development (OECD) and the U.S. Environmental Protection Agency (USEPA) (Parvez et al., 2006; Zeng et al., 2017) and is widely applied to investigate the toxicity effects of chemical compounds and mixtures (Backhaus et al., 2000a; Escher et al., 2017; Vighi et al., 2009). This method allows ease, reproducibility, speed and cost effectiveness of application and recently has been indicated as a good candidate for first screening tests in a tiered approach for the hazard assessment of complex waste materials (Deprez et al., 2012; Weltens et al., 2012, 2014).

Considering that the co-occurrence of chemicals is more likely than their presence alone in the environment, we investigated the toxicity of mixtures and the nature of their potential interactions. Experimental and predictive toxicities of binary mixtures were compared by applying the CA and IA models to verify potential interactions among compounds. It was proven that using binary mixtures allowed for the best understanding of combined effect issues (Deneer, 2000). At the chosen concentrations, the Microtox® test was highly suitable.

2. Materials and methods

2.1. Tested compounds and mixtures

The five tested compounds are: benzyl dimethyl dodecyl ammonium chloride [BAC-12]; decyl trimethyl ammonium bromide [C10TAB]; didecyl dimethyl ammonium chloride [DADMAC-10]; hexadecyl trimethyl ammonium chloride [ATMAC-16] and tetradecyl trimethyl ammonium bromide [C14TAB]) (Table S1).

These QACs, well known for their antibacterial properties, were selected for their potential harm to aquatic organisms, their widespread use and their occurrence in effluents from WWTPs (Zhang et al., 2015).

All chemicals were purchased in the highest available purity from Sigma-Aldrich (BAC-12:>99%; ATMAC-16: >98%; C14TAB: >99% and C10TAB: >98%) and from Dr. Ehrenstorfer GmbH, Augsburg – Germany (DADMAC-10: >98%).

Stock solutions were prepared in a saline solution (NaCl 2%) according to Vighi et al. (2009). The pH, ranging between 6.60 and 7.08, fell within the range (6.00-8.50) reported in the procedure DIN EN ISO 11348–3. The tested concentrations of single compounds varied between 0.004 mg L^{-1} and 25 mg L^{-1}.

Ten binary mixtures and a multi-compound mixture were also tested.

QACs have been found in effluent-receiving waterbodies at concentrations ranging from tens to hundreds µg L^{-1} (Ferrer and Furlong, 2001; Kreuzinger et al., 2007; Olkowska et al., 2013), thus to reproduce realistic conditions, we combined the selected compounds at low concentrations, corresponding to their individual IC_{10} value. The compounds were mixed according to a fixed ratio design at equitoxic ratios, as follows:

- Binary Mixture 1 (BM1): BAC-12 + DADMAC-10
- Binary Mixture 2 (BM2): BAC-12 +ATMAC-16

- Binary Mixture 3 (BM3): BAC-12 + C14TAB
- Binary Mixture 4 (BM4): BAC-12 + C10TAB
- Binary Mixture 5 (BM5): DADMAC-10 + ATMAC-16
- Binary Mixture 6 (BM6): DADMAC-10 + C14TAB
- Binary Mixture 7 (BM7): DADMAC-10 + C10TAB
- Binary Mixture 8 (BM8): ATMAC-16 + C14TAB
- Binary Mixture 9 (BM9): ATMAC-16 + C10TAB
- Binary Mixture 10 (BM10): C14TAB + C10TAB
- Multi-compound Mixture (MM): BAC-12 + DADMAC-10 + ATMAC-16 + C14TAB + C10TAB

Total concentrations were systematically varied with an adequate dilution factor keeping constant the concentration ratio of the compounds in the mixtures. The minimum and maximum concentrations corresponded to ∑0.01TU and ∑50TU, respectively, with 1TU= IC10 (TU=Toxic Unit).

2.2. Testing procedure

All tests were performed according to the testing conditions and operating protocol of the Microtox® system operating manual, Acute Toxicity Basic Test procedures (Azur Environmental 1998), which is based on the measurement of the reduction in the bioluminescence emitted by the marine bacterium *Aliivibrio fischeri* when exposed to toxic agents. Frozen, dry bacteria and reconstitution solution were purchased from Ecotox LDS s.r.l. (Milan, Italy). The luminescence inhibition (f_a=affected fraction) was measured using a Microtox® Model 500 Analyzer in acute mode. Bacteria were exposed to the test chemicals for 5 and 15 minutes. The temperature during the exposure was 15 °C. Due to the negligible difference between the results obtained from the different exposure times, only the 15-minute exposure data have been reported and used for the

final elaborations. Toxicity tests were carried out in duplicate on a control and nine different concentrations of stock solutions from a serial dilution in 2% NaCl solution (20 °C). In all tests, we referred to the nominal (= initial) concentrations of QACs. Each test was performed at least twice with a total of 4 replicates for each concentration tested .

2.3. Statistical analysis

The probability of a toxicity event, I (bioluminescence inhibition), as a function of the chemical concentration, c, and of a vector of parameters, (β_1; β_2), was obtained using the Weibull non-linear regression model (eq. 1):

$$I = (c, \beta) = 1 - \exp\{-\exp\{\ln(\beta_2(\ln(c) - \ln(\beta_1))\}\} \quad \text{[eq. 1]}$$

Thus, all the concentration–response relationships for the single toxicant and for their mixtures were estimated.

To check the goodness-of-fit of the selected model, the analysis of the statistical parameters of the estimated regression coefficients was performed. The comparison of different non-linear regression models on the basis of analysis of goodness-of-fit with the log likelihood functions and the Akaike Information Criterion (AIC) gave negligible differences among models, and the Weibull model provided good estimates for β_1 and β_2 parameters. The analysis of the residues of the applied model was also performed by applying the normality hypothesis using the Kolmogorov-Smirnov test. In all cases, the null hypothesis of normality was not rejected (null hypothesis of normality are rejected if the *p-value* is <0.05) (Tables S1a-S1d).

All the statistical analyses and the I_x (100 * I %) values, with the related confidence intervals at 95%, were obtained using R® software (R Core Team, 2015; drc package: Ritz and Streibig, 2005).

2.4. Predictive models for mixture toxicities

Concentration Addition (CA) and Independent Action (IA) models were applied to predict the effects of the tested mixtures (ECx_{mix}). These are commonly used for mixtures of chemicals when the different compounds have the same or different mode/mechanism of action (Loewe and Muischnek 1926; Bliss 1939). Both approaches evaluate mixture toxicities in an additive way: additivity of concentrations (CA) and additivity of effects (IA). The underlying idea of CA's mathematical concept is that if chemicals that have the same mechanism of toxicity are mixed at an equitoxic ratio, they may be considered as the same chemical towards the same biological target. For a multi-compound mixture, the mathematical CA concept can be expressed as follows (Berenbaum, 1985):

$$\sum_i^n \frac{C_i}{EC_{xi}} = 1 \qquad \text{[eq. 2]}$$

where n is the number of compounds in the mixture causing the x % of the total effect; c_i is the concentration of the i^{th} component; and ECx_i is the concentration of the respective component that produces the same effect when applied singularly.

The fraction c_i/ECx_i represents the Toxic Units (TUs) of each component (Sprague, 1970), and the concentration of each component of the mixture is represented as a fraction of the equi-effective individual concentration. Consequently, if the sum of the TUs is 1, the behaviour of the mixture is additive.

The IA concept is based on the idea that the toxicity of each component in a mixture is not influenced by the toxicity of the other compounds, assuming that they act differently. Thus, single responses are added, and the probability that chemicals act simultaneously on the same target should be subtracted. This mathematical concept is expressed as follows (eq. 3):

$$E(C_{mix}) = 1 - \prod_{i=1}^{n}(1 - E(C_i)) \qquad \text{[eq. 3]}$$

where $E(C_{mix})$ is the predicted effect of the total concentration of the mixture; C_{mix} is the total concentration of the mixture; and $E(Ci)$ is the effect that the i^{th} compound would have singularly.

2.5. Model Deviation Ratio (MDR)

To evaluate potential deviations of the experimental toxicities from predictions, the accuracy of the predictive approaches was quantified by applying the Model Deviation Ratio (MDR) (Belden et al., 2007) to the CA and IA models. The MDR is the ratio between the observed and the predicted mixture toxicities. A compliance with the predictive approaches is assumed if the experimental values fall within half or double the predicted value ($0.5 \leq MDR \leq 2$).

3. Results and discussion

The concentrations of the single QACs and their mixtures causing decreases of 10% and 50% of the bioluminescence emitted by bacteria (IC_{10}; IC_{50}) and their confidence interval values at 95% were derived and reported in Tables 2 and 3, respectively. In both cases, results are shown in decreasing order of toxicity (increasing value of IC_{50}).

From the obtained results, the analysis of the statistical parameters gave a good estimate of the effective concentrations. All the regression coefficients obtained using the Weibull mathematical model were statistically significant, as the null hypothesis that the effect I is not linked to the concentration c was rejected. The *t-values* indicated that all the obtained β_1 and β_2 parameters were significantly (*p-value*<0.05) distant from zero.

3.1. Single QAC toxicity

The resulting IC_{50} values for almost all the tested surfactants were below 1 mg L^{-1} (Table 1). The most toxic compounds are BAC-12 and DADMAC-10, with resulting IC_{50} values of 0.17±0.03 mg L^{-1} and 0.40±0.06 mg L^{-1}, respectively, and IC_{10} values of 0.07±0.03 mg L^{-1} and 0.08±0.03 mg L^{-1},

respectively. The C14TAB and the ATMAC-16 also show a very high toxicity that was on the order of hundreds of μg L^{-1}. The least toxic compound is the C10TAB, with IC_{50} and IC_{10} values of 2.83±0.7 mg L^{-1} and 0.91±0.3 mg L^{-1}, respectively. To the best of our knowledge, no IC_{50} data for C10TAB acting on *A. fischeri* is present in the literature; however, the results obtained for the other QACs tested are in good agreement with data from previous studies (Garcia et al., 2001; Tezel, 2009).

Table 1 Toxicity values (in increasing order of IC_{50}) for the studied QACs (mg L^{-1}; mean ± 95% confidence intervals in brackets), slope values as the ratio between IC_{80} and IC_{20} values and statistical parameters of the selected regression models.

	IC_{10}	IC_{50}	*Slope*	Mod. par		*Std. Error*	*t-value*	*p-value*	*n-1*
BAC-12	0.07 [0.05; 0.08]	0.17 [0.14; 0.2]	4.78	β_1	0.13	0.011	11.99	<0.001	63
				β_2	-1.30	0.16	-7.85	<0.001	
DADMAC-10	0.08 [0.04; 0.11]	0.40 [0.33; 0.46]	5.73	β_1	0.55	0.045	12.27	<0.001	38
				β_2	1.10	0.136	8.32	<0.001	
C14TAB	0.19 [0.11; 0.28]	0.74 [0.63; 0.84]	4.10	β_1	0.96	0.075	12.73	<0.001	33
				β_2	1.4	0.207	6.76	<0.001	
ATMAC-16	0.47 [0.37; 0.56]	0.99 [0.92; 1.06]	1.58	β_1	1.10	0.039	29.69	<0.001	55
				β_2	2.50	0.293	8.53	<0.001	
C10TAB	0.91 [0.62; 1.21]	2.83 [2.11; 3.55]	6.42	β_1	2.00	0.209	9.56	<0.001	46
				β_2	-1.10	0.185	-5.76	<0.001	

According to the relationship between the QACs' molecular characteristics and their related acute toxicity on aquatic organisms reported in previous studies, their toxicity decreases with increasing alkyl chain length above 14 carbon atoms (Jing et al., 2012; Qiu et al., 2013; Zhu et al., 2010, Cheng and Jiang, 2006). On the contrary, below 14 carbon atoms, the opposite trend is reported: toxicity and alkyl chain length are positively correlated (Dorn et al., 1993; Isomaa et al., 1986; Scaife, 1985; Wong et al., 1997). This justifies the lower toxicity of C10TAB to *A. fischeri* compared to C14TAB. Conversely, the further increase of the alkyl chain length in ATMAC-16 resulted in a decrease of toxicity to *A. fischeri.*

However, several other molecular descriptors, e.g., the polarizability tense of the molecule, the positive net atomic charge on a hydrogen atom and the entropy, contribute to the overall toxicity of QACs (Collina et al., 2007; Franzetti et al., 2008; Zhu et al., 2010). The high toxicity of BAC-12 and DADMAC-10, both of which have long alkyl chains (21 and 22 carbon atoms, respectively), could be explained by the presence of a benzyl group, as a substitute for a methyl group, in the alkyl chain (Garcia et al., 2001) of BAC-12 and by the peculiar double chain structure of DADMAC-10 (Yoshimatsw and Hiyama, 2007). Geometric and topological structure gives double chain QACs, such as DADMAC-10, a higher capacity for crossing cell membranes (Jing et al., 2012).

Table 1 includes slope values, calculated as the ratio between IC_{80} and IC_{20}. Except for ATMAC-16, all the compounds show a similar slope for the concentration-response curves with IC_{80}/IC_{20} values ranging between 4.10 and 6.42, suggesting similar modes of action (MoA) for the tested compounds towards *A. fischeri* (Backhaus et al., 2004; Geiger et al., 2016). Only the slope of the concentration-response curve of ATMAC-16 is different, where $IC_{80}/IC_{20} = 1.58$.

To investigate the MoA of the selected compounds, the Quantitative Structure–Activity Relationship (QSAR) equations specifically developed to predict the effect of polar and non-polar narcotics on this organism (Vighi et al., 2009) were applied. Non-polar narcosis, also called baseline toxicity, is the toxic effect exerted by an inert chemical without a specific mode of action towards an organism and is related to hydrophobicity, which is defined on the basis of the n-octanol/water partitioning coefficient (K_{ow}). Polar narcosis is generally slightly stronger than non-polar narcosis, although it is not related to a specific mode of action (less inert chemicals). On the basis of the Verhaar classification scheme and its updates (Verhaar et al., 1992; Tremolada et al., 2004, Enoch et al., 2008), compounds can also be classified as reactive or as specifically acting chemicals. Reactive chemicals show an enhanced response relative to the baseline toxicity but react unselectively with common chemical structures in biomolecules. In contrast, the toxicity of specifically acting chemicals is due to interactions with specific receptor molecules.

As shown in Fig. 1, the tested compounds are more toxic than non-polar narcotics (inert chemicals) and polar narcotics (less inert chemicals). In fact, the observed experimental IC_{50} values, expressed as Log($1/IC_{50}$) (mmol L^{-1}), are all above the line representing the polar narcotic toxicity (Fig. 1). For the C10TAB, the value of Log K_{ow} was calculated using the KOWWIN™ v1.68 model in the EPI (Estimation Program Interface) Suite™ program that estimates the log octanol-water partition coefficient by an atom/fragment contribution method.

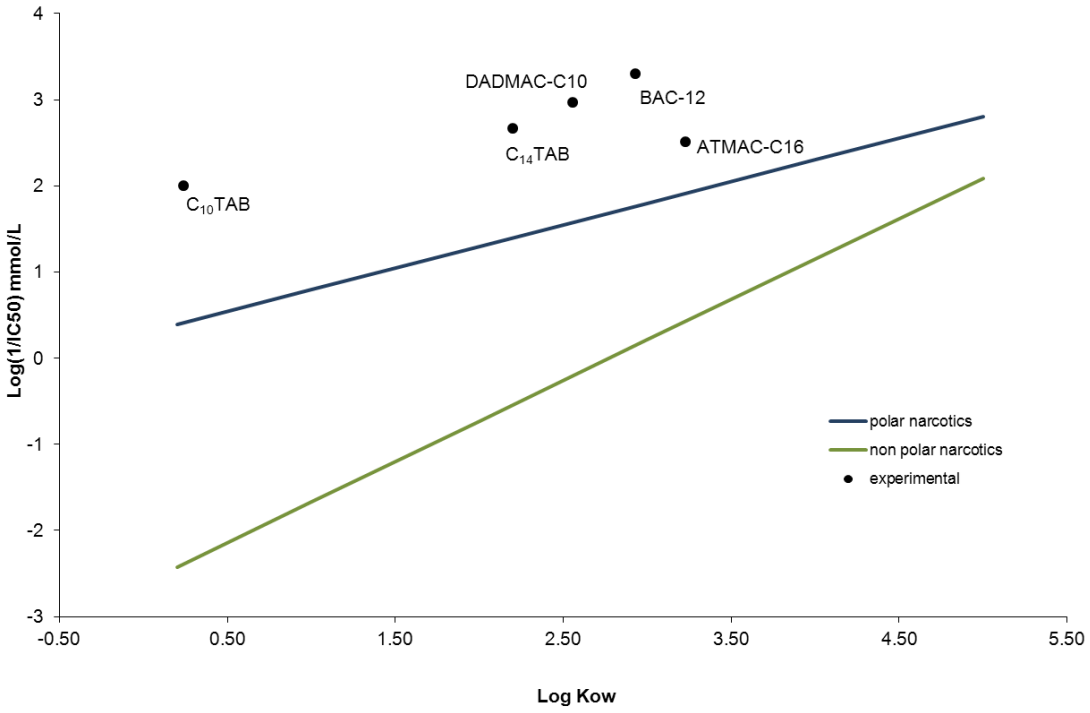


Fig. 1. Log($1/IC_{50}$) (in mmol L^{-1}) as a function of Log K_{ow} for the selected QACs. Blue and green curves represent the regression line between the toxicological endpoint for *A.fischeri* (bioluminescence) and the K_{ow} values for polar and non-polar narcotic compounds, respectively (Vighi et al., 2009).

Table S3 reports the QSAR predictions of the IC_{50} (mg L^{-1}) values toward *A. fischeri* and the toxicity ratio (TR) between the estimated polar or non-polar narcosis IC_{50} and the experimental IC_{50} for each QAC (TR_1= $IC_{50nonpolnarcotic}/IC_{50experimental}$; TR_2= $IC_{50polarnarcotic}/IC_{50experimental}$). From the analysis of TR_2, the toxicity of ATMAC-16 towards *A. fischeri* seems to be of polar narcotic-type. However, the observed difference in TR_2 could fall within the normal range of uncertainties potentially affecting the results, namely variability of literature data of K_{ow} values and uncertainties of QSAR model results (Tremolada et al., 2004).

The specific target of QACs, as they are biocides, are bacteria. Their toxic action against bacterial cells is mainly due to the ionic interactions among the cationic quaternary nitrogen, the head groups of the acidic phospholipids of the membrane and the negatively charged structural and enzymatic bacterial proteins (Gilby and Few, 1960; Takasaki et al., 1994; Maillard, 2002; Gilbert and Moore, 2005). Membranes are destabilized, leading to a leakage of intracellular low-molecular-weight material, the degradation of proteins and nucleic acids (Salton 1968) and rapid cell lysis (Chapman, 2003). According to this MoA and the Verhaar classification scheme, the selected QACs seem to act as aspecific reactive compounds. Only ATMAC-16 seems to have a lower reactivity toward the cell membrane of *A. fischeri*, likely due to the greater length of its hydrophobic tail, as previously discussed. In Jing et al. (2012), ATMAC-16 showed one of the highest values of a molecular descriptor inversely related to the capacity of the molecules to enter the cell membrane namely the molecular total connectivity index (T_{con}). Such lower capacity could explain the lower reactivity of ATMAC-16 on *A. fischeri*.

The knowledge of the MoA of substances is one of the basic concepts of predicting the toxicity of mixtures. Mixtures can be made of compounds acting in a similar or dissimilar MoA, or more likely both. By definition, the toxicity of mixtures of compounds with a similar MoA should be well described by the CA model, and the final combination effects are expected to be larger than the effect of each mixture component applied singularly. However, this is only the case for substances

that do not interact and that do not influence the MoA of each other, namely substances acting in additive way. The CA approach can also be applied when no information is available on the mode of action of the toxicants, which involves an acceptable and pragmatic, conservative approach (Kortenkamp et al., 2009).

In this study, all the single QACs, except ATMAC-16, seem to exert their toxicity towards *A. fischeri* similarly, thus the CA model is indicated as the most suitable approach for the prediction of their combined toxicity. Conversely, the effect of binary mixtures containing ATMAC-16 should be predicted using the IA approach.

For the selected QACs, a high acute toxicity is also reported toward other aquatic organisms (algae, *Daphnia*, and fish), with IC_{50} values mostly below 1 mg L^{-1} (Farrell et al.,1998; Garcia et al., 2001; Jing et al., 2012; Kreuzinger et al., 2007; Lavorgna et al., 2016; Sandbacka et al., 2000; Zhu et al., 2010). Many of the acute toxicity values for *A. fischeri* are on the same order of magnitude as those for algae and fish and one order of magnitude above those for daphnids. It should be highlighted that the reported literature data often referred to homologs of different alkyl chain lengths, so direct comparisons are not always correct. Sensitivity towards a single QAC may vary from one species to another. For example, the acute toxicity of C14TAB is 91 µg L^{-1} for *D. magna* (Sandbacka et al., 2000), 180 µg L^{-1} for algae (Zhou et al., 2010), 740 µg L^{-1} for *A. fischeri* (this study) and 2.51 mg L^{-1} for fish (Sandbacka et al., 2000). For other QACs, such as ATMAC-16, the toxicity is on the same order of magnitude (hundreds of µg L^{-1}) for all the tested organisms, except daphnids (tens of µg L^{-1}). However, toxicity data for this class of compounds is still fragmentary, especially regarding their effects from long term exposure (chronic toxicity).

The comparison of acute toxicity data from *A. fischeri* and other hierarchical levels of aquatic systems allows us to assert the high sensitivity of microorganisms to the contamination of QACs. The high sensitivity of *A. fischeri* was also observed by Ortiz de Garcìa et al. (2014) for different categories of contaminants, such as pharmaceutical and personal care products. Other authors

reported on the comparability of results for *A. fischeri* tests and other aquatic organism assays (Di Nica et al., 2017a; Kaiser, 1998; Parvez et al., 2006).

Bacteria play important ecological roles in all ecosystems, e.g., the nutrient regeneration, but they are not included as representative test organisms for the assessment of toxic effects of pollutants in aquatic environments. In fact, the procedures for the environmental risk assessment of new and existing chemicals (EC, 2003) are currently based on assessing toxicity effects for algae, *Daphnia* and fish, as organisms representative of the main trophic levels of aquatic ecosystems (primary producers, primary consumers, and secondary consumers). Their exclusion as a test organism could lead to the adoption of insufficient protective measures for the aquatic environment in some cases.

3.2. Mixture toxicity results

The IC_{50} for binary mixtures span from 0.33±0.04 mg L^{-1} (BM1) to 4.37±0.50 mg L^{-1} (BM7), and the multi-compound mixture was in the same range (IC_{50} = 1.90±0.20 mg L^{-1}), as shown in Table 2.

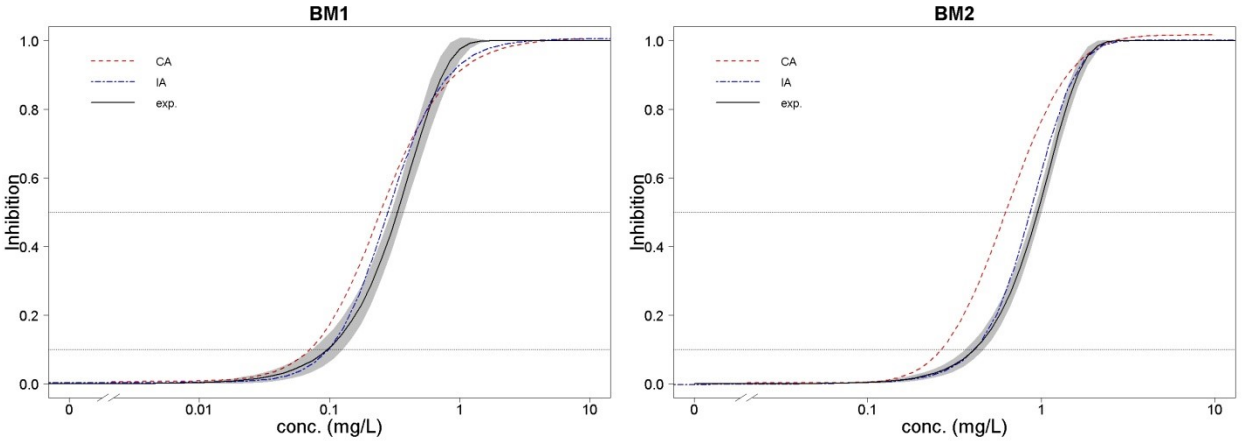
Table 2 Mean values (in increasing values of IC_{50}) and confidence intervals of IC_{10} and IC_{50} (sum of the chemicals in equitoxic mixture) in 15 min acute Microtox test, slope (IC_{80}/IC_{20} ratios) and statistical parameters of the selected regression model.

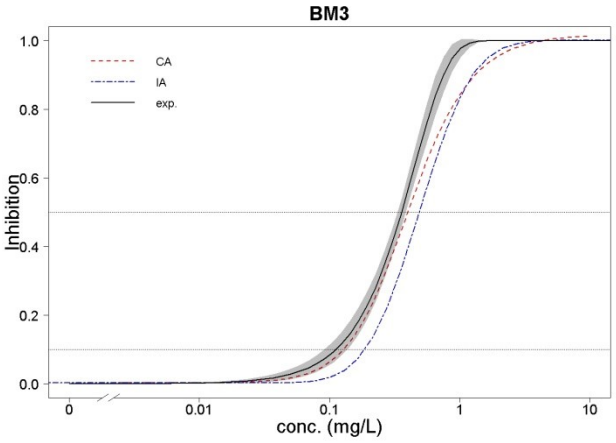
	IC_{10} (mg L^{-1})	IC_{50} (mg L^{-1})	slope	Parameter estimates		Std. Error	*t-value*	*p-value*	*n-1*
BM1	0.10 [0.07 - 0.12]	0.33 [0.30 - 0.37]	3.68	β_1	0.424	0.024	17.545	<0.001	24
				β_2	1.518	0.146	10.368	<0.001	
BM3	0.11 [0.09 – 0.14]	0.36 [0.33 - 0.38]	3.38	β_1	0.449	0.018	25.319	<0.001	33
				β_2	1.621	0.132	12.299	<0.001	
BM8	0.33 [0.22 - 0.44]	0.81 [0.71 - 0.92]	2.56	β_1	0.968	0.068	14.306	<0.001	32
				β_2	2.100	0.356	5.902	<0.001	
BM2	0.41 [0.36 - 0.46]	0.95 [0.90 - 1.00]	2.40	β_1	1.120	0.028	40.106	<0.001	22
				β_2	2.251	0.133	16.937	<0.001	
BM4	0.10 [0.04 - 0.15]	0.98 [0.80 - 1.16]	11.43	β_1	1.536	0.143	10.766	<0.001	27
				β_2	0.811	0.083	9.816	<0.001	
BM6	0.35 [0.27 - 0.42]	1.10 [1.02 - 1.18]	3.34	β_1	1.372	0.048	28.418	<0.001	27

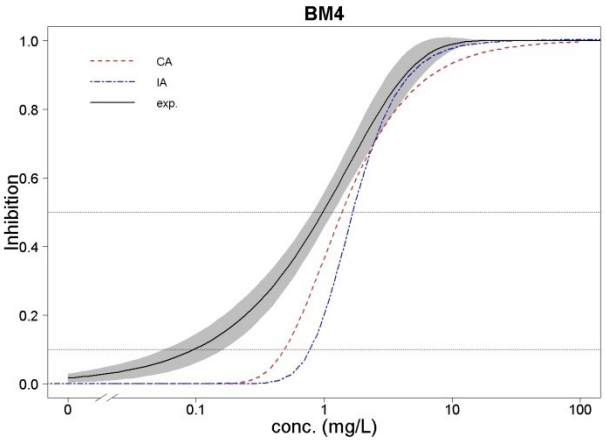
				β_2	1.638	0.125	13.143	<0.001	
BM5	1.13 [1.05 - 1.20]	1.58 [1.51 -1.64]	1.74	β_1	1.423	0.029	48.946	<0.001	25
				β_2	-3.571	0.335	-10.643	<0.001	
BM9	0.87 [0.65 -1.09]	2.42 [2.20 - 2.63]	2.91	β_1	2.946	0.136	21.624	<0.001	33
				β_2	1.848	0.203	9.108	<0.001	
BM10	0.38 [0.28 - 0.49]	2.58 [2.37 - 2.79]	7.38	β_1	3.735	0.135	27.644	<0.001	19
				β_2	0.988	0.056	17.796	<0.001	
BM7	1.108 [0.79- 1.43]	4.37[3.87- 4.87]	5.00	β_1	5.678	0.295	19.251	<0.001	27
				β_2	1.228	0.098	12.521	<0.001	
MM	0.68 [0.54 - 0.82]	1.89 [1.74 - 2.05]	2.92	β_1	2.308	0.096	23.944	<0.001	34
				β_2	1.845	0.160	11.520	<0.001	

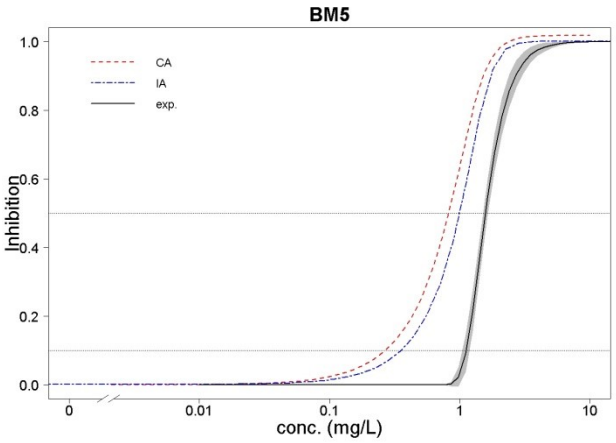
3.3. Predictability of mixture toxicities

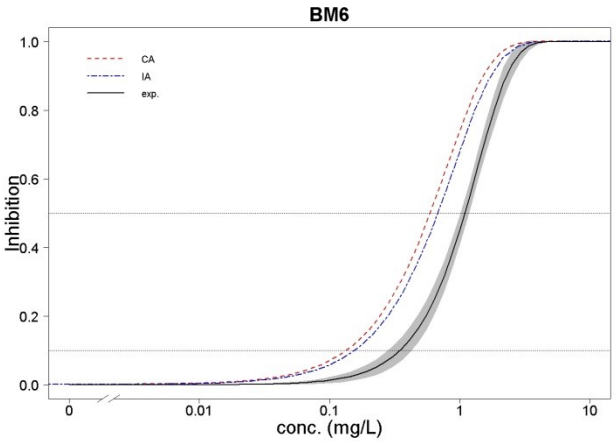
To evaluate the predictability of mixture toxicities from the knowledge of single chemical parameters, the experimental concentration-response curves (CRCs) for the BMs and MM were compared with those predicted using the CA and IA models. Fig. 2 shows the experimental CRCs for combinations of QACs acting additively, i.e. in good agreement with predictions (BM1; BM2; BM3; BM8; BM9; BM10 and MM), or deviating from the conceptual expectations, i.e. more than additive (BM4) and less than additive (BM5, BM6 and BM7) mixture toxicities, along with their 95% confidence intervals. In Table S4, the predicted IC_{10} and IC_{50} values are reported.

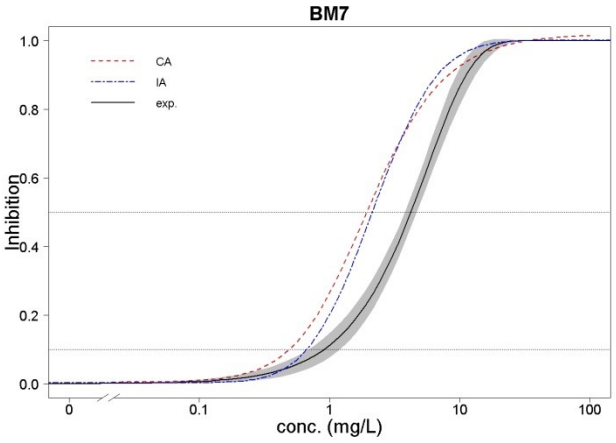


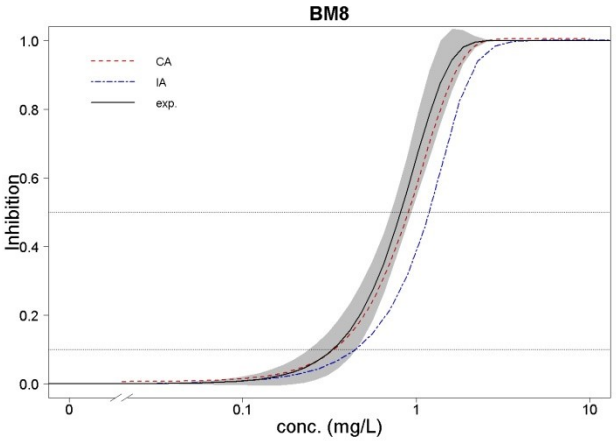

BM3
CA
IA
exp.
Inhibition
conc. (mg/L)


BM4
CA
IA
exp.
Inhibition
conc. (mg/L)


BM5
CA
IA
exp.
Inhibition
conc. (mg/L)


BM6
CA
IA
exp.
Inhibition
conc. (mg/L)


BM7
CA
IA
exp.
Inhibition
conc. (mg/L)


BM8
CA
IA
exp.
Inhibition
conc. (mg/L)

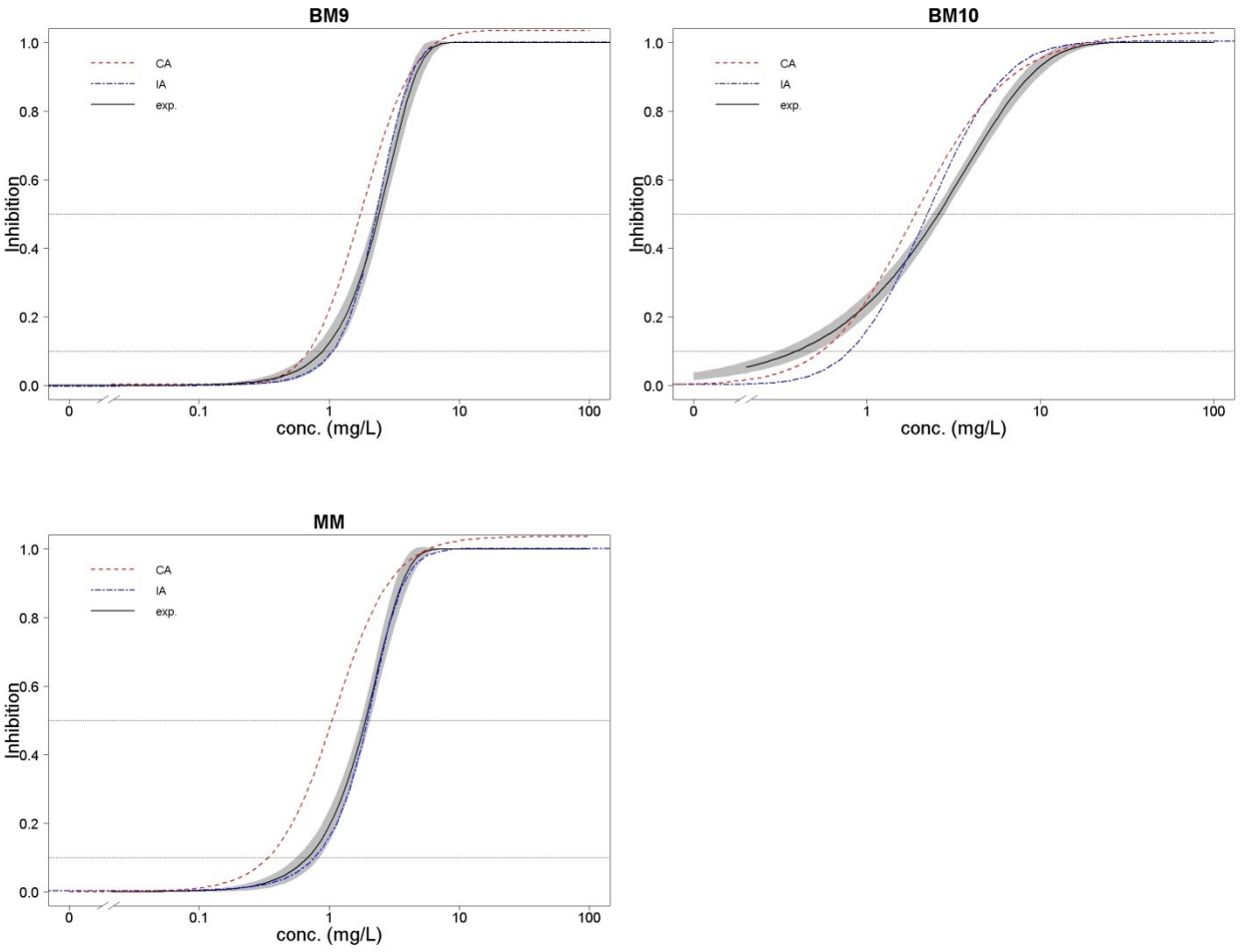


Fig. 2. Experimental (black line) and predicted toxicity curves (from CA and IA models in red and in blue lines, respectively) for the binary and multi-compound mixtures of QACs (in shadow are the interval confidences at 95% of experimental responses; in dotted horizontal lines are the intersections at IC_{10} and at IC_{50}).

In all cases, the two mathematical models gave similar predictions for the mixture toxicity, in agreement with reported literature observations (Backhaus et al., 2004; Barata et al., 2006; Carbajo et al., 2015; Cedergreen et al., 2008; Syberg et al., 2008; Villa et al., 2012; 2014). The obtained prediction windows, as IA/CA ratio, are quite small, ranging between 1.07 (BM7) and 1.80 (MM) for the IC_{50}. The occurrence of a small prediction window can be explained by the similar shape and

slope of the concentration-response curves of individual compounds and by the low number of compounds in the mixtures (Dresher and Bodeker, 1995; Backhaus et al., 2004).

The good fit of CA and IA models to the observed results for the majority of the mixtures (7 out of 11) is in agreement with the high predictive power of CA and IA models demonstrated in previous studies for approximately 80% or more of experimental results, with no significant deviations from expectations for mixtures of chemicals, such as pharmaceuticals, pesticides and other toxicants tested on *A. fischeri*, daphnids and algae (Backhaus et al., 2000a,b; Belden et al., 2007; Cedergreen, 2014).

In the 3.1 section, the similarity of the MoA of the selected QACs on *A. fischeri* have suggested the suitability of CA approach for the prediction of the joint toxicity for almost all the tested mixtures, except for mixtures containing ATMAC-16 (BM2, BM5, BM8, BM9 and MM) which should be better predicted by IA. Nevertheless, the IA model seems to provide a better prediction only for BM2 and MM. However, as mentioned above, the differences between the two predicted concentration-response curves are very small, and a general similarity in the prediction of the experimental responses can be assumed for the tested mixtures.

For the remaining binary mixtures, some deviations from the expected values were observed. In particular the predictive models overestimated the overall toxicity for BM5, BM6 and BM7 and underestimated toxicity for BM4 at lower concentrations (Fig. 2).

These deviations from additivity concepts might depend on interactions among the components. For the first three combinations, the observed deviations could be attributed to the presence of DADMAC-10 in the mixtures. A possible explanation can be found in the double-chain structure of DADMAC-10 (see Table S1). Del Burgo et al. (2006) found that mixtures containing a cationic double-chain and a cationic single-chain surfactant lead to the formation of pre-vesicle and vesicle nano-aggregates at very low concentrations. These could prevent the interaction of the QAC and

negatively charged substances of cells and could be responsible for the lower toxicity of mixtures containing DADMAC-10.

On the contrary, the BM1 that contained also the DADMAC-10 in combination with BAC-12 showed an additive behaviour. The structure of BAC-12, containing a benzyl group, could prevent the formation of vesicles aggregates, which would explain the different result obtained for BM1.

For BM4, the reasons why at concentration below IC50 the toxicity is more than additive are not clear. An increase of deviations from predictions from 1.51 (at IC_{50}) to 5.1 (at IC_{10}) fold occurred at concentration levels which could be of environmental concern (hundreds of $\mu g\ L^{-1}$).

These findings agree with the final report by Kortenkamp et al. (2009) on the state of the art on mixture toxicity, pointing out that rare cases of synergism are very specific to a single combination of chemicals and their concentrations.

3.4. Quantification of the predictive accuracy of CA and IA models by Model Deviation Ratio (MDR)

The compliance (or deviation) of the observed toxicities with CA and IA predictions was evaluated by calculating the MDR for the experimental dataset (Table S5). The MDR indicates a perfect fit for the models (additive behaviour) when MDR is 1. An underestimation of the real toxicity of the mixture (toxic effects higher than predicted) yield a MDR <1, and an overestimation of toxicity (toxic effects lower than predicted) yield a MDR >1. In agreement with previous authors (Belden et al., 2007; Carbajo et al., 2015; Cedergreen, 2014; Geiger et al., 2016) a two-fold deviation threshold is assumed to denote the conformity of the observed toxicity to CA and IA predictions ($0.5 \leq MDR \leq 2$). As reported by Cedergreen (2014), when MDR values are even slightly within these values, the existence of potential interactions cannot be excluded.

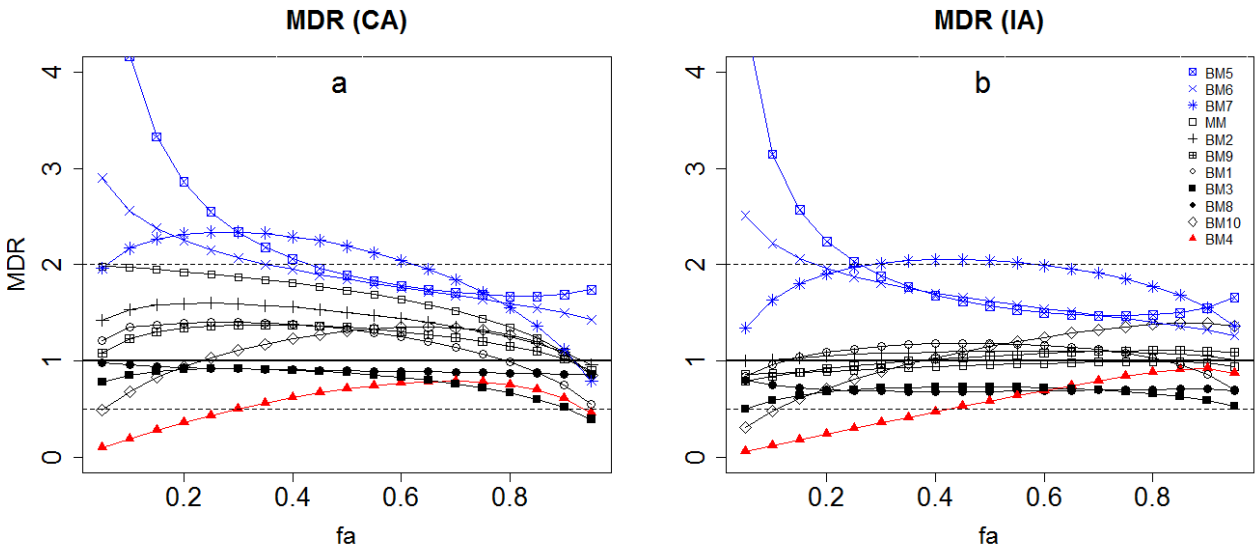


Fig. 3. MDR for CA (a) and IA (b) for growing fractions of bioluminescence inhibition (f_a) for the studied binary and multi-compound mixtures. The dotted grey lines indicate the range of compliance (0.5-2).

Using this approach, most tested mixtures (BM1, BM2, BM3, BM8, BM9, BM10 and MM), in black in Fig. 3, exhibit the additive behaviour of the single compounds at all f_a levels, where MDR values range between 0.5 and 2 for both models (Table S4). For BM4 (in red in Fig. 3), the MDR values for CA predictions are below 0.5 up to the IC_{30}, highlighting a potential synergistic action between the compounds at low concentrations.

BM5, BM6 and BM7 (in blue in Fig. 3) behave differently, with MDR values >2, until at an f_a level of approximately 0.5 for the CA model. The MDR applied to the IA, which is more suitable to evaluate antagonistic interactions, highlights that the compounds in BM5 and BM6 could have antagonistic interactions at low concentrations, approximately up to f_a of 0.25-0.30, resulting in values over or near 2. The MDR values applied to the IA model for BM7 are close to 2 for f_a levels of between approximately 0.20 and 0.70.

For the MM, no deviations from predictions were observed, and a higher compliance with the IA assumptions was obtained. As stated by Backhaus (2014), synergistic and antagonistic effects are rare in multi-compound mixtures and more likely to occur in mixtures with two or three compounds. In our case, the effect of DADMAC-10 and BAC-12/C10TAB are counterbalanced by the presence of other combinations of QACs acting in an additive way. From the analysis of the Toxic Units (TUs) of the mixture compounds, it can be observed that at the IC_{50} level, the toxicity of the MM to *A. fischeri* was mostly driven by the presence of BAC-12 and ATMAC-16, which together contributed approximately 53% of the sum of the TUs. Our results confirm the hypothesis that the presence of several compounds in the mixtures mitigates the less relevant synergistic or antagonistic interactions of a few compounds, leading an overall toxicity closer to an additive MoA and to the predictions from the CA and IA models (Backhaus, 2014).

Where observed, the interactions among QACs in mixtures could be concentration dependent. At low concentrations, greater deviations were observed, and the significance of the deviations decreases with increasing concentrations until toxicity tends toward additivity. This could be because specific compounds exert their specific action when cells are less affected by the toxicants (González-Pleiter et al., 2013). We believe that at low concentrations, single toxicants start to alter the functionality of cells by neutralizing the proton motive force of membranes through inhibition of ATP synthesis and membrane-associated enzyme activity, such as the electron transport chain (Denyer and Hugo, 1977; Maillard, 2002). At higher concentrations, the toxicity could increase aspecifically as cells are already heavily affected (González-Pleiter et al., 2013), meaning that the cell membranes are structurally disrupted and that the leakage of cytoplasmic contents leads to a rapid cell death. As observed by Geiger (2016), in tests with binary combinations of pharmaceuticals at higher concentrations, compounds could act in a narcotic MoA.

Interactions among toxicants at low concentrations, particularly the synergistic ones, were also observed by other authors in studies testing different types of active substances in pharmaceutical

and personal care products using different bioassays, including the Microtox® test (Carbajo et al., 2015; Di Nica et al., 2017b; Ge et al., 2010; Gonzalez-Pleiter et al., 2013; Rosal et al., 2010; Rodea-Palomares et al., 2010; 2012; Magdaleno et al., 2015). As reported by Marx et al. (2015), no general statement can be made on the inverse proportionality between synergism and concentration because of the limited number of studies and the dependencies between results from a particular combination of substances and from a chosen test organism.

Despite their high reliability, CA and IA models have some limitations. First, by definition they are built on the assumption that no interaction occurs among components in the mixtures. Then, processes, such as uptake, transportation, metabolism and excretion of the substances, that could have some effect on combined toxicity, cannot be addressed (Geiger et al., 2016). The observation of synergistic and antagonistic interactions is limited to the empirical observation of deviation of the experimental effects from the predicted ones, and a general schematization cannot be made. Thus, when CA and/or IA models are used in environmental risk assessment, the observed deviations should be evaluated on a case-by-case basis (Kortenkamp et al., 2009).

4. The potential risk of QACs in aquatic ecosystems

The widespread use of QACs in hospitals, households and industrial products raises concerns about the continuous release of QACs into the environment and the potentially harmful effects of QACs to aquatic ecosystems. Despite the high mean rate of removal in WWTPs (over 90%) (Clara et al., 2007; Kreuzinger et al., 2007; Martinez-Carballo et al., 2007), their residual loads can easily reach different environmental compartments because of their continuous input (Kümmerer et al., 1997; Merino et al., 2003; Martinez-Carballo et al., 2007; Li et al., 2014). Some authors studied the occurrence of QAC residues in surface waters (Ding and Tsai, 2003; Ferrer and Furlong, 2001; Kreuzinger et al., 2007; Olkowska et al., 2013), in sediments of surface waters (Ferrer and Furlong, 2002; Lara-Martín et al., 2010; Chiaia-Hernandez et al., 2013), and in seawater (Bassarab et al., 2011), where QACs were detected at a wide range of concentrations.

For instance, Martinez-Carballo et al. (2007) reported MECs of BAC-12 in Austrian wastewaters from hospitals and laundries at 2.8 mg L^{-1} and 2.1 mg L^{-1}, respectively. Olkoska et al. (2013) found 0.1 mg L^{-1} of BAC-12 in reservoir surface water near the city of Gdansk, Poland. Lower, but detectable, concentrations were reported in effluents from wellness centres (46 µg L^{-1} for BAC-12 and 18 µg L^{-1} for DADMAC-10), in WWTP effluents and in surface water. According to Martinez-Carballo et al. (2007), the concentrations of the most toxic compound, BAC-12, were 4 and 2 µg L^{-1} in WWTP effluents and freshwater bodies, respectively. Lower values were found for the other tested QACs: 0.85 µg L^{-1} for DADMAC-10 and 1.1 µg L^{-1} for ATMAC-16 in WWTP effluents and 0.15 µg L^{-1} for DADMAC-10 in surface water in Austria (Clara et al., 2007; Martinez-Carballo et al., 2007).

The flowrate of the receiving waterbody is the chief factor in determining the concentration of QACs after effluent discharge, and when the flowrate is too low to dilute the input of QACs to safe levels, critical situations may arise. It is difficult to perform effluent polishing to further remove QACs.

Apart from the variability of environmental concentrations, another factor affecting the hazardousness of chemicals is the slope of the concentration-response curve. Toxicants with sharper curves (lower IC_{80}/IC_{20} ratios) are relatively easier to manage as small decreases in concentration involve large decreases in their potential hazard. In the case of the tested mixtures, this must be considered in light of the toxicity mechanisms of the QACs. Nearly all the BMs containing C10TAB (3 out of 4 mixtures) showed a weaker slope (higher IC_{80}/IC_{20} ratio) and higher toxicity than other mixtures at low concentrations (i.e., IC_{10} levels) (see Fig. 2 and Table 2), particularly BM4 and BM10. It seems that C10TAB, the least toxic compound, exerts an important influence on the slope of mixtures so that large decreases in concentration would be needed to significantly decrease their toxic response.

Although their occurrence has been detected in different environmental compartments, studies on MECs in aquatic systems are still very limited and very fragmented. The available data might be not representative of the actual conditions in European waterbodies.

The analysis of acute ecotoxicity data from this study and from the literature reveals that a potential environmental risk cannot be excluded. Additionally, considering the continuous input of QACs to the environment, aquatic organisms are likely to be exposed for their entire life.

Different QACs are normally present at the same time giving a higher overall toxicity, and the current regulations for effluent discharge generally refer to a comprehensive concentration of "total surfactants". In this study, nearly all the tested mixtures acted additively, and their toxic effect was approximately related to the sum of single compound concentrations. Consequently, the real risk posed by QACs may be much higher, even 1000 times higher, than the risk posed by individual compounds (Backhaus and Faust, 2012).

5. Conclusions

All the selected QACs showed a considerable acute toxicity to *A. fischeri*, both as individual compounds and in different combinations of binary mixtures, and most of the IC_{50} values was below 1 mg L^{-1}.

The studied compounds act as aspecific reactive chemicals toward *A. fischeri*, except ATMAC-16 which exhibits polar-narcotic behaviour.

The application of CA and IA approaches to binary and multicomponent mixtures confirms the high predictive power of the ecotoxicological theoretical models.

In some cases, knowledge of the toxicity of single compounds is not sufficient to predict the aquatic toxicity of binary mixtures. A synergistic effect was observed at environmentally relevant concentrations (on the order of a few μg L^{-1}).

Because no deviations from additivity concepts were observed, the results from the 5-QAC mixture confirm a buffering effect for the less relevant interacting combinations.

When considering the relevant acute effects on aquatic organisms, a potential risk cannot be excluded. QACs have the potential to be identified as priority substances, and further studies on their environmental occurrence are highly needed.

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SUPPORTING INFORMATION

Toxicity of Quaternary Ammonium Compounds (QACs) as single compounds and mixtures to aquatic non-target microorganisms: experimental data and predictive models

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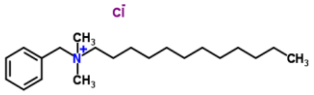
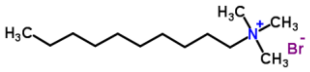
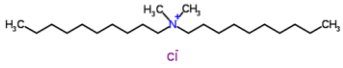
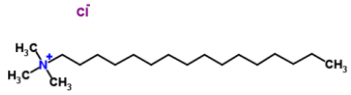
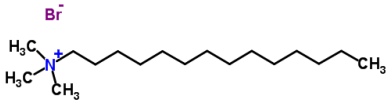
Contents

Supporting information shows: Main physical-chemical parameters of the studied QACs **(Table S1);** Results of application of the log likelihood and the Akaike Information Criterion (AIC) functions to different non-linear regression models for single QACs **(Table S2a)** and for mixtures **(Table S2b)**; Compared non-linear regression models **(Table S2c)**; Results of the Kolmogorov-Smirnov test applied to the regression models for single QACs and for their mixtures **(Table S2d)**; Experimental and Predicted IC_{10} and IC_{50} (CA and IA models) with the 95% confidence intervals **(Table S3);** Values of the Model deviation ratios (MDRs) for the tested mixtures of QACs **(Table S4**).

Table 1. Main physical-chemical parameters of the tested compounds (QACs are shown in alphabetical order according to their nomenclature).

	CAS number	Molecular formula	Structural formula	Molecular weight ($g \cdot mol^{-1}$)	Solubility ($mg\ L^{-1}$)	Log K_{OW} (25°C)
BAC-12	139-07-1	$C_{21}H_{38}Cl.N$		339.99	22.47*[a]	2.93[b]
C10TAB	2082-84-0	$C_{13}H_{30}Br.N$		280.29	9992*	0.24*
DADMAC-10	7173-51-5	$C_{22}H_{48}Cl.N$		362.08	700[c]	2.56[c]
ATMAC-16	112-02-7	$C_{19}H_{42}Cl.N$		320.00	440[d]	3.23[e]
C14TAB	1119-97-7	$C_{17}H_{38}Br.N$		336.39	200000[f]	2.20[b]

a: Yang, dissertation, 2007 (Estimated Episuite); b: Zhu et al., 2010; c: Tezel, dissertation, 2009; d: Boethling, 1994; e: Hansch et al.,1995; f: Chemblink, on line database: http://www.chemblink.com/products/1119-97-7.htm; *Water Solubility Estimate from Log Kow (EPI Suite™ -WSKOWWIN™ v1.42); *Log Kow (EPI Suite™ - KOWWIN™ v1.68).

Table S2a. Analysis of goodness-of-fit for different regression models based on the log likelihood functions and the Akaike Information Criterion (AIC) for single QACs.

	BAC-12		**DADMAC 10**		**C14TAB**		**ATMAC-16**		**C10TAB**	
Mod.	LogLik	IC Lack fit	LogLik	IC Lack fit	LogLik	IC Lack fit	LogLik	IC Lack fit	LogLik	IC Lack fit
W1.2	52.494	-98.988	25.687	-45.375	24.125	-42.250	44.852	-83.704	28.071	-50.142
W1.3	53.033	-98.065	27.972	-47.945	24.137	-40.274	45.019	-82.038	28.205	-48.411
W1.4	53.425	-96.851	28.510	-47.020	27.374	-44.747	45.159	-80.317	29.483	-48.966
W2.2	49.160	-92.320	30.647	-55.294	26.512	-47.025	49.147	-92.293	24.618	-43.236
W2.4	52.165	-94.329	30.690	51.380	28.074	-46.148	49.178	-88.356	28.633	-47.266
LN.2	51.407	-96.815	28.854	-51.708	25.458	-44.917	47.300	-88.601	27.000	-48.000
LN.3	53.016	-98.032	29.314	-50.627	25.642	-43.284	47.301	-86.603	27.859	-47.719
LN.4	53.496	-96.992	29.659	-49.319	27.813	-45.627	47.377	-84.755	29.314	-48.628
L.4	51.437	-92.874	30.839	-51.678	28.493	-46.987	49.130	-88.260	28.413	-46.826
L.5	51.661	-91.321	30.825	-49.650	28.497	-44.993	49.596	-87.192	28.690	-45.380
LL.2	51.199	-96.398	29.065	-52.131	26.336	-46.673	47.258	-88.516	-14.975	35.950
LL.3	52.583	-97.165	29.817	-51.633	26.407	-44.813	47.286	-86.573	24.402	-40.803
LL.4	53.115	-96.230	30.034	-50.068	28.145	-46.290	47.332	-84.665	29.306	-48.613
LL.5	53.449	-94.899	30.992	-49.983	28.379	-44.758	50.435	-88.871	29.484	-46.968

Table S2b. Analysis of goodness-of-fit for different regression models based onthe log likelihood functions and the Akaike Information Criterion (AIC) for mixtures of QACs.

	BM1		**BM2**		**BM3**		**BM4**		**BM5**	
Mod.	LogLik	IC Lack fit	LogLik	IC Lack fit	LogLik	IC Lack fit	LogLik	IC Lack fit	LogLik	IC Lack fit
W1.2	37.066	-68.132	33.001	-60.001	50.055	-94.110	30.581	-55.163	41.617	-77.233
W1.3	37.191	-66.383	33.421	-58.842	50.275	-92.550	30.967	-53.934	42.557	-77.113
W1.4	37.207	-64.415	34.464	-58.927	57.361	-104.721	31.915	-53.830	43.539	-77.077
W2.2	32.834	-59.669	42.316	-78.632	49.810	-93.619	29.591	-53.182	33.875	-61.749
W2.4	34.481	-58.962	43.158	-76.316	52.901	-95.802	31.054	-52.109	35.621	-61.241
LN.2	35.689	-65.379	38.486	-70.972	50.885	-95.770	31.371	-56.742	37.211	-68.422
LN.3	36.705	-65.411	38.486	-68.973	52.134	-96.268	31.502	-55.004	37.800	-67.599
LN.4	36.730	-63.459	38.684	-67.367	55.972	-101.944	31.720	-53.440	38.653	-67.307
L.4	33.544	-57.088	43.960	-77.920	53.637	-97.274	30.014	-50.028	35.728	-61.457
L.5	34.610	-57.219	47.123	-82.245	55.137	-98.274	30.273	-48.546	38.198	-64.397
LL.2	35.602	-65.205	39.428	-72.856	53.124	-100.247	31.692	-57.384	37.422	-68.843
LL.3	36.474	-64.947	39.470	-70.939	54.178	-100.356	31.723	-55.446	38.147	-68.295
LL.4	36.548	-63.096	39.545	-69.089	57.410	-104.820	31.792	-53.584	39.253	-68.507
LL.5	37.237	-62.473	48.137	-84.273	57.622	-103.242	31.908	-51.815	43.416	-74.833

	BM6		**BM7**		**BM8**		**BM9**		**BM10**	
Mod.	LogLik	IC Lack fit	LogLik	IC Lack fit	LogLik	IC Lack fit	LogLik	IC Lack fit	LogLik	IC Lack fit
W1.2	36.191	-66.381	36.932	-67.863	28.258	-50.517	52.984	-99.968	33.505	-61.010
W1.3	36.636	-65.273	39.639	-71.278	28.460	-48.920	53.912	-99.825	37.472	-66.944
W1.4	39.190	-68.379	41.155	-72.310	29.129	-48.258	56.818	-103.636	37.598	-65.195
W2.2	44.634	-83.269	39.060	-72.119	28.983	-51.966	59.203	-112.406	34.946	-63.891
W2.4	47.375	-84.749	42.978	-75.956	31.161	-52.322	60.175	-110.350	37.434	-64.868
LN.2	40.354	-74.708	40.007	-74.014	29.040	-52.079	56.488	-106.976	37.327	-68.653
LN.3	40.520	-73.039	40.191	-72.383	29.043	-50.086	56.786	-105.572	37.480	-66.960
LN.4	42.766	-75.531	42.205	-74.409	30.042	-50.084	59.037	-108.076	37.514	-65.029
L.4	48.146	-86.292	42.848	-75.697	31.966	-53.932	60.202	-110.404	35.621	-61.242
L.5	49.411	-86.822	42.883	-73.765	32.178	-52.356	60.217	-108.433	36.233	-60.466

LL.2	41.595	-77.190	39.290	-72.579	29.187	-52.374	56.461	-106.922	36.888	-67.775
LL.3	41.596	-75.192	39.655	-71.311	29.189	-50.379	57.109	-106.218	37.133	-66.267
LL.4	43.155	-76.311	42.251	-74.503	30.466	-50.932	58.892	-107.784	37.468	-64.936
LL.5	49.725	-87.449	42.597	-73.194	31.888	-51.777	60.141	-108.282	37.534	-63.067

MM	Mod.	**W1.2**	**W1.3**	**W1.4**	**W2.2**	**W2.4**	**LN.2**	**LN.3**
	LogLik	43.229	43.944	43.946	47.660	49.041	47.026	47.031
	IC Lack of fit	-80.458	-79.888	-77.891	-89.320	-88.081	-88.052	-86.061
	Mod.	**LN.4**	**L.4**	**L.5**	**LL.2**	**LL.3**	**LL.4**	**LL.5**
	LogLik	47.239	49.104	49.112	47.115	47.183	47.547	49.597
	IC Lack of fit	-84.477	-88.209	-86.225	-88.230	-86.366	-85.093	-87.194

In Tables S2a –S2b W1.2, W1.3, W1.4, W2.2, W2.4, LN.2, LN.3, LN.4, L.4, L.5, LL.2, LL.3, LL.4, LL.5 are the compared two, three and four-parameter Weibull (W), Log Normal (LN), Logistic (L) and Log Logistic (LL) dose-response models as in table S1c:

Table S2c. The compared regression models.

Weibull (W)		Log Normal (LN)	
W1.2	$f = exp(-exp(\beta 2(ln(c) - ln(\beta 1)))$	LN.2	$f = \varphi(\beta 2(ln(c) - ln(\beta 1)))$
W1.3	$f = 0 + (\beta 3 - 0)exp(-exp(\beta 2(ln(c) - ln(\beta 1)))$	LN.3	$f = \beta 3(\varphi(\beta 2(ln(c) - ln(\beta 1)))$
W1.4	$f = \beta 4 + (\beta 3 - \beta 4)exp(-exp(\beta 2(ln(c) - ln(\beta 1)))$	LN.4	$f = \beta 4 + (\beta 3 - \beta 4)(\varphi(\beta 2(ln(c) - ln(\beta 1)))$
W2.2	$f = 1 - exp(-exp(\beta 2(ln(c) - ln(\beta 1)))$		
W2.4	$f = \beta 4 + (\beta 3 - \beta 4)(1 - exp(-exp(\beta 2(ln(c) - ln(\beta 1))))$		
Logistic (L)		Log Logistic (LL)	
L.4	$f = \beta 4 + \frac{\beta 3 - \beta 4}{(1 + exp(\beta 2(ln(c) + ln(\beta 1)))}$	LL.2	$f = \frac{1}{1 + exp(\beta 2(ln(c) + ln(\beta 1))}$
L.5	$f = \beta 4 + \frac{\beta 3 - \beta 4}{(1 + exp(\beta 2(ln(c) + ln(\beta 1)))^{\beta 5}}$	LL.3	$f = 0 + \frac{\beta 3 - 0}{1 + exp(\beta 2(ln(c) + ln(\beta 1))}$
		LL.4	$f = \beta 4 + \frac{\beta 3 - \beta 4}{1 + exp(\beta 2(ln(c) + ln(\beta 1))}$
		LL.5	$f = \beta 4 + \frac{\beta 3 - 0}{(1 + exp(\beta 2(ln(c) + ln(\beta 1)))^{\beta 5}}$

Table S2d. Results of Kolmogorov-Smirnov test applied to the selected models for single QACs and their mixtures.

	Mod.	ks.test *p-value*		Mod.	ks.test *p-value*		Mod.	ks.test *p-value*		Mod.	ks.test *p-value*
BAC-12	W1.2	0.141	BM1	W2.2	0.861	BM6	W2.2	0.567	MM	W2.2	0.858
DADMAC-10	W2.2	0.820	BM2	W2.2	0.754	BM7	W2.2	0.485			
C14TAB	W2.2	0.419	BM3	W2.2	0.676	BM8	W2.2	0.887			
ATMAC-16	W2.2	0.753	BM4	W2.2	0.820	BM9	W2.2	0.711			
C10TAB	W1.2	0.926	BM5	W1.2	0.462	BM10	W2.2	0.956			

Table S3. Experimental and predicted (CA and IA) IC_{10} and IC_{50} values (mg L^{-1}) for the tested mixtures of QACs along with the 95% confidence interval in brackets.

	IC10 (CA)	IC10 (IA)	IC10 (exp.)	IC50 (CA)	IC50 (IA)	IC50 (exp.)
BM1	0.07 [0.05 - 0.09]	0.10 [0.07 - 0.12]	0.10 [0.07- 0.12]	0.25 [0.18 - 0.34]	0.28 [0.20 - 0.36]	0.33 [0.30 - 0.37]
BM3	0.13 [0.08 - 0.14]	0.19 [0.11 - 0.21]	0.11 [0.09 - 0.14]	0.41 [0.29 - 0.54]	0.49 [0.33 - 0.64]	0.36 [0.33 - 0.38]
BM8	0.35 [0.19 - 0.47]	0.44 [0.23 - 0.71]	0.33 [0.22 - 0.44]	0.90 [0.80 - 1.02]	1.19 [0.90 - 1.43]	0.81 [0.71 - 0.92]
BM2	0.27 [0.22 - 0.34]	0.41 [0.33 - 0.42]	0.41 [0.36 - 0.46]	0.63 [0.55 - 0.76]	0.87 [0.75 - 0.97]	0.95 [0.90 - 1.00]
BM4	0.49 [0.36 - 0.64]	0.78 [0.66 -0.92]	0.10 [0.04 - 0.15]	1.38 [1.01 - 1.92]	1.68 [1.38 - 1.88]	0.98 [0.80 - 1.16]
BM6	0.14 [0.06 - 0.23]	0.16 [0.05 - 0.30]	0.35 [0.27 - 0.42]	0.59 [0.45 - 0.73]	0.68 [0.42 0.94]	1.10 [1.02 - 1.18]
BM5	0.27 [0.18 - 0.37]	0.36 [0.20 - 0.53]	1.13 [1.05 - 1.20]	0.84 [0.76 - 0.92]	1.00 [0.81 - 1.19]	1.58 [1.51 -1.64]
BM9	0.71 [0.57 - 0.93]	1.03 [0.80 - 1.26]	0.87 [0.65 - 1.09]	1.79 [1.40 - 2.31]	2.30 [1.89 - 2.66]	2.42 [2.20 - 2.63]
BM10	0.56 [0.37 - 0.61]	0.81 [0.55 - 0.67]	0.38 [0.28 - 0.49]	1.97 [1.49 - 2.72]	2.24 [1.72 - 2.83]	2.58 [2.37 - 2.79]
BM7	0.51 [0.37 - 0.61]	0.68 [0.53 - 0.78]	1.11 [0.79 - 1.43]	1.99 [1.53 - 2.70]	2.12 [1.66 -2.66]	4.37 [3.87- 4.87]
MM1	0.34 [0.24 - 0.37]	0.78 [0.42 - 0.92]	0.68 [0.54 - 0.82]	1.10 [0.95 - 1.40]	1.98 [1.51 - 2.31]	1.89 [1.74 - 2.05]

Table S4. Values of the Model Deviation Ratios (MDRs) for CA and IA for the tested mixtures of QACs at growing ICx .

		MDR				MDR	
		CA	IA			CA	IA
BM1	IC_{10}	1.35	0.96	BM5	IC_{10}	**4.17**	**3.15**
	IC_{25}	1.40	1.12		IC_{25}	**2.55**	**2.03**
	IC_{50}	1.33	1.18		IC_{50}	1.89	1.57
	IC_{75}	1.07	1.08		IC_{75}	1.69	1.47
BM3	IC_{10}	0.85	0.59	BM9	IC_{10}	1.23	0.84
	IC_{25}	0.92	0.70		IC_{25}	1.36	0.95
	IC_{50}	0.88	0.73		IC_{50}	1.35	1.05
	IC_{75}	0.72	0.68		IC_{75}	1.20	1.10
BM8	IC_{10}	0.96	0.75	BM10	IC_{10}	0.68	**0.48**
	IC_{25}	0.92	0.69		IC_{25}	1.03	0.81
	IC_{50}	0.90	0.68		IC_{50}	1.31	1.15
	IC_{75}	0.88	0.70		IC_{75}	1.32	1.35
BM2	IC_{10}	1.53	1.01	BM7	IC_{10}	2.17	1.63
	IC_{25}	1.60	1.07		IC_{25}	2.34	1.97
	IC_{50}	1.50	1.10		IC_{50}	**2.19**	**2.04**
	IC_{75}	1.30	1.09		IC_{75}	1.71	1.85
BM4	IC_{10}	**0.19**	**0.12**	MM	IC_{10}	1.97	0.87
	IC_{25}	**0.43**	**0.30**		IC_{25}	1.90	0.90
	IC_{50}	0.71	0.58		IC_{50}	1.73	0.96
	IC_{75}	0.78	0.84		IC_{75}	1.44	0.99
BM6	IC_{10}	**2.56**	**2.22**				
	IC_{25}	**2.15**	1.87				
	IC_{50}	1.85	1.62				
	IC_{75}	1.64	1.44				

MDRs are the ratio between the experimental and the predicted ICx (CA or IA). Bold text shows MDRs out of the 2X thresholds.

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CHAPTER VIII

Synthesis and general conclusions

Synthesis and general conclusions

Chapter VII summarizes the main results obtained from topics addressed in this study (chapters III to VII) highlighting the general final conclusions and the future perspectives.

The overall aim of this research activity was to study the toxic effects towards bioluminescent aquatic microorganisms (*Aliivibrio fischeri*) of selected emerging pollutants, as single toxicants and as different combinations of mixtures. Particularly, the predictability of effects produced by several combinations of pharmaceuticals and quaternary ammonium compounds by means of the knowledge of the single toxicity parameters of mixture components was evaluated (component based approach). To do this the classical eco-toxicological predictive models, Concentration Addition (CA) and Independent Action (IA), were applied. The existence of deviations from conceptual expectations (additivity of concentrations and additivity of responses) was also investigated.

In a first step of the research activity, a prioritization methodology for the ranking of pollutants according to their environmental risk and the subsequent selection of compounds of greatest concern was developed (Chapter III). Particularly, this methodology was implemented for veterinary medicinal products (VMPs) by means of the development of a risk indicator (RANKVET) on the basis of the specific EU requirements for the VMPs authorization process. The usefulness of RANKVET was tested on 48 VMPs largely used in Italy. This tool has proved useful for several applications: ranking of VMPs based on risk for aquatic and soil compartments, and/or also with reference to a specific typology of treated species or husbandry practices. This can allow the adoption of appropriate risk mitigation measures or the implementation of targeted monitoring plans. The application of a risk-based prioritization approach has proven to be a useful approach to focus management efforts on chemicals of the greatest concern. In the past, several criteria for chemical selections (e.g. for monitoring purposes) have been adopted. Most of them were mainly based on consumption data. This criterion

has a logical validity since is supposed that active substances (a.s.) used in large amounts have a greater potential to reach in higher amounts the environment. Nevertheless, considerations on other parameters, such as physical-chemical properties of a.s., the dose administered in target animals, or the percentage of excretion, etc., allow to increase the probability to find an a.s. in the environment. Additionally, some chemicals already at low concentration of exposure may be highly toxic towards non-target organisms; thus consider only exposure characteristics may be not sufficient for an appropriate selection of chemicals of concern. Combination of exposure and effects data becomes the most suitable methodology. Nevertheless, one of the greater limitations in the use of risk-based indicators, including RANKVET, is related to the scarcity of chronic eco-toxicological toxicity data in literature. Consequently, for most of compounds, including pharmaceuticals, it is hard to calculate accurate PNEC values.

In a second step the toxic effects of a list of 10 pharmaceutical active compounds (PhACs) were assessed as single pollutants towards the bioluminescent bacterium *A. fischeri* (Microtox® test) (Chapter IV). Results indicated a moderate toxicity for the majority of PhACs with IC_{50} values ranging from 12.1 mg L^{-1} for chlortetracycline to >1700 mg L^{-1} for amoxicillin. The application of Quantitative Structure-Activity Relationship (QSAR) models specifically developed for *A. fischeri* revealed a polar narcotic mode of action (MoA) for the majority of PhACs on the test organism, with exception of the antiepileptic carbamazepine that acted as non-polar narcotic compound and the antibiotic chlortetracycline that showed an enhanced toxicity respect to baseline toxicity, maybe ascribed to a more specific mechanism of action on bacteria. Comparison of toxicity results with a collection of acute toxicity data from organisms representative of the three main trophic levels (algae, *Daphnia* and fish) for surface waters showed a sensitivity of *A. fischeri* comparable to other aquatic species. The moderate difference in sensitivity among the analysed aquatic species and the relative moderate acute toxicity gave some indications about a narcotic nature of the MoA of the studied PhACs also for other aquatic species (an exception was represented by chlortetracycline). Comparison between acute

toxicity data, including those obtained in this study, and PhAC residue levels measured in EU surface water bodies gave indications on the absence of acute risk for aquatic life at least considering individual pollutants. Nevertheless, the assessment of combined effects relating to their simultaneous presence becomes necessary in order to investigate the real risk for the environment.

The study of the main concentration-response curve parameters for single toxicants allowed the application of the component-based approach for the assessment of mixture effects (Chapters V and VI). Comparison between predicted mixture toxicities provided by the application of theoretical models (CA and IA) and experimental results for binary combinations of veterinary pharmaceuticals, mixed according to individual IC_{10} values, showed the presence of deviations from additivity expectations for the majority of the tested combinations, particularly in direction of synergistic responses at lower concentrations. For these combinations occurrence of interactions between components was supposed; this hypothesis was confirmed and quantified by means of the application of the Combination Index method. Anyway, concentrations of PhACs giving synergistic interactions were above the environmentally realistic concentrations. On the contrary, the multicomponent mixture of PhACs combined according individual values of PNEC showed no effect on *A. fischeri* suggesting that, at least for short time of exposure and for microorganisms, these are protective of aquatic systems even if pharmaceutical residues are simultaneously present in environment. Lastly, CA and IA models provided a good prediction of experimental results for binary combinations of human pharmaceuticals mixed in a ratio corresponding to individual IC_{50} values.

Results obtained with the last study (Chapter VII) proved a high toxicity of quaternary ammonium compounds (QACs) on *A. fischeri* and a comparable sensitivity of test organism to other aquatic species. Comparison of toxicity results with the few available data on measured environmental concentrations showed the existence of a potential acute risk for aquatic life already for exposure to single pollutants. Application of QSAR models indicated an unspecific reactivity of the majority of QACs to *A. fischeri,* with the exception of the hexadecyl trimethyl ammonium chloride (ATMAC-16) that

acted as polar narcotic compound. The experimental results from the majority of binary and multicomponent mixtures of QACS, combined at individual IC_{10} values, were well predicted by theoretical CA and IA models. Anyway, for 4 of the tested binary combinations, some deviations (in direction of synergistic and antagonistic responses) whose extent was greater at lower concentrations were observed. In particular synergistic interactions were observed at environmentally relevant concentrations (order of $\mu g\ L^{-1}$).

Results above synthesized lead us to the following general conclusions:

In mixture studies the substantially comparable predictive power of the two mathematical models (CA and IA) not always lead to accurate predictions of combined effects, particularly in the case of pharmaceutical active compounds, suggesting that in this case the knowledge of single toxicity parameters is not always sufficient for a good estimates of mixture effects. Where observed, the presence of interactions, their nature (synergistic or antagonistic) and extent is strongly dependent on the specific combination of toxicants and on the effect levels under considerations. For the same combination of toxicants, synergistic interactions at low effect levels may become strongly antagonistic at high effect levels. Occurrence of interactions is, substantially, independent of the mode of action of toxicants, in fact narcotic or unspecific reactive acting chemicals can strongly interact synergistically on *A. fischeri*.

The observed deviations from additivity expectations are present in mixtures of few components (binary combinations), whereas an overall additivity response is observed with the increase of the number of mixture components. These results confirmed the buffering hypothesis of multicomponent mixtures versus the influence of few synergistic or antagonistic interactions, i.e. the influence of few interacting chemicals seems to be neutralized by other not interacting compounds. Anyway, for this

purpose further investigations are needed as, from literature, studies of multicomponent mixtures are comparatively scarce.

LIST OF ABBREVIATIONS

AMX: amoxicillin

ASA: acetylsalycilic acid

AIC: Akaike Information Criterion

AF: Assessment Factor

ANPA: Italian Environment Protection Agency

ATMAC-16: hexadecyl trimethyl ammonium chloride

a.s.: active substance

BAC-12: benzyl dimethyl dodecyl ammonium chloride

BM: Binary Mixture

BZF: bezafibrate

CA: Concentration Addition

CAS: Chemical Abstract Service

CBZ: carbamazepine

CESIO: European Committee of Organic Surfactants and their Intermediates

CFF: caffeine

CHEMS-1: Chemical Hazard Evaluation for Management Strategies

CI: Combination Index

CTC: chlortetracycline

CRC: Concentration Response Curve

C10TAB: decyl trimethyl ammonium bromide

C14TAB: tetradecyl trimethyl ammonium bromide

DA: Dose Addition

DADMAC-10: didecyl dimethyl ammonium chloride

DCF: diclofenac sodium

Dm: median-effect dose

DMSO: dimethyl sulfoxide

Dow: octanol/water distribution coefficient

DTx: disappearence time for x% of a compound

EC: emerging contaminant

ECx: effective concentration of a substance that causes x% of response

EMA: European Medicinal Agency

EINECS: Inventory of Commercial Chemical Substances

ETR: exposure-toxicity ratio

EURAM: European Union Risk Ranking Method

fa: effect level

FEDESA: European Federation of Animal Health

FSM: furosemide

GMP: Good Manufacturing Practice

HWW: hospital wastewater

HPV: High Production Volume Chemicals

IA: Independent Action

IBU: ibuprofen

ICx: inhibition concentration of a substance that causes x% of response

Kd: partition coefficient soil/water

Koc: Soil Organic Carbon-Water Partitioning Coefficient

Kow: octanol/water partition coefficient

ks.test: Kolmogorov-Smirnov test

L: Logistic

LL: Log Logistic

LN: Log Normal

LC50: median lethal concentration

LOEC: Observable Effect Concentration

MDR: Model Deviation Ratio

MEC: Measured Environmental Concentration

MM: Multicomponent Mixture

MoA: Mode of Action

NSAIDs nonsteroidal anti-inflammatory drugs [

NOEC: No Observable Effect Concentration

OECD: Organization for Economic Co-operation and Development

PCP: personal care product

PEC: Predicted Environmental Concentrations

PhAC: Pharmaceutical Active Compound

PKa: acid dissociation constant

PNEC: Predicted No Effect Concentrations

POP: persistent organic pollutant

PPCP: pharmaceutical and personal care product

QAC: Quaternary Ammonium Compound

QSAR: Quantitative Structure-Activity Relationship

RQ: risk quotient

SAA: Surface Active Agent

SCHER: Scientific Committee on Health and Environmental Risks

SMT: sulfamethizole

STP: Sewage Treatment Plants

Tcon: total connectivity index

TR: toxicity ratio

TU: Toxic Unit

U.S.EPA: U.S. Environmental Protection Agency

USGS: United States Geological Survey

VICH: Veterinary International Conference on Harmonization

VMP: Veterinary Medicinal Product

VSDB: Veterinary Substances DataBase

W: Weibull

WWTP wastewater treatment plant