UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Università degli Studi di Padova

Dipartimento di **Biomedicina Comparata ed Alimentazione**

SCUOLA DI DOTTORATO DI RICERCA IN: Scienze Veterinarie
INDIRIZZO: Unico
CICLO XXVI

Sublethal Effects of Some Veterinary Antibacterials on Freshwater Organisms

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“Some people never go crazy.

What truly horrible lives they must lead.”

(C. Bukowsky)

CONTENTS:

Abstract

The problem of the presence of pharmaceuticals in the environment and of their possible effects on ecosystems has been evidenced only in the last two decades, thanks to the progresses in analytical chemistry, and also to the progressive refinement of toxicological bioassays, leading to the detection of more subtle effects of pharmaceuticals on model organisms.
In the last decade, progresses have been made also from the legislative point of view, introducing the duty of accurate evaluation of environmental impact for all newly commercialized drugs. Nevertheless, there is still a lack of knowledge about the environmental effects of pharmaceuticals.
In the veterinary field, there are particular concerns about the environmental impact of livestock mass treatments (prophylactic/metaphylactic) which are administered for some days, at full dose and to a wide number of animals. Consequently, large quantities of active substances are released into the environment.
Aim of this thesis, presented as a collection paper, was the evaluation of some sublethal effects of antibacterials, employed in mass treatments, on freshwater organisms. This in order to obtain a more in depth picture of the risks they could pose to the aquatic environment.
The sensitivity of *Daphnia curvirostris* and *Daphnia magna* toward 10 antibacterials [enrofloxacin (EFX), ciprofloxacin(CPX), sulfaguanidine (SGD), sulfadiazine (SDZ), sulfamethazine (SMZ), sulfaquinoxaline (SQO), sulfaclozine (SCZ), sulfamerazine (SMA), sulfadimethoxine (SDM) and trimethoprim (TMP)] and some of their binary mixtures, was compared. Furthermore, a tentative prolonged-toxicity test (lasting 13 days) was settled up in order to evidence toxic responses with drug concentrations that were uneffective in the classic 48h immobilization test. Results showed that *D.curvirostris* was more sensitive than *D.magna* to the majority of compounds (7 out of 10). Lowest 48h EC_{50s} were obtained with EFX (4.3 mg L^{-1} in *D.curvirostris*) and SGD (6.2 mg L^{-1} in *D.magna*). The toxicity of paired compounds was always additive or less than additive. In the prolonged-toxicity test mortality and/or reproduction inhibition were constantly observed. It was concluded that: (1) *D.curvirostris* could be a suitable model for the evaluation of acute toxicity of antibacterials; (2) the toxicity of EFX and SGD should be given special attention (3) the concentration addition is usually a reasonable worst case estimation of the environmental impact of antibacterial mixtures.
Some veterinary antibacterials which may contaminate the aquatic environment due to their use in livestock and/or aquaculture mass treatments were evaluated for their effects on swimming activity in *D.magna* (primary consumer) and *Poecilia reticulata* (secondary consumer). Results showed that the chosen endpoint may call to the attention of ecotoxicology some compounds otherwise negligible, based on lethality test.
Sublethal effects of TMP were then evaluated in four freshwater organisms: *Pseudokirchneriella subcapitata* and *Lemna minor* (growth inhibition), *D.magna* (reproduction and growth inhibition) and *P.reticulata* (swimming activity inhibition). TMP showed varying levels of toxicity in the four test performed, with NOEC for the various endpoints in the range of 3.12 to 25 mg L^{-1}. The compound was active on *P. reticulata* at concentration ≥50 mg L^{-1} causing inhibition of swimming activity. *L. minor* was more sensitive than unicellular algae to TMP, with a NOEC of 12.5 mg L^{-1}. The lowest NOEC (3.12 mg L^{-1}) was obtained in *D. magna* reproduction test and then a Risk Quotient of <0.03 was calculated by comparing the PNEC (31.2 µg L^{-1}) and the TMP concentrations usually detected in freshwater (<1 µg L^{-1}). It was concluded that while TMP concentrations normally detected in surface water are below those able to evoke appreciable biological effects in the various aquatic organisms, TMP concentrations in aquaculture and hospital effluents can be one to three orders of magnitude higher. Furthermore, the co-occurrence and additive effects of other antifolic agents should be taken into account for a cautious risk assessment of the drug.

Multigenerational tests on *D.magna* were performed exposing two subsequent generation of the crustacean to EFX, his metabolite CPX and TMP. In F1 with respect to F0, both for growth and reproduction, a worsening trend of the response with EFX, a similar response with CPX and an attenuating trend with TMP was observed. Furthermore, the lowest EC_{20} for reproduction inhibition (1.27 mg L^{-1}) was calculated for F1 exposed to EFX. However, other experimentations, longer and more complex, are necessary in order to confirm that EFX is more hazardous to daphnids than CPX and TMP. EC_{50s} measured for the three assayed antibacterials were in the 6.49-36.53 mg L^{-1} range and therefore environmental unrealistic, except in case of exceptional contaminations that may occur in relation to poorly controlled wastewaters from pharmaceutical factories or excessive use of prophylactic treatments in aquaculture.

Riassunto

La presenza di molecole farmacologicamente attive nell'ambiente è un problema che è stato sollevato solamente negli ultimi due decenni grazie, da un lato, all'affinamento delle tecniche analitiche e, dall'atro, alla messa a punto di bioassay sempre più sensibili. Nell'ultimo decennio inoltre, sono stati fatti notevoli passi avanti anche dal punto di vista normativo introducendo l'obbligo di un'accurata valutazione dell'impatto ambientale per tutti i nuovi principi attivi immessi in commercio. Tuttavia le conoscenze relative agli effetti dei farmaci nell'ambiente sono ancora piuttosto limitate.

In campo veterinario particolare preoccupazione in relazione alla sicurezza ambientale destano i trattamenti di massa che vengono effettuati a scopo profilattico/metafilattico coinvolgendo un gran numero di animali che vengono trattati a dose piena per alcuni giorni con conseguente notevole rilascio di principi attivi nel suolo o nelle acque.

Lo scopo di questa tesi, presentata sottoforma di *collection paper*, è stato quello di indagare alcuni effetti subletali di antibatterici, impiegati nei trattamenti di massa, su organismi acquatici, al fine di giungere ad una valutazione più accurata dei rischi che questi contaminanti possono comportare per il comparto dulciacquicolo.

La sensibilità di *Daphnia curvirostris* e *Daphnia magna* è stata confrontata attraverso l'impiego di 10 antibatterici [enrofloxacina (EFX), ciprofloxacina (CPX), sulfaguanidina (SGD), sulfadiazina (SDZ), sulfametazina (SMZ), sulfachinossalina (SQO), sulfaclozina (SCZ), sulfamerazina (SMA), sulfadimetossina (SDM) e trimetoprim (TMP)] e di alcune loro miscele binarie. Inoltre, è stato condotto un test preliminare di tossicità prolungata (della durata di 13 gg), al fine di evidenziare effetti a concentrazioni che non avevano prodotto risposte a 48h col classico test di immobilizzazione. I risultati hanno permesso di osservare che *D.curvirostris* è più sensibile di *D.magna* alla maggior parte dei composti (7 su 10). Le più basse EC_{50} sono state ottenute con EFX (4.3 mg L^{-1} in *D.curvirostris*) e SGD (6.2 mg L^{-1} in *D.magna*). La tossicità delle miscele binarie è stata sempre additiva o sub additiva e i test prolungati hanno permesso di osservare in tutti i casi mortalità e/o inibizione della riproduzione. Si è concluso che: (1) *D.curvirostris* può essere un modello adatto per la valutazione della tossicità degli antibatterici; (2) particolare attenzione dovrebbe essere prestata alla tossicità di EFX e SGD; (3) generalmente il concetto di semplice additività è una stima cautelativa dell'impatto ambientale delle miscele di antibatterici.

Sono stati valutati gli effetti di alcuni antibatterici sull'attività natatoria di *D.magna* e *Poecilia reticulata*. I risultati hanno permesso di osservare che l'end-point selezionato, a differenza dei test di letalità, consente di richiamare l'attenzione sulla tossicità ambientale di alcuni composti.

Alcuni effetti subletali del TMP sono stati misurati in quattro organismi d'acqua dolce: *Pseudokirchneriella subcapitata* and *Lemna minor* (inibizione dell'accrescimento), *D.magna* (inibizione della riproduzione e della crescita) e *P.reticulata* (inibizione dell'attività natatoria). Il TMP ha mostrato diversi livelli di tossicità nei diversi organismi con NOEC che variavano da 3,12 a 25 mg L^{-1}. Il composto era attivo su *P.reticulata* alla concentrazione ≥50 mg L^{-1} provocando un'inibizione dell'attività natatoria. La NOEC più bassa (3,12 mg L^{-1}) è stata ottenuta nel test d'inibizione della riproduzione con *D. magna*, quindi, il confronto tra la PNEC (31,2 µg L) e le concentrazioni normalmente ritrovate nelle acque dolci (<1 µg L^{-1}) ha permesso di calcolare un quoziente di rischio <0,03. Si è concluso che mentre le concentrazioni di TMP normalmente rilevate nelle acque superficiali non sarebbero in grado di provocare effetti biologici apprezzabili nei vari organismi acquatici, le concentrazioni di TMP impiegate in acquacoltura e ritrovate negli scarichi ospedalieri possono raggiungere livelli sino a tre ordini di grandezza superiori. Inoltre per un'analisi del rischio sufficientemente cautelativa la copresenza di TMP ed altri antifolici ed i possibili, conseguenti, effetti additivi dovrebbero essere tenuti in considerazione.

Test multi generazionali sono stati effettuati su *D.magna* esponendo due generazioni consecutive a EFX, al suo metabolita CPX ed a TMP. In F1 rispetto ad F0 è stato osservato, sia sull'accrescimento che sulla riproduzione: un peggioramento degli effetti con EFX, risposte simili

con CPX, un'attenuazione degli effetti con TMP. Inoltre, l' EC_{20} più bassa, relativa all'inibizione della riproduzione, è stata determinata per gli F1 esposti a EFX; tuttavia, disegni sperimentali più lunghi e complessi si rendono necessari al fine di confermare la maggior pericolosità, per i daphnidi, di EFX rispetto a CPX e TMP. Le EC_{50} misurate nei tre test variavano da 6,49 a 36,53 mg L^{-1} e sono, dunque, da considerarsi irrealistiche dal punto di vista ambientale salvo casi eccezionali di contaminazione che si possono verificare in occasione di scarso controllo dei reflui provenienti da stabilimenti farmaceutici o in relazione all'abuso di trattamenti profilattici in acquacoltura.

Chapter 1

General Introduction

Mirco Dalla Bona.

1 Veterinary Antibacterials in the environment

The problem of the presence of pharmaceuticals in the environment and of their possible effects on ecosystems has been evidenced only in the last two decades, thanks to the progresses in analytical chemistry, allowing to detect drug concentrations in the various environmental compartments at ppt (part per thousands) levels (Kümmerer, 2009a), and also to the progressive refinement of toxicological bioassays, leading to the detection of more subtle effects of pharmaceuticals on model organisms. In the meantime, annual global spending on medicines is growing constantly (Rehman et al., 2013), both because of the aging of population in developed countries and because of the extension of medical care and increasing access to medicines supported through a range of government policies and programs in developing countries. In these latters there is also an increasing demand of veterinary pharmaceuticals due to the rapid expansion of intensive breeding facilities (Thornton, 2010).

Among veterinary pharmaceuticals, antibacterials are of particular concern for the environment not only because of the large employed quantities but also because of their responsibility in the spreading of bacterial resistance (Heuer et al., 2011) (EEA, 2010). Actually, in farm animals they are often used for prophylactic/metaphylactic treatments, which being usually performed at full dosage and in large groups of animals, lead to a not negligible environmental drug load. This is even more true for aquaculture treatments, where after treating fish with medicated feed a direct discharge of effluents in the surrounding water streams usually occurs (Boxall et al., 2012; Kümmerer, 2009b).

Concerns for antibacterials regard not only the spreading of bacterial resistance but also the possible effects on the ecological fitness of non-target organisms which in the long run can lead to the loss of biodiversity and to the deterioration of ecosystems.

With Regulation 2005/183/EC (EC, 2005) the European Community, with the aim of preserving public health, has definitively banned the growth promoting use of antibacterials, while the FDA has made some steps in this direction by reducing the use of enrofloxacin in poultry (Marshall and Levy, 2011) and forbidding the growth promoting use of cephalosporins since fifth of April 2012 (FDA, 2008). Nevertheless, the worldwide consumption of antibacterials is growing annually by about 4% (Hamad, 2010).

Antibacterials administered to animals are generally eliminated in feces and urine and those intended for food producing animals (80-90% of the total) are partially eliminated unmodified (30-90% of the dose) (Sarmah et al., 2006). Manure and slurry are usually used for the fertilization of agricultural fields and if not adsorbed to the organic matter and/or minerals of the soil, may leach into the ground water or runoff to the surface water because of their hydrophilicity (Tarazona et al., 2009). Furthermore, livestock prophylactic/metaphylactic treatments in aquaculture involve the direct transfer of substantial amounts of medication into surface water. Indeed, It is reported that only 20-30% antibacterials are ingested by fish

and the remaining 80-70% reach the environment, and that even the antibacterials ingested by aquatic animals may be excreted as such or as metabolites (Vijayakumaran, 1997).

Studies designed to assess the presence and the environmental fate of veterinary antibacterials have determined their presence in surface waters, ground waters and even drinking water (Boxall et al., 2012; Kümmerer, 2009b; Santos et al., 2010), typically in the nanogram per liter-few microgram per liter concentration range.

A contamination source, previously underestimated, is wastewater from pharmaceutical factories. These are expanding rapidly in Asian countries like China, India and Viet Nam where environmental regulations are less restrictive and in some cases not fully observed (Larsson et al., 2007; Rehman et al., 2013); as a result, surprisingly high concentrations of pharmaceuticals have been occasionally detected in the water environment of these countries. More specifically, a study on effluents from a water treatment plant of 90 pharmaceutical factories in Patancheru (India) has shown worrying levels of antibacterials, including 31 mg L-1 of Ciprofloxacin and 900 μg L-1 of Enrofloxacin (Larsson et al., 2007), while another monitoring study has shown up to 2 mg L-1 of Trimethoprim in a shrimp farming pond in Viet Nam (Le and Munekage, 2004).

Based on consumption data, probability of reaching the aquatic environment and toxicity veterinary pharmaceutical have been evaluated in order to identify those compound that may represent a threat for environmental health (Boxall et al., 2003; Capleton et al., 2006; Kim et al., 2008); these studied have brought to attention some antibacterials as needing a more in depth evaluation of exotoxicity.

2 Environmental Risk Assessment (ERA) of Veterinary Pharmaceuticals: Background and Limitations

In the early 90s, growing concern about the environmental effects of pharmaceuticals has led to debates designed to meet the urgency of regulations in the European Community aimed at mitigating consumption of pharmaceuticals also on the basis of their environmental hazard. First guidelines for environmental risk assessment of veterinary drugs were drawn up in 1997 (EMEA, 1997) and followed by VICH GL 6 and VICH GL 38 guidelines (VICH, 2000, 2004) with the aim of harmonizing US, EU and Japan guidelines. These guidelines are not mandatory but are generally recognized and followed by the various parties.

From the legal point of view, the first European document regulating the marketing of veterinary pharmaceuticals appeared in 1981 with Directive 81/851/EEC (EEC, 1981). Later on, in 2001, it was substituted by Directive 2001/82/EC (EC, 2001), introducing the necessity of environmental risk evaluation for veterinary drugs. The latter was subsequently amended by Directive 2004/28/EC pointing to the concept of risk/benefit balance, i.e. an evaluation of the positive therapeutic effects of the veterinary medicinal product in relation to the environmental risks.

Environmental Risk Assessment (ERA) for Veterinary Pharmaceuticals (VP) is depicted by VICH GL6 (Phase I) and VICH GL 38 (Phase II) guidelines. Determination of PEC (Predicted Environmental Concentration) in soil and water is requested in Phase I, and it is realized assuming a worst case scenario, where the drug is administered to the whole target population and no metabolization or degradation occurs. However, a decision tree allows the exclusion from ERA of products that will be used only in non-food animals or to treat a small number of animals in a flock or herd, thereby contributing negligibly to the environmental drug load. As a consequence, only those VP intended for mass treatments undergo ERA.

If the PEC, calculated using a mathematical model, is less than 100 μg Kg^{-1} in soil or less than 1 μg L^{-1} in water, the product is considered safe for the environment and the ERA may stop in Phase I (VICH, 2000). Otherwise, the VP should advance the Phase II, where environmental fate and effects on non-target organisms are evaluated. In tier A of Phase II the determination of acute EC50, on animal models from different trophic levels (for the water compartment: unicellular algae, crustaceans and fish), is requested in order to calculate a PNEC (Predicted No Effect Concentration) by applying an assessment factor (1000 for crustacean and fish, 100 for algae) to the EC50s. A Risk quotient (RQ) is then obtained from the PEC/PNEC ratio. At Tier A, if the RQ is <1 for all taxonomic levels tested, the assessment should normally stop (VICH, 2004).

Various authors have criticized the use of acute tests for such evaluation because, in the environment, exposition of non-target organisms to drug residues is usually chronic and involve multiple generations (Dietrich et al., 2010; Fent et al., 2006; Santos et al., 2010). Furthermore, an AF of 1000 is considered inadequate for those substances with a very high acute/chronic toxicity ratio (Schmitt et al., 2009). Considering that, in the aquatic compartment only, hundreds of pharmacologically active molecules have been detected so far (Kümmerer, 2010), often in complex mixtures (Gros et al., 2010), another unavoidable limitation of the guidelines is that possible interactions with other pharmaceuticals are not taken into account. Indeed, the summation of the toxicity of single molecules may lead to an alteration of the ecosystem even when the concentrations of the single molecules may be considered safe (Backhaus et al., 2008).

3 Some Recent Approaches in Ecotoxicology.

In recent years, the need for more detailed studies on the effects of VP toward the environment has led researchers to develop toxicity studies which take into account the interactions between the molecules, identify more subtle changes in the fitness of organisms that make up the ecosystem and estimate with greater sensitivity long-run effects on populations.

3.1 Mixture toxicity

The evaluation of ecotoxicological effects of contaminant mixtures is realized using two different approaches (Altenburger et al., 2013). Concentration addition works on the premise that chemicals act in a similar fashion. It assumes that every chemical in a mixture contributes to the overall effect in proportion to its concentration, even at levels below those known to cause effects. An alternative reference for predicting the effects of mixtures of compounds with different mode of action is the concept of response addition (Backhaus et al., 2008), also known as independent action, Bliss independence or effect multiplication. The concept of response addition is based on the assumption that the combination effect EA,B of a mixture is the product of the individual activities of its components, EA x EB. Various studies have been performed using the two approaches with more or less complex mixtures of pharmaceuticals (Backhaus et al., 2008, 2000; Christensen et al., 2006; Cleuvers, 2003; De Liguoro et al., 2009). However, while interactions between the active ingredients are known in human and veterinary pharmacology, the combined effects of mixtures of VP on non-target organisms is still far from being elucidated, and further studies are needed for this purpose (Backhaus et al., 2000).

3.2 Behavioural Effects and Videoanalisis.

With the recent developments in image analyses and computer sciences, there are now powerful tools that can substitute or complement traditional behavioral observation tools. As a result, behavior alterations are regarded with growing interest by ecotoxicologists. Behavioral alterations of animals represent a response of the whole organism which integrates internal and external factors and may give information about the effects of contaminants both at individual and population level. The exposition to different environmental stresses, as for example the exposition to contaminants, may induce behavioral alterations in sensitive species and influence ecologically relevant activities such as food gathering, mating activity and predators avoidance (Kane et al., 2004)

Video tracking, by definition, is the tracking of moving objects and the monitoring of their activities by image sequences obtained from video cameras. It is an automatized procedure that determines animal position over time and gives the resulting tracks with a large array of data such as distance travelled, speed or space used (Delcourt et al., 2013).

3.3 Multigenerational tests.

VP are continuously released into the aquatic compartment where they may remain at low concentrations for prolonged periods of time. Consequently, the exposition of water organisms to these molecules concerns not only their whole life cycle but also multiple generations. Indeed, at environmentally realistic concentrations some compounds may exert their deleterious effects on water organisms only over several generations, leading over time to the loss of the more sensitive species. Long-term toxicity studies, especially assessments conducted on several generations are therefore necessary to more accurately predict the possible impact of VP on the aquatic ecosystem (Fent et al., 2006; Kim et al., 2012).

4 Aim and Outline of this Thesis

In this thesis sublethal effects of some veterinary antibacterials on freshwater organisms are presented. Compounds were selected taking into account their large usage volume and their relative environmental stability.

In Chapter 2, after testing the lethal effects of ten veterinary antibacterials on two freshwater crustaceans (*Daphnia magna* and *Daphnia curvirostris*) a general comparison of the sensitivity of the two organisms was done. Some of the assayed compounds were then selected taking into account the probability of co-presence in the aquatic environment and 8 binary tests (4 on each species) were performed. Finally, using the same two organisms, the three more toxic compounds were evaluated for their effects on reproductions using a specially designed prolonged toxicity test.

In Chapter 3, effects of 6 antibacterials on the swimming activity of *Poecilia reticulata* (secondary consumer) and *D.magna* (primary consumer) were evaluated. Traveled distance was the measured parameter.

In Chapter 4, the effects of trimethoprim on the swimming activity of *P.reticulata* were evaluated more in depth using scaled concentrations of the compound. Then, other sublethal effects of trimethoprim (growth inhibition and/or reproduction inhibition) were measured on two primary producers (*Lemna minor* and *Pseudokirchneriella subcapitata*), a primary consumer (*D.magna*) and a secondary consumer (*P.reticulata*)

In Chapter 5, lethal and sublethal effects of Trimethoprim, Enrofloxacin and Ciprofloxacin on two generations of *D.magna* were studied.

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Chapter 2

The sensitivity of *Daphnia magna* and *Daphnia curvirostris* to 10 veterinary antibacterials and to some of their binary mixtures

Mirco Dalla Bona, Vincenzo Di Leva, Marco De Liguoro.

Submitted

Abstract

Aim of this study was to evaluate the suitability of *Daphnia curvirostris* for the acute toxicity test usually performed on *Daphnia magna*, and to compare the sensitivity of the two species toward 10 antibacterials [enrofloxacin (EFX), ciprofloxacin(CPX), sulfaguanidine (SGD), sulfadiazine (SDZ), sulfamethazine (SMZ), sulfaquinoxaline (SQO), sulfaclozine (SCZ), sulfamerazine (SMA), sulfadimetoxine (SDM) and trimethoprim (TMP)] and some of their binary mixtures. Furthermore, a tentative prolonged-toxicity test (lasting 13 days) was settled up in order to evidence toxic responses with drug concentrations that were uneffective in the classic 48h immobilization test. Results showed that *D.curvirostris* was more sensitive than *D.magna* to the majority of compounds (7 out of 10). Lowest 48h EC_{50s} were obtained with EFX (4.3 mg L^{-1} in *D.curvirostris*) and SGD (6.2 mg L^{-1} in *D.magna*).

The toxicity of paired compounds was always additive or less than additive. In the prolonged-toxicity test mortality and/or reproduction inhibition were constantly recorded. It was concluded that: (1) *D.curvirostris* could be a suitable model for the evaluation of acute toxicity of antibacterials since its sensitivity is generally greater than that of *D.magna*; (2) if adequately validated, the prolonged test could represent an alternative to the classic reproduction test; (3) the toxicity of EFX and SGD should be given special attention as the two compounds, in the prolonged test, showed to be active at concentrations of 0.9 mg L^{-1} and 2.5 mg L^{-1}, respectively; (4) the concentration addition is usually a reasonable worst case estimation of the environmental impact of antibacterial mixtures.

Keywords: *Daphnia curvirostris*; *Daphnia magna*; Sulfonamides; Fluoroquinolones; Comparison; Mixtures.

1 Introduction

Medicines have an important role in the treatment and prevention of diseases in both humans and animals (Boxall, 2004) and, in the last decades, tons of physiologically active chemicals have been produced worldwide. Yet, it was only in the mid 90s with advances in analytical techniques that important knowledge on environmental contamination by these compounds grew (Santos et al., 2010).

Antibacterials used in intensive farming may contaminate the aquatic environment from livestock waste treatment plants or through runoff from manure-treated farmlands (Boxall, 2003). Moreover,

treatments used in aquaculture have a high potential to impact the aquatic environment, because they are added directly to the environment (Weston, 1996).

Fluoroquinolones (FQs) and Sulfonamides (SAs) are two classes of antimicrobials that are largely used both in human and in veterinary medicine. The use of SAs in veterinary medicine is well established, due to their efficacy on both bacterial and protozoa, and to their relatively low cost. FQs have recently gained particular interest in the veterinary field and are very important in human medicine, especially for the treatment of urinary infections. As a consequence, both SAs and FQs have been repeatedly detected in surface waters (Gunnarsson et al., 2009; Santos et al., 2010) and some of them as, for example, enrofloxacin (EFX), ciprofloxacin (CPX) and sulfadiazine (SDZ) have been given high priority for detailed risk assessment (Kim et al., 2008).

The waterflea *Daphnia magna* is largely employed in aquatic ecotoxicology due to its high sensitivity to a wide range of pollutants and to the relative easiness of culture and maintenance in laboratory. Even if many studies show that other daphnids are more sensitive than *D.magna* to various contaminants (Bossuyt and Janssen, 2005; Canton and Adema, 1978; Versteeg et al., 1997) *D.magna* is still considered the reference cladoceran for risk assessment in aquatic ecotoxicology. Koivisto and coll. (1995) suggested that when *D.magna* is used to estimate safe toxicant concentrations for zooplankton, the hazard may be underestimated since smaller species are more susceptible than *D.magna* to the transfer of toxicants across the integument. Consequently, the use of smaller crustaceans in environmental risk assessment may lead to a more cautious risk evaluation. Furthermore, in order to preserve biodiversity and to generate ecotoxicological data that better represent the effects of pollutants in specific environments, the careful selection and use of indigenous species should be promoted (Harmon et al., 2003). Biodiversity plays a crucial role in environmental preservation and the current rate of loss of species is not sustainable, in the long run, without functional collapses (Rockström et al., 2009).

Daphnia curvirostris is a small cladoceran widespread in southern and eastern Europe (Ishida et al., 2006; Mura and Brecciaroli, 2003; Petrusek et al., 2005), also due to its high adaptability to water salinity variations (Petrusek et al., 2005). There is a lack of data regarding the sensitivity of *D.curvirostris* to pollutants and, to our knowledge, no attention has yet being paid to the effects of pharmaceuticals on this cladoceran.

Aim of this study was to compare the sensitivity of *D.curvirostris* and *D.magna* to the toxicity of 10 antibacterials: EFX, CPX, SDZ, sulfaguanidine (SGD), sulfamethazine (SMZ), sulfaquinoxaline (SQO), sulfaclozine (SCZ), sulfamerazine (SMA), sulfadimethoxine (SDM) and trimethoprim (TMP), that are largely used in veterinary medicine. Acute toxicity tests were performed and then some compounds were also assayed in combination (binary tests), taking into account the probability of their co-presence in the aquatic environment. Furthermore, based on the results of the acute toxicity tests,

those compounds likely to be hazardous at environmentally relevant concentrations were also evaluated, on both species, using a tentative prolonged-toxicity test.

2 Materials and methods

2.1 Culture conditions

Ephippia of *D.magna* were originally provided by ECOTOX (Milano, Italy). A single clone culture was selected based on the correct sensitivity to potassium dichromate (ISO, 1996) which was then rechecked periodically. A single clone culture of *D.curvirostris* was instead obtained from wild specimens collected in Rosolina (Rovigo, Italy; GPS coordinates (WGS84): 45.138666698153 lat 12.3240131618937 lon) and identified using taxonomic keys (Petrusek et al., 2005). The organisms were maintained in ADaM (Klüttgen et al., 1994a, 1994b) at 20±1°C, with a 16h light (2.6 μE m^{-2} s^{-1}): 8h dark photoperiod. They were fed three times per week with *Scenedesmus dimorphus* ($8x10^5$ cells mL^{-1}). The alga was cultured in 2L ADaM enriched with 3 g of sterilised poultry dung and suspended by bubbling filtered air. Before it was fed to the Daphnia culture, the chlorophyte was filtered through a 50 μm laboratory test sieve (Endecotts LTD, London, England), centrifuged at 3000 *g* for 10 min, resuspended in 25% ADaM medium at a concentration of $2x10^8$ cells mL^{-1} and stored at 4±1°C.

2.2 Chemicals

Analytical grade compounds were purchased from Sigma-Aldrich (Milano, Italy) and were of the following minimum purity: SDZ 99%, SGD 99%, SMA 99%, SDM 98%, SMZ 99%, SQO 95%, TMP 98%, EFX 98%, CPX 98% SCZ 99% . When necessary, their solubilisation in ADaM was achieved by bringing back the pH of the medium to the original value (8.0) using 1M HCl or NaOH. Conductivity and total dissolved oxygen (TDO) were measured using an YSI 85 multi-parameter probe (YSI Incorporated, Yellow Spring, OH, USA). According to already published data (Bortoluzzi, 2012; De Liguoro et al., 2009) the 48h degradation of the above mentioned compounds at the various exposure conditions used in the tests is always lower than 20%. Therefore, test results were based on nominal concentrations (OECD 202, 2004).

2.3 Acute tests

In order to assess the 48h EC_{50}, immobilization tests were carried out following the OECD 202 guideline (OECD, 2004). Before each test, daphnids were fed for about 1h with dried Spirulina powder (15 mg in 100 mL of ADaM) and then incubated at the same conditions (temperature and photoperiod) used for culturing. After 48h incubation, the number of immobile specimens was recorded. *D.curvirostris* was exposed to a 5 concentrations series of each of the ten antibacterials (EFX, CPX, SGD, SDZ, SMZ, SQO, SCZ, SMA, SDM and TMP) while, to avoid redundancy, *D.magna* was exposed only to EFX, CPX, and SCZ since raw toxicity data for the other compounds (SGD, SDZ, SMZ, SQO, SMA, SDM and TMP) were available from a previous study performed in our laboratory (De Liguoro et al., 2009). Then, in both species, the additivity of some compounds (TMP+SDZ, TMP+SDT, TMP+SQO and EFX+CPX) was evaluated, the likelihood of their co-presence in the aquatic environment considered. In these binary immobilization-tests, the concentration-response relationship (five exposition levels) was analyzed for three selected combination ratios equidistantly distributed on the additivity line of isobologram (Altenburger et al., 1990) connecting individual EC_{50s}.

2.5 Tentative prolonged-toxicity tests

Tentative prolonged-toxicity tests were performed using those compounds (ECX, CPX and SGD) that showed, at least in one species, a 48h EC_{50} lower than 20 mg L^{-1}. Daphnids were exposed to the highest concentrations that produced no effects in the immobilization test (*D.curvirostris*: EFX 0.9 mg L^{-1}, CPX 5.0 mg L^{-1}, SGD 3.2 mg L^{-1}; *D.magna*: EFX 9.0 mg L^{-1}, CPX 12.5 mg L^{-1}, SGD 2.5 mg L^{-1}).

For each assayed compound, 15 daphnids were allocated individually in 150 mL jars containing 50 mL of the solution to be tested. Other 15 daphnids were kept in pure ADaM and used as controls. The crustaceans were incubated for 13 days at the conditions (photoperiod and temperature) used for culturing. Every other day, test solutions were renewed and food was provided (*S.dimorphus*: $8x10^5$ cells mL^{-1}). Daphnids were checked daily in order to assess mortality, time to bear the first egg and offspring (newborns were removed and counted).

2.6 Data analysis

The evaluation of 48h EC_{50s} was performed by non linear regression [log(agonist) vs. response - Variable slope] using GraphPad Prism® 5.02 (La Jolla, California, USA). EC_{50s} comparisons were realized with T-test when distribution was normal and variances were homogeneous. Otherwise, Mann-Whitney test or Wilcoxon test were used. The additive toxicity of the binary mixtures was

evaluated according to the method described by Tallarida (Tallarida, 2006). In prolonged toxicity tests the survival rate was obtained using Kaplan-Meier curves. Hazard ratios were calculated by Cox regression using R software (R Development Core Team, 2011) with the survival package (Therneau and original Splus->R port by Thomas Lumley, 2011). When mortality was lower than 50%, differences in 1st egg bearing were analyzed using the above mentioned methods while reproduction inhibition was compared using T-test (R Development Core Team, 2011).

3 Results

3.1 Water quality parameters

In all tests the recorded values were always within the following ranges: pH 7.6 - 8.2, O_2 7.54 - 8.18 mg L^{-1} , Conductivity 1016 - 1069 µS cm^{-1}.

3.2 Acute tests with single compounds

No immobilization were recorded in the controls. The calculated EC_{50s} spanned three orders of magnitude, varying from 4.3 mg L^{-1} (EFX) to 421.1 mg L^{-1} (SDM) in *D.curvirostris* and from 6.2 mg L^{-1} (SGD) to 312.2 mg L^{-1} (SCZ) in *D.magna.* The calculated EC_{50} was significantly different in the two species for all compounds. With seven antibacterials (EFX, CPX, SGD, TMP, SMZ, SQO and SCZ) *D.curvirostris* showed to be more sensitive than *D.magna* (Figure 1).

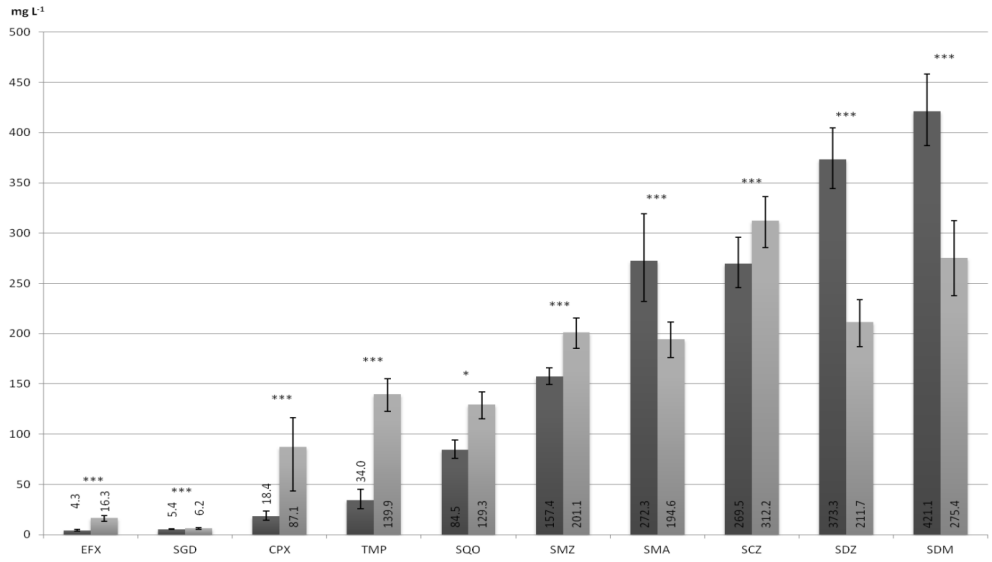

Figure 1. Immobilization test: EC_{50} of single compounds on *D. curvirostris* (dark bars) and *D. magna* (light bars). * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$

3.3 Binary tests

No immobilization were recorded in the controls. The effects of paired compounds were always simply additive with the exceptions of TMP+SDZ in *D.magna* (Figure 2 g) and EFX+CPX both in *D.magna* and in *D.curvirostris* (Figure 2 d, h), where sub-additive effects were observed at one combination ratio. Isobolograms for all binary tests are reported in Figure 2.

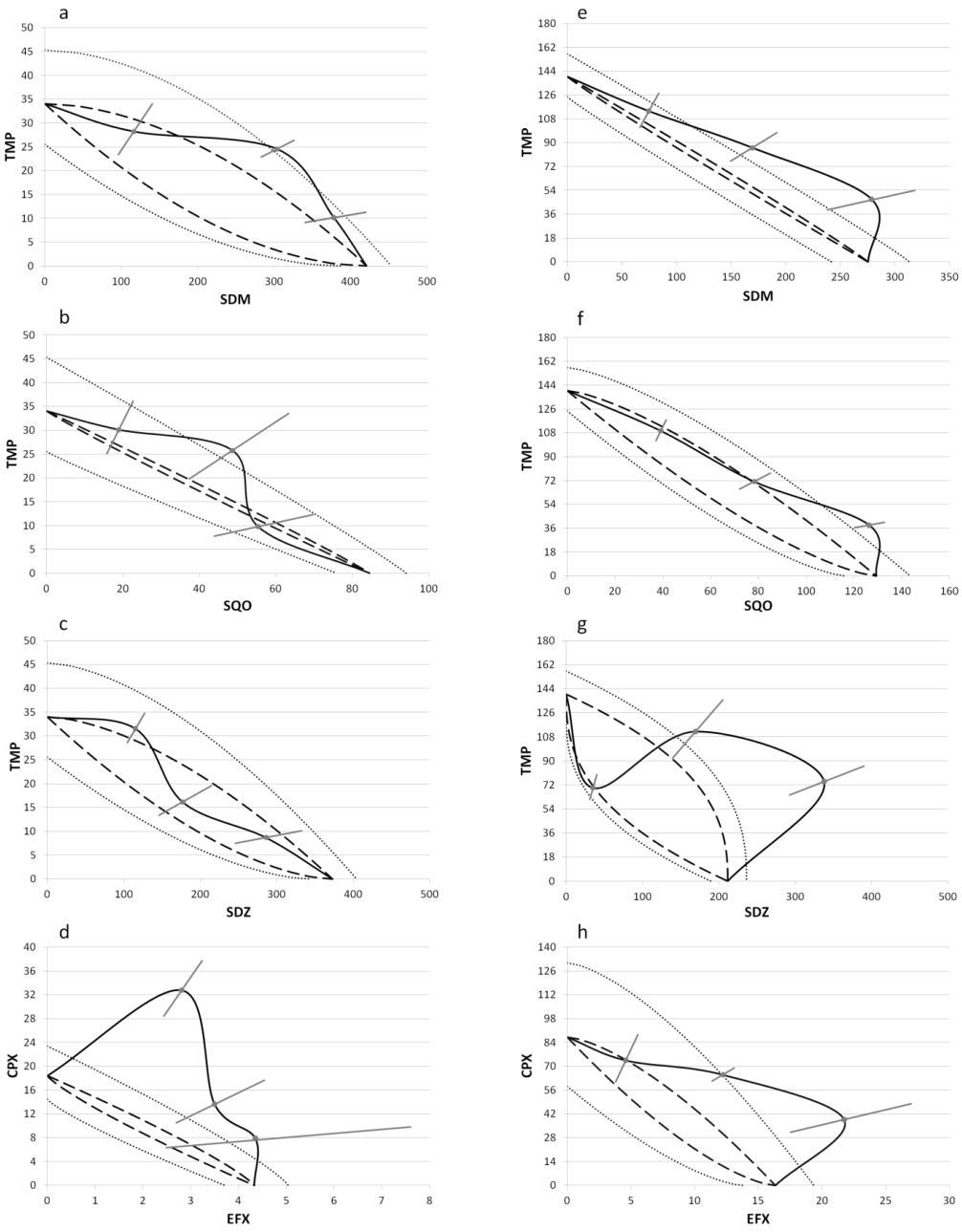

Figure 2. Immobilization test: isobolograms of some binary mixtures assayed on *D. curvirostris* (a,b,c,d) and *D. magna* (e,f,g,h). The additivity curves (dashed lines) are sourrounded by 95% C.I. (dotted lines). EC_{50}s of mixtures with their 95% C.I. are shown in gray. Concentrations are in mg L^{-1}.

As a decision rule, deviation from additivity is detected only if the confidence intervals for the effect concentrations of the combined substances do not overlap the confidence belt of the additivity curves

3.4 Prolonged toxicity test

In the prolonged test few mortalities (1 *D.magna*, 3 *D.curvirostris*) were recorded in the controls. Significant differences in survival curves (Figure 3) were observed for *D.magna* exposed to EFX or SGD and for *D.curvirostris* exposed to CPX or SGD (Table 1). CPX in *D.magna* and EFX in *D.curvirostris* elicited a mortality rate lower than 50%. Consequently, sub-lethal effects on 1st egg bearing and reproduction were taken into consideration. Hazard ratios, with confidence intervals, for the delay in 1st egg bearing are reported in Table 1. While both *D.magna* exposed to CPX and *D.curvirostris* exposed to EFX showed a significant inhibition of newborn production: 19% ($p < 0.05$) and 65% ($p < 0.01$) respectively.

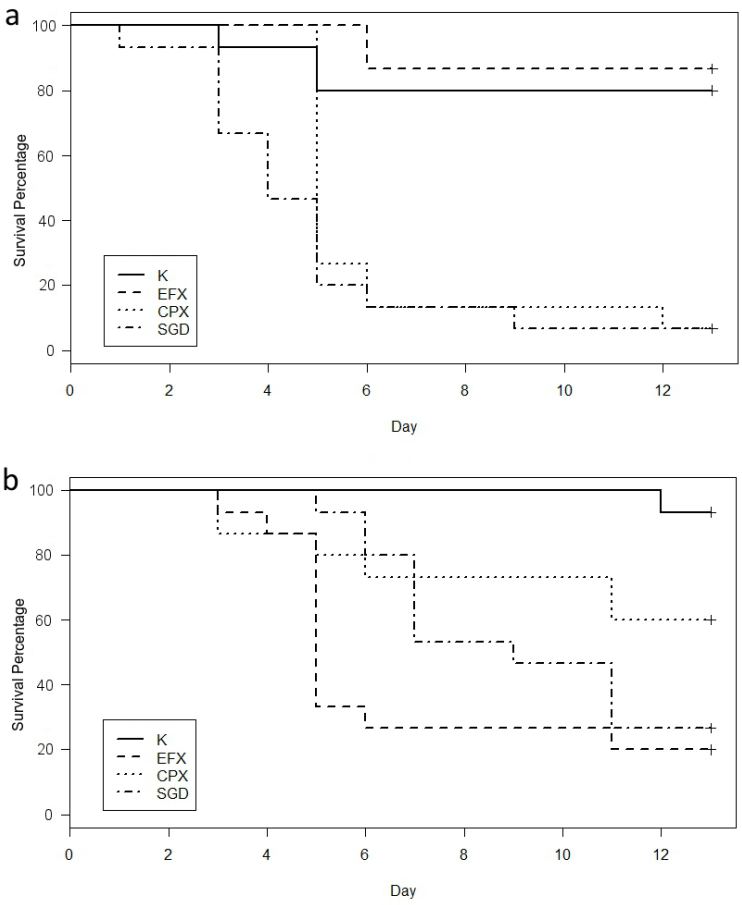

Figure 3. Subacute tests: survival percentage (Kaplan - Meier) of *D.curvirostris* (a) and *D.magna* (b) exposed to enrofloxacin, ciprofloxacin and sulfaguanidine.

Table 1. Prolonged-toxicity test: hazard ratios for survival and 1st egg bearing with the respective 95% confidence intervals and p values. ** = p < 0,01 and *** = p < 0,001.

		1st egg bearing			Survival			
Compound	**Species**	**Hazard Ratio**	**CI 95%**	**p Value**	**Hazard Ratio**	**CI 95%**		**p Value**
Enrofloxacin	*D.magna*				30.6	3.95 -	238	0.00106**
	D.curvirostris	0.451	0.185 - 1.10	0.0800	0.586	0.0980-	3.51	0.0559
Ciprofloxacin	*D.magna*	0.284	0.120 - 0.673	0.00419 **	7.99	0.961 -	66.4	0.0545
	D.curvirostris				8.31	2.34 -	29.5	0.00106**
Sulfaguanidine	*D.magna*				16.2	2.08 -	127	0.00780 **
	D.curvirostris				13.1	3.69 -	46.3	0.0000684***

4 Discussion

In the immobilization tests *D.curvirostris* showed to be significantly more sensitive than *D.magna* to the majority of compounds (7 out of 10). This is in agreement with data from other studies involving daphnids smaller than *D.magna* (Bossuyt and Janssen, 2005; Manar et al., 2012; Versteeg et al., 1997). The high sensitivity of *D.curvirostris* may be due to physiological aspects linked to its small body size, as the high rate of chemicals uptake, due to the great ratio of surface to volume, and the low bio-dilution of compounds (Koivisto, 1995). However, the difference in sensitivity of the two species to the tested antibacterials was always within a factor of 5, which is in general agreement with the observations of Bossuyt and Janssen (2005) on copper lethality in *Daphnia* genus. Furthermore, some studies comparing the sensitivity of *D.magna* and *Daphnia pulex* have found no significant differences between the two species (Canton and Adema, 1978; Lilius et al., 1995). Consequently, the size alone cannot justify the different sensitivity of *D.magna* and *D.curvirostris*.

Data regarding the acute immobilization of *D.magna* exposed to different suphonamides and FQs are available (Kim et al., 2010, 2007; Park and Choi, 2008; Robinson, 2009) and are in general accordance with those obtained in this study. On the other hand, to our knowledge, no pharmaceutical has ever been tested on *D.curvirostris*.

In both species, the toxicity of the two tested FQs was higher than that of the majority of SAs. Indeed, both EFX and its metabolite CPX are deemed priority as environmental contaminants (Kim et al., 2008). Furthermore, their use in veterinary medicine is criticized since FQs are important drugs in human medicine and there is concern about the spreading of bacterial cross-resistance (Cabello, 2006; Martinez et al., 2006). However, taking into account the wide and consolidated use in veterinary medicine, the possible simultaneous presence in the aquatic environment, the low biodegradability, and the toxicity of some compounds (i.e. SGD), the environmental impact of SAs should be considered as well (Baran et al., 2011).

It is worth noting that only for *D.curvirostris* EFX EC_{50} was lower than 10 mg L^{-1} and TMP and SQO EC_{50s} were lower than 100 mg L^{-1}. These two thresholds were established by EC Regulation 1272/2008 to classify the contaminants of the aquatic environment, according to their acute toxicity. As a consequence, the use of acute toxicity data gained from the less sensitive *D.magna* would lead to a misclassification of the three compounds.

The prolonged toxicity test was settled up as a tentative midway between the acute immobilization test (OECD 202, 2004) and the official reproduction inhibition test (OECD 211, 1998) with the aim of detecting, in reasonable time, effects that would not be observed within 48h.

Even if the assayed concentrations were those still unable to cause any immobilization in the acute tests, they elicited lethal and/or sub-lethal effects in all these prolonged tests. In four tests the survival probability was significantly lower than that of controls (even more than 30 times lower for *D.magna* exposed to EFX, see Table 1). In the remaining two tests mortality was lower than 50% while the reproduction (1st egg bearing and/or offspring generated in the firsts two clutches) was significantly affected. Therefore, the prolonged test was able to detect lethal and sub-lethal effects that were not detectable with the acute test but are still deleterious for the population growth rate of the *Daphnia* genus.

Compounds assayed in binary tests were selected based on the probability of their co-presence in the aquatic environment. Indeed, while the co-presence in the aquatic environment of EFX+CPX could be a consequence of the photolysis of EFX (Knapp et al., 2005; Li et al., 2011; Sturini et al., 2010), the co-presence of TMP+SDZ, TMP+SDT and TMP+SQO may be a direct consequence of their use as pharmaceutical associations, particularly in aquaculture where drugs have a high potential to impact the aquatic environment (Weston, 1996). In the two species, no substantial differences in response to the binary tests were found (Figure 2). As already observed in previous studies (De Liguoro et al., 2010, 2009), the toxicity of the paired compounds was mainly additive. In some circumstances (Figure 2 d, f, g, h) sub-additive toxicity was recorded but only at one combination ratio. These results confirm that concentration addition is often a reasonable worst case estimation of the environmental impact of antibacterial mixtures (Boedeker et al., 1993; De Liguoro et al., 2009).

5 Conclusions

D.curvirostris could be a suitable model for the evaluation of acute toxicity since its sensitivity is generally greater than that of *D.magna.* Moreover, this tiny crustacean, being naturally present in the water ponds of northern Italy (Stoch, 1995), can give a realistic picture of the effects of pollutants in that specific environment. Concentrations of antibacterials unable to cause adverse responses during short term exposure (48h) were constantly able to provoke either lethal and/or reproductive

effects in the prolonged test, confirming that acute test are not always predictive of the environmental impact of xenobiotics. If adequately validated, the prolonged test could represent an alternative to the classic reproduction test, which is more time consuming. The toxicity of EFX and SGD should be given special attention as the two compounds, in the prolonged test, showed to be active at concentrations of 0.9 mg L^{-1} and 2.5 mg L^{-1}, respectively. In tests with antimicrobials mixtures, paired compounds showed only additive or sub-additive effects, confirming that concentration addition is often a reasonable worst case estimation of the environmental impact of antibacterial mixtures (Boedeker et al., 1993; De Liguoro et al., 2009).

Acknowledgments

We gratefully acknowledge the contribution of Ioana Catalina Arrigo and Silvia Galuppo. This investigation was funded by grants from the University of Padua (ex60% 2012).

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Chapter 3

Effects of veterinary drugs on swimming activity in two freshwater organisms

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Published in: Trends in Veterinary Sciences, pp 97 - 101, 2013

Summary

Alterations in swimming activity may influence ecologically-relevant performances such as predation avoidance, prey capture, growth, stress resistance, mating and longevity. The evaluation of swimming activity support toxicological investigation with endpoints other than traditional LC_{50s}, and may aid in investigating the environmental relevance of low-level exposures and in determining lowest NOELs and LOELs. In this paper, some veterinary antibacterials which may contaminate the aquatic environment due to their use in livestock and/or aquaculture mass treatments are evaluated for their effects on swimming activity in *Daphnia magna* (primary consumer) and *Poecilia reticulata* (secondary consumer). Results show that the chosen endpoint may call to the attention of ecotoxicology some compounds otherwise negligible, based on lethality test.

1 Introduction

Behavioural changes represent integrate responses of the whole organism. These altered responses may be associated with reduced fitness and survival, resulting in adverse consequences at the population level (Bridges, 1997).

Until recently, behavioural endpoints have been slow to be integrated in aquatic toxicology, not only because of the poor understanding of their consequences on ecologically relevant aspects as predation avoidance, prey capture, growth and reproduction, but also because it was difficult to obtain quantifiable and reproducible data (Kane *et al*, 2005).

Recent improvements in computer and video automation have made possible significant progress in the ease, utility and affordability of obtaining, interpreting and applying behavioural endpoints in a variety of applications, including aquatic toxicity tests. Currently, after exposing an aquatic organism to a substance, it is possible to acquire a film and then analyse it, by means of a videotracking software, in order to accurately evaluate the swimming activity through graphical and statistical elaboration of data.

Acute toxicity tests while demanding little time and labour are generally less sensitive than the chronic ones. Their sensitivity can be improved by evaluating sublethal effects such as the behavioural ones. All in all, the acute toxicity (lethality) of antibacterials toward non-target organisms as crustaceans and, mainly, fishes occurs only at concentrations higher than 100 mg/L, and substances with an EC_{50} higher than this threshold are considered safe for the aquatic environment (Regulation 1272/2008/EEC).

In this paper some methodologies are described which can be used for swimming activity evaluation of fishes and crustaceans after their exposition to antibacterials. And it is shown that significant

effects of these drugs on swimming activity may be detected also at concentrations unable to cause any lethality of the test organisms.

2 Materials and methods

In accordance to the Fish, Acute Toxicity Test (OECD 203, 1992), the following compounds were tested at concentration of 100 mg/L (limit test) on Poecilia reticulata: Enrofloxacin (EFX), its metabolite Ciprofloxacin (CFX), Trimethoprim (TMP), Sulphamethazine (SMZ), Sulphaguanidine (SGD) e Sulphaquinoxaline (SQO). As no lethality was recorded after 96 h, the text was extended to 14 days (OECD 204, 1984). At the end of this prolonged toxicity test, before sacrificing animals with an overdose of MS-222, each group of 7 fish, allocated in a small rounded tank (20 cm diameter) and in standard conditions of light and temperature, was filmed from above for 12 minutes using a digital video camera JVC EVERIO GZ-MS100E, and the middle part (2min) of each video sequence analysed on a PC AcerAspire M3201, using Swistrack4.0 (Lochmatter *et al.*,2008). Then the spatial coordinates (2D) of movements were exported to the Excel (Microsoft®) software in order to perform graphical and statistical elaborations.

Based on the results of this test the following compounds were selected to be tested also on *Daphnia magna*: EFX, TMP, SGD, and SQO. Ten daphnids (6 days old) were allocated in Roux flasks (75 cm^2) at 20±1° C and with a 16 h photoperiod (200 lux), and exposed for 24h to a single compound dissolved in ADaM medium (Klüttgen *et al.*, 1994) at 100mg/L concentration. At the end of the test, each daphnid was frontally filmed for 5 minutes (see above) and then the middle part (2 minutes) of the video sequence analysed on PC Acer Aspire M3201 using the Kinovea software (freeware). For all tests statistical analysis was done by ANOVA followed by Dunnett's test, using the SPSS Statistics ® 17.0 application. This experimentation was authorised by the Italian Ministry of Health (decree n. 175/2010-B).

3 Results

No lethality was observed in the tests. In Figure 1, box-plots of swimming activity of fish (a) and daphnids (b) exposed to the various compounds are shown. With the exception of SMZ (in fish) and SGD (in daphnids), all the drugs tested have elicited a statistically significant reduction of the travelled distance. In Figure 2, as an example, video trackings of controls and of subjects exposed to TMP are compared, both for *P.reticulata* (a) and *D.magna* (b).

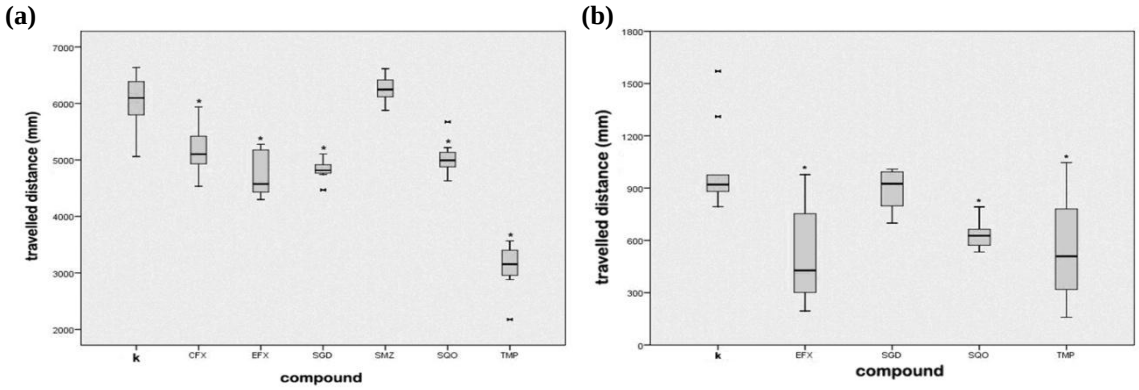

Figure 1. Box-plots showing the distance travelled in 2 minutes by 7 specimens of *Poecilia reticulata* **(a)** and by 10 specimens of *Daphnia magna* **(b)**, for control group (k) and for groups exposed to the antibacterial compounds (100 mg/L). Bowties are outliers, asterisks indicate significant difference from k ($P < 0.01$).

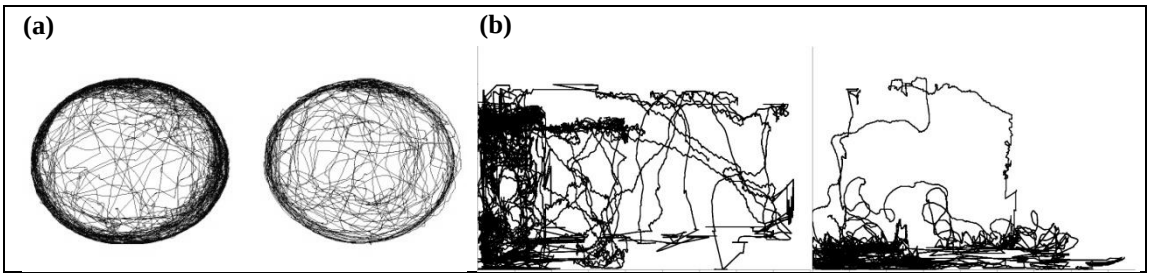

Figure 2. (a) Two minutes path of 7 specimens of *Poecilia reticulata* (filmed from above): control group and group exposed for 14 days to 100 mg/L of TMP are compared. **(b)** Two minutes path of 10 specimens of *Daphnia magna* (frontally filmed): control group and group exposed for 24 h to 100 mg/L of TMP are compared.

4 Discussion

Based on results from algal toxicity tests (EC_{50}<100mg/L), TMP is already classified as 'Harmful to aquatic organisms'and 'Possibly able to cause long-term adverse effects in the aquatic environment' (Phrases R42 and R43). However, as frequently happens, the acute lethality test on *Brachydanio rerio* (Sorensen *et al.*, 2000) gave negative results pointing to a NOEC for fish of 100 mg/L (Chemwatch, 2009). On *P.reticulata* this data would have been confirmed in our test, but by referring to the sublethal endpoint (swimming activity inhibition) the exposition to 100 mg/L is not devoid of effects. As a matter of fact, the protocol for the acute lethality test (OECD 203, 1992) recommends the importance of recording also any sublethal effect. Thus, it will be important, in the near future, to evaluate the same endpoint with scaled concentrations of TMP in order to determinate both the NOEC and the EC_{50} with confidence limits.

Swimming activity inhibition has been recorded also with other 5 out of 6 tested compounds, for which also, though with less urgency than with TMP, some more detailed study may be recommended. It is worth to note that in this study the evaluation of more refined components of

swimming that could increase the sensitivity of the tests, has not been considered. These are accelerations, freezings, angles of turns, horizontal and vertical distribution, predator avoidance etc. In Figure 2, for example, it is possible to note that control group, compared to the TMP exposed one, swims more periferically (*P.reticulata*) or has a higher tendency to move in the water column (*D.magna*).

OECD protocols for both acute (immobilisation) and chronic (reproduction) tests on *D.magna* require the use of newborn specimens. However, in our pioneristic approach to the evaluation of the effects of acute exposure to drugs on swimming activity, we have chosen to use 6 days old daphnids. This was done because, at this age, they are developed enough to be filmed with a conventional video camera but not yet ready for reproduction. Indeed, reproduction in *D.magna* causes a physiological fluctuation of swimming activity which would unavoidably interfere with test results. The test was quick and easy to perform and showed, at least with TMP, to be more sensitive than the traditional immobilisation test (OECD 202, 2004) where the calculated EC_{50} was 149 mg/L (De Liguoro *et al.*, 2009).

Altogether our results confirmed that swimming activity is a valuable endpoint in aquatic toxicology and indicate the need of deeper studies on TMP toxicity. It is not clear, indeed, if the strong effect on swimming activity is only the consequence of the metabolic disturbance on folate synthesis caused by the drug, or might be related to some direct effects on the nervous system of aquatic organisms. It should be noted, on this regard, that TMP is largely used both in human and veterinary medicine. Furthermore, is use is extended to the aquaculture facilities where non negligible input of the compound can be directly released to the aquatic environment when metaphylactic treatments are in place. Moreover, TMP is poorly biodegradable, having a long (>22 days) environmental half-life for primary degradation (Chemwatch, 2009) and its effects in the aquatic environment may add up to those from other antifolic compounds (sulphonamides) that have been frequently detected in surface waters and are as well quite persistent in the environment (De Liguoro *et al.,* 2010).

Aknowledgments

This investigation was funded by grants from the University of Padua (ex60% 2011).

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Chapter 4

Sublethal effects of trimethoprim on four freshwater organisms

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Published in: Ecotoxicology and environmental safety 82, 114-121, 2012

Abstract

Sublethal effects of trimethoprim (TMP) were evaluated in four freshwater organisms: *Pseudokirchneriella subcapitata* and *Lemna minor* (growth inhibition), *Daphnia magna* (reproduction and growth inhibition) and *Poecilia reticulata* (swimming activity inhibition). Cytochrome P4501A induction was also evaluated in *P. reticulata*. TMP showed varying levels of toxicity in the four test performed, with NOEC for the various endpoints in the range of 3.12 to 25 mg L^{-1}. The compound was active on *P. reticulata* at concentration ≥50 mg L^{-1} causing inhibition of swimming activity. In the same organism an induction of CYP1A protein, mainly in kidney, gills and intestine, was also detected. *L. minor* was more sensitive than unicellular algae to TMP, with a NOEC of 12.5 mg L^{-1}. The lowest NOEC (3.12 mg L^{-1}) was obtained in *D. magna* reproduction test and then a Risk Quotient of <0.03 was calculated by comparing the PNEC (31.2 µg L) and the TMP concentrations usually detected in freshwater (<1 µg L^{-1}). However, based on recently reported data, it was concluded that while TMP concentrations normally detected in surface water are below those able to evoke appreciable biological effects in the various aquatic organisms, TMP concentrations in aquaculture and hospital effluents can be one to three orders of magnitude higher. Furthermore, the co-occurrence and additive effects of other antifolic agents should be taken into account for a cautious risk assessment of the drug.

Keywords: Trimethoprim, *P. subcapitata*, *D. magna*, *P. reticulata, L. minor*, Sublethal toxicity.

1. Introduction

Trimethoprim (TMP), the earliest antibacterial diaminopyrimidine introduced for clinical use, is still widely used as a sulfonamide potentiator both in human and veterinary medicine.

A plethora of veterinary medicinal products containing TMP are currently available on the market, and most are intended for routine mass treatments (prophylactic/metaphylactic) of various food producing species. For these reasons, the environmental load of TMP is substantial and, as a consequence of its mobility in soil and slow biodegradation (Chemwatch, 2009), releases of the compound from livestock waste treatment plants or through runoff from manure-treated farmlands may contaminate the aquatic environment. Furthermore, TMP may be directly released to surface waters as its use is extended to aquaculture (Boxall *et al*., 2003).

The environmental inputs from veterinary use of TMP add to those coming from human use. The main route for transportation of antibiotics used in human medicine to the environment is via sewage treatment plants, and TMP has been shown to withstand sewage water treatment, with almost 100% of the environmental load transferred to the final effluent (Lindberg, 2005).

In fact, TMP recently has been detected in various aquatic systems usually at concentration up to few μg L^{-1} (Kummerer, 2009; Santos *et al*., 2010) and occasionally at concentrations up to 2 mg L^{-1} (Le and Munekage, 2004), and an evaluation of its impact in aquatic organisms seems advisable. To date, mainly lethal effects of TMP have been studied on aquatic model organisms and generally they are not elicited by environmentally realistic concentrations. Kim *et al*. (2007) indicated a LC_{50} > 100 mg L^{-1} both for *D. magna* and the Japanese medaka fish *Oryzias latipes*, while Halling-Sørensen *et al*. (2000) found an EC_{50} of one order of magnitude lower (17.8 mg L^{-1}) but only for the growth inhibition of activated sludge bacteria, which are among target organisms for TMP effects. However, a thorough evaluation of environmental impact should also take into account sub-lethal effects. For example, Binelli *et al.* (2009) have shown that TMP is able to inflict DNA damage on Zebra mussel hemocytes at 2.9 μg L^{-1} concentration.

The aim of this work was to evaluate the sublethal effects of TMP on various aquatic organisms representing distinct trophic levels: the green alga *Pseudokirchneriella subcapitata* and the duckweed *Lemna minor* (primary producers), the microcrustacean *Daphnia magna* (primary consumer) and the teleostean fish *Poecilia reticulata* (secondary consumer). Effects on growth and cell surface area (*P. subcapitata*), frond development (*L. minor*), reproduction and growth (*D. magna*), and swimming activity (*P. reticulata)* were evaluated and, based on the lowest measured no observable effect concentration (NOEC), an aquatic predicted no effect concentration (PNEC), using an Assessment Factor (AF) of 100 (von der Ohe *et al*., 2011), and an hazard quotient were determined. Furthermore, in *P. reticulata* the CYP1A expression was evaluated since CYP1A was recently shown to contribute to the formation of reactive intermediates of TMP (Damsten *et al*., 2008).

2. Materials and Methods

2.1 Test organisms

2.1.1 P. subcapitata

The algae used for the tests were from axenic cultures of *P. subcapitata* (strain UTEX 1648) in mid exponential growth phase. The cultures were maintained in Bold Basal Medium (BBM; Nichols, 1973) suspended by bubbling filtered air, at 24±1°C and under continuous illumination (90±10% $\mu Es^{-1}\ m^{-2}$).

2.1.2 D. magna

Only young daphnids (<24h old) obtained from the second to fifth brood were used in the test. The organisms were derived from a single clone cultured and maintained in Aachener *Daphnien* Medium (ADaM:hardness 193 mg $CaCO_3$ L^{-1}; Klüttgen *et al.,*1994a,b) at 20±1° C, with a 16-h light (200 lux): 8-h dark photoperiod. They were fed every other day with *Scenedesmus dimorphus* ($8x10^5$ cells mL^{-1}). The alga was cultured in 500 mL ADaM medium enriched with 0.75 g of sterilized poultry dung and suspended by bubbling filtered air. Before it was fed to the *Daphnia* culture, the chlorophyte was filtered through a 50 µm laboratory test sieve (Endecotts LTD, London, England), centrifuged at 1072 *g* for 10 min, resuspended in 25% ADaM medium at a concentration of $2x10^8$ cells mL^{-1} and stored at 4°C.

2.1.3 L. minor

The duckweed *L. minor*, originally obtained from the field, was first sterilized as indicated by Jang *et al*. (2007): plants were immersed in 70% ethanol for 30 s and 10% sodium-hypochloride solution for 10 s and next rinsed in sterile water three times. Then the duckweed was axenically cultured for about three months in 100 mL of BBM at 24±2°C and at a light intensity of 8000±15% lux provided by fluorescent tubes (OSRAM L 18W/21-840). From a single isolated plant, randomly selected from the culturing flask, a stock culture was grown for three weeks before the beginning of the tests.

2.1.4 P. reticulata

Fingerlings, 10-15 days old, were obtained from a local breeder (Ecopolis, Venezia, Italy) and kept for about 4 months in 40 L glass aquaria with suitable reconstituted water (pH ~7.5, O_2 > 8 mg/L, total hardness dH 20±2) prepared by adding 2 g L^{-1} of synthetic marine salt to dechlorinated tap water. Fish were kept under standardised conditions (T 24±1°C, 12-h light photoperiod) and fed daily with the following balanced formulation: 40% actigran + 40% spirucell + 20% artemin (AQUAEL, Warszawa, Poland). Less than 1 % mortality per week was observed in all stocks used.

When the fish reached an average length of about 2 cm, 42 subjects were randomly collected from the stock and transferred, in groups of 7, to 6 small tanks containing the same reconstituted water, where they were kept unfed and acclimatised for 24 h pending the assay (OECD, 1992). To minimize disturbances, direct measurement of the fish intended for the test was not performed. So, another 42 subjects (not intended for the test) were collected from the same stock and weighed in order to get indication of the average weight of fish at the beginning of the experiment.

2.2 Chemicals

TMP (98% pure) was purchased from Sigma-Aldrich (Milano, Italy). Where necessary, the solubilization was achieved by bringing back the pH of the medium to the original value using 1M HCl. The pH was measured using a BASIC 20 pH meter (CRISON, Carpi, Italy). For the preparation of the ADaM for daphnids and of the reconstituted water for fish, the synthetic marine salt 'Ocean Fish' (PRODAC, Cittadella, Italy) was used; all the other ingredients used for the preparation of the various medium formulations were of analytical quality and were purchased from Sigma-Aldrich.

2.3 Toxicity tests

For each test an adequate volume of stock solution of TMP was prepared just prior to use by dissolving the drug in the pertinent nutrient medium at a concentration equals to or higher than the maximum concentration to be tested; then the stock solution was accordingly diluted in the nutrient medium to obtain the various concentrations used for the test.

2.3.1 Algal growth inhibition test

The toxicity of TMP to the freshwater green alga *P. subcapitata* was evaluated using the 797.1050-Algal acute toxicity test (CFR US-EPA, 2005). BBM was prepared by adding the appropriate stock solutions (Nichols, 1973) to Milli-Q water and was then autoclaved. Algal inocula corresponding to 10,000 cells mL^{-1} were grown in 125 mL Erlenmeyer flasks containing 50 mL of either BBM alone (controls) or BBM and TMP. First, based on preliminary results, five different concentrations in the range of 6.25-100 mg L^{-1} were tested (Test 1). Then, in order to reach about 100% growth inhibition, two higher concentrations (200 and 400 mg L^{-1}) were tested (Test 2). The tests were carried out in triplicate and in axenic conditions (Environment Canada, 2007). The flasks were incubated on a shaking (~100 RPM) apparatus at the same conditions (light, temperature) used for culturing. After 96 h the algal growth was measured by counting the cells in a Burker chamber. In order to measure the average cell surface area, images of ~ 100 algal cells from each tested concentration were acquired using a digital photo camera (Nikon coolpix S3000) mounted on the microscope eyepiece, pre-processed using Adobe Photoshop 7.0 software and then analyzed using NIS-Elements Br software. At the end of the tests, algistatic effects were differentiated from algicidal effects by subculturing in 50 mL of BBM a 0.5 mL aliquot of each test solution containing growth-inhibited algae. The subcultures were incubated for a period of up to 9 days under the environmental conditions used in the test and were discontinued as soon as growth occurred.

2.3.2 Reproduction test (D. magna)

The *D. magna* reproduction test was performed in accordance with the OECD Guideline 211 (OECD, 1998). The ADaM medium was used for the controls and the dilution of test solutions. First, based on preliminary results, five different concentrations in the range of 0.39-6.25 mg L^{-1} were tested (Test 1). Then, in order to reach about 100% reproduction inhibition, three higher concentrations (12.5, 25 and 50 mg L^{-1}) were tested (Test 2). For each of the concentrations tested and for the control, 10 young daphnids (<24 h old) were allocated individually in 150 mL sterile polystyrene vessels containing 50 mL of solution and incubated at the same conditions (light, temperature) used for culturing. During the tests, solutions were renewed every other day, the neonates removed and counted, and the feed (*S. dimorphus*, $8x10^5$ cells mL^{-1}) supplied. Old (48-h) solutions from each concentration were pooled and monitored for pH, conductivity and dissolved oxygen using YSI 85 Multiparameter Instrument (YSI Incorporated, Yellow Springs, OH, USA). Effects on growth were evaluated as follows. After collecting the 60 individuals for the test, another sixty randomly selected juveniles (<24h old and born to the same mothers) were transferred in clean ADaM, fixed by progressively adding ethanol to a 70% concentration, and their length measured (from the top of the eye to the base of the tail spine) by means of a DMD108 Digital Microimaging Device (Leica Microsystems, Milano, Italy). This was done to get the approximate medium length of daphnids at the beginning of the test. The same procedure was repeated on the surviving daphnids at the end of the test and the average daily growth rate calculated over the 21-d period.

2.3.3 Growth inhibition test (L. minor)

This test was performed in accordance with the OECD guideline 221 (OECD, 2006). Roux flasks (75 cm^2), equipped with vented caps, were filled with 100 mL of either BBM alone (controls) or BBM and TMP. Based on preliminary experiments, five concentrations of TMP (Table 1) were tested, in triplicate. The test started by introducing 9 *L. minor* fronds into each flask. Only plants with two or three fronds were chosen. The flasks were incubated at the same conditions (light, temperature) used for culturing. During the exposure period, the colonies were photographed daily using the Nikon coolpix S3000, the frond number counted and the total frond area measured using 'imageJ' free software. At the end of the test, after photographing, fronds from each flask were weighed (fresh weight).

Table 1. Applied Guidelines and trimethoprim (TMP) concentrations used in the various tests.

Organism	Test	Guideline	TMP concentration (mg L^{-1})
P.subcapitata	Growth inhibition	797.1050 CFR US-EPA	6.25; 12.5; 25; 50;100; 200; 400
D.magna	Reproduction	OECD 211	0.39; 0.78; 1.56; 3.12; 6.25; 12.5; 25; 50
L.minor	Growth inhibition	OECD 221	6.25; 12.5; 25; 50;100
P.reticulata	Prolonged toxicity	OECD 203/204	6.25; 12.5; 25; 50;100

2.3.4 Fish Toxicity Test (P. reticulata)

This semi-static test lasted 14 days and was performed in accordance with the OECD Guideline 203 (OECD, 1992) and 204 (OECD, 1984). The same reconstituted water used for stock maintenance in the aquaria was used for the controls and for the preparation of test solutions. The assayed concentrations were based on results from preliminary tests, and are reported in Table 1. For each of the five concentrations and for the control, seven fish (estimated average weight: 137 ± 20 mg) were allocated in small, round glass tanks containing 1 L of solution, and incubated at the same conditions (light, temperature) used for stock maintenance. During the test, feed (14 mg of the formula used for stock maintenance) was supplied daily to each group. At 84-h intervals fish were transferred to a clean tank containing a renewed solution. Old (84-h) solutions from each concentration level were collected and monitored for pH, conductivity and dissolved oxygen using the YSI 85 Multiparameter Instrument.

At the end of the test, in order to study the effects of TMP on swimming activity (total travelled distance), fish groups of each tank were filmed for 12 minutes. All videos were acquired using a JVC EVERIO GZ-MS100E digital video-camera and the middle part (2 minutes) of each video sequence analysed on a PC Acer Aspire M3201, using Swistrack 4.0 (Lochmatter *et al*., 2008) and Microsoft Excel 2007 software, to calculate the total distance travelled in 2 minutes by each fish. Video acquisition settings were as follows: frame rate 25 FPS, view from above, shot distance 50 cm, Focal length 2.2 mm, Field of view 56.7°, light intensity 150 ± 20 lux. Finally, fish were sacrificed with a lethal dose of tricaine (Sandoz, Italy), measured (total length and weight) and prepared for immunohistochemistry (see below). This experimentation was authorized by the Italian Ministry of Health (decree n. 175/2010-B).

2.4 Immunohistochemistry (IHC)

Fish were fixed with 4% paraformaldehyde in physiologically buffered saline (PBS) (0.1 M, pH 7.4) at 4°C overnight, washed in PBS, dehydrated through a graded series of ethanol and embedded in paraffin. Consecutive sections were cut at a thickness of 4 µm using a microtome. Immunohistochemical staining was performed using the Envision system (goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled complex, Dakocytomation, Italy). Before applying the primary antibodies, endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 in PBS. Non-specific binding sites were blocked by incubating the sections in normal goat serum (Dakocytomation). Sections were then incubated with the primary polyclonal rabbit CYP1A antiserum, overnight at 4 °C, at a dilution of 1:200. The antibody was raised against peptides 190-202 and 282-296 of rainbow trout CYP1A (Biosense laboratories, Norway). The immunoreactive sites were visualized using a freshly prepared solution of 10 mg of 3.3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, Italy) in 15 mL of a 0.5M Tris buffer at pH 7.6, containing 1.5 mL of 0.03% H_2O_2. To ascertain structural details, sections were counterstained with Mayer's haematoxylin. The specificity of the immunostaining was verified by incubating sections with: (i) PBS instead of the specific primary antibody; (ii) preimmune sera instead of the primary antiserum; (iii) PBS instead of the secondary antibodies; (iv) antiserum pre-absorbed with an excess of the respective synthetic peptide (3 µg μL^{-1}).

2.5 Chemical analysis

To check for the stability of the compound, samples of freshly prepared and 48-h-old (*D. magna* Reproduction Test), 84-h-old (*P. reticulata*, Prolonged Toxicity Test), 96-h-old (*P. subcapitata*, Growth Inhibition Test) and 7 days old (*L. minor* Growth Inhibition Test) solutions were collected. As the media did not interfere with the analysis, extraction and purification were unnecessary. Samples were simply filtered through a Phenex-RC 0.20 µm filter (Phenomenex, Castelmaggiore, Italy) and then analyzed by means of HPLC, with UV detector set at 268 nm. The HPLC system (Jasco, Tokyo Japan) consisted of a PU-980 HPLC pump equipped with an LG-980-02 ternary gradient unit, a UV-975 detector and an AS-950 autosampler. The analytical column was a Zorbax XDB C18 (Agilent Technologies, USA). The mobile phase consisted of (A) KH_2PO_4 25 mM brought to pH 3 with orthophosphoric acid + (B) acetonitrile (85+15), operating in isocratic condition at 0.5 mL min^{-1} flow. Injection volumes of 50 µL and column operating at room temperature (21±1°C) were used. The linearity of response was verified in the 0.050–6.25 mg L^{-1} range. In order to match this concentration range, samples were accordingly diluted in HPLC mobile phase before injection, when necessary.

2.6 Data Analysis

Data were analyzed using Toxstat 3.4 (Western Ecosystems Technology, Inc., Cheyenne, WY): after verifying normality (X^2 test) and homogeneity of variance (Bartlett's test), no observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) values were derived by analysis of variance (ANOVA) and t-test with the Bonferroni adjustment or Dunnett's test. In all tests, the level of significance was set at $p<0.05$. Using the Graph Pad Prism 5.04 software, data were best fitted to a four-parameter (*L. minor* and *P.* reticulata) or five- parameter (*P. subcapitata* and *D. magna*) logistic dose-response model and then the EC_{50s} and EC_{20s} were determined.

3. Results

3.1 HPLC analysis

Calibration curves obtained with TMP spiked water samples were linear over the entire concentration range (0.050–6.25 mg L^{-1}) with a correlation coefficient always >0.965. Measured concentrations of TMP in freshly prepared test solutions were within the range 91-106% of nominal. Degradation rates measured by comparing peaks of fresh solutions to peaks of old solutions were always ≤ 7%. In the chromatograms no peaks attributable to metabolites of the compound were observed. As the concentration of TMP was maintained within ±20% of the nominal concentration, test results were based on nominal values.

3.2 P. subcapitata growth inhibition test

Statistically significant ($p<0.05$) effects on algal growth were recorded at TMP concentrations ≥25 mg L^{-1}. EC_{20} and EC_{50} are reported in Table 2, together with the estimated NOEC and LOEC. The growth inhibition curve is shown in Fig. 1 (a). No significant pH variations were recorded during the 96-h incubation period. The average cell surface area of algae exposed to the various TMP concentrations was not significantly different from the control values (22±6 and 23±5 μm^2). In all the subcultures obtained from the test solutions containing growth-inhibited algae, recovery of algal growth was observed within one week (algistatic effect).

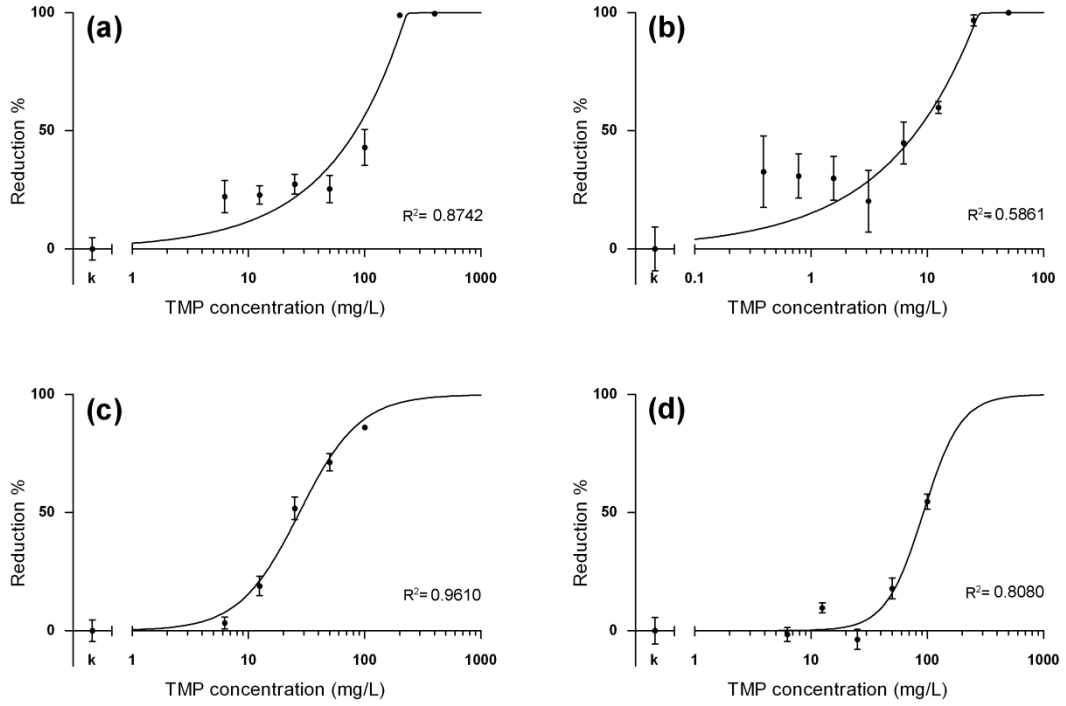

Figure 1. Trimethoprim (TMP) reduction percentage of, *P. subcapitata* growth (a), *D. magna* reproduction,(b) *L. minor* fresh weight (c) and *P. reticulata* swimming activity (d). Reduction percentages were calculated from the ratio between the observed responses and the control mean. Error bars represent standard error of the mean.

Table 2. NOEC, LOEC and ECs of trimethoprim (TMP) measured (mg L^{-1}) on the four test organisms.

Organism	Endpoint	NOEC	LOEC	EC_{20} (95% C.L.)	EC_{50} (95% C.L.)
P.subcapitata	Growth (cells number)	12.5	25	23.73 (12.81-43.93)	83.80 (59.81-117.40)
D.magna	Reproduction (offspring number)	3.12	6.25	2.25 (1.30-3.90)	8.21 (5.93-11.36)
L.minor	Growth (fresh-weight)	6.25	12.5	11.98 (9.65-14.87)	27.43 (24.03-31.31)
P.reticulata	Swimming (travelled distance)	25	50	55.31 (46.48-65.82)	92.66 (83.11-103.30)

3.3 D. magna reproduction test

In the reproduction tests, monitored water quality parameters ranged as follows: pH 7.7–8.2, conductivity 1016–1170 µS cm^{-1}, and dissolved oxygen 8.4–9.0 mg L^{-1}. No mortality was observed in the control groups, with an average of 126 (test 1) and 130 (test 2) neonates per female. Significant ($p<0.05$) effects on reproduction were observed at concentrations of 6.25 mg L^{-1} and above while significant effects on the average daily growth were observed only at concentrations of 25 and 50 mg L^{-1}. At this latter concentration, reproduction was totally inhibited and only 50% survival was recorded (Table 3). The reproduction (neonates per female) inhibition curve is shown in Fig.1 (b)

Table 3. *Daphnia magna* reproduction tests with trimethoprim (TMP): survival, average daily growth and offspring production.

Nominal concentration (mg L^{-1})	Survival (%)	Average daily growth (length, µm)	Total number of neonates	Neonates per female (mean ± sd)
Test 2				
50	50	60.3±9.5[a]	0	0[a]
25	80	120.2±4.4[a]	34	4.25±8.15[a]
12.5	100	161.2±12.1	521	52.10±10.11[a]
Control	100	168,3±8.1	1298	129.80±16.35
Test 1				
6.25	100	143.3±21.8	702	70.20±34.97[a]
3.125	90	145.7±8.0	834	92.67±45.32
1.562	80	142.4±9.0	698	87.25±37.46
0.781	90	140.2±13.5	761	84.56±38.51
0.395	90	142.7±16.1	761	84.56±56.89
Control	100	146.7±14.2	1256	125.60±36.83

[a] significantly different from the control ($p<0.05$)

3.4 L. minor growth inhibition test

Effects on frond number were significant at concentrations ≥25 mg L^{-1} while those on fresh weight were significant ($p<0.05$) at concentrations ≥12.5 mg L^{-1}. NOEC, LOEC and ECs for this

endpoint are reported in Table 2. Growth inhibition curve is shown in Fig. 1 (c). No significant pH variations were recorded during the 7 days incubation period.

3.5 P. reticulata Prolonged Toxicity Test

Monitored water quality parameters ranged as follows: pH 7.7–8.6, conductivity 4243-4469 µS cm^{-1}, and dissolved oxygen 5.9–7.2 mg L^{-1}. No lethality was recorded in this test. The average total distances travelled in 2 minutes by fish exposed to TMP concentrations of 50 and 100 mg L^{-1} (4441±676 and 2250±331 mm, respectively) were significantly different ($p<0.05$) from the controls (5518±822 mm). NOEC, LOEC and ECs for this endpoint are reported in Table 2. The swimming activity inhibition curve is shown in Fig.1 (d), while in Fig. 2 a box plot of swimming activity is presented. Average body length, weight and fitness index (length/weight) of fish exposed to all TMP treatments were not significantly different from the control values (25.2±1.4 mm; 157±18 mg; 0.16 mm/mg).

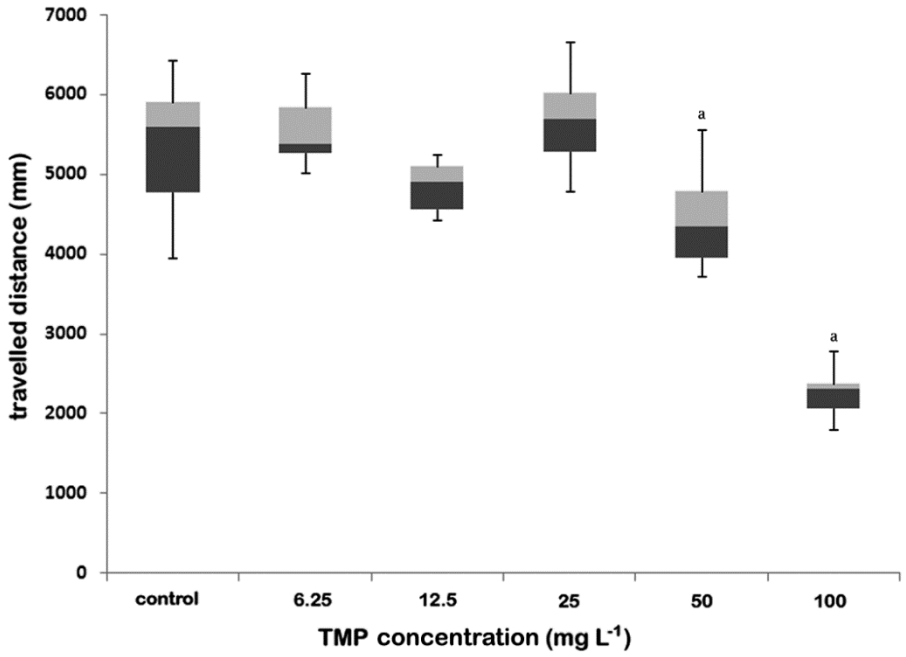

Figure 2. Box Plot for swimming activity of groups of seven fish (*Poecilia reticulata*) exposed to different concentrations of trimethoprim (TMP). Median, interquartile range, and maximum and minimum values are shown. a : significantly different from the control ($p<0.05$)

The anti-CYP1A antibody exhibited positive immunostaining in the parenchyma of liver, in the epithelia of skin, gills, kidney, pharynx and intestine (Fig. 3), in endothelial cells of vessels surrounding skeletal muscle fibres and in melano-macrophage centres located in the parenchyma of thymus. In the liver, a faint immunostaining was diffusely detectable in the cytoplasm of hepatocytes as well as in the endothelial cells of hepatic arteries and veins. In the kidney, a CYP1A immunoreactivity was found in the epithelial cells of the tubules, whereas the glomeruli were

immunonegative. In skeletal muscle, immunopositivity was detectable in endothelial cells of vessels surrounding skeletal fibres. In the epithelia of kidney (Fig.3A), gills (Fig.3C) and intestine (Fig.3E), the highest expression of CYP1A protein was detected in animals incubated at the concentration of 100 mg L^{-1}, whereas the lowest expression was observed in control animals (Fig.3B, D, F). In these tissues a gradual decrease of CYP1A expression was detectable comparing animals incubated at the concentrations of 100, 50, 25, 12.5, 6.25 mg L^{-1}, respectively. The specificity of immunostaining was verified as all controls were negative.

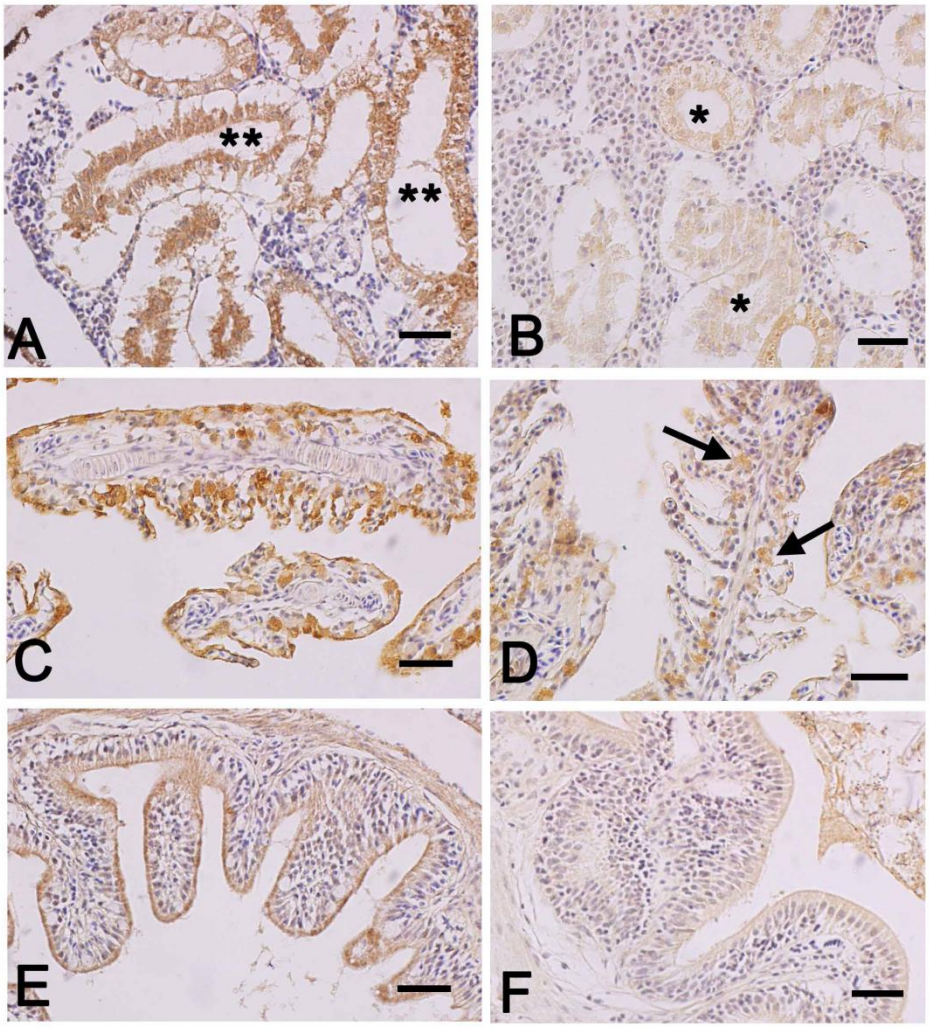

Figure 3. Immunohistochemical localization of CYP1A in *P.reticulata*. All panels are counterstained with Mayer's haematoxylin. A) Kidney of an animal exposed to 100 mg L^{-1} of TMP which exhibits a strong CYP1A immunostaining in the cytoplasm of the epithelial cells of tubules (asterisks). B) Kidney of an animal from control group which exhibits a faint CYP1A immunostaining in the cytoplasm of the epithelial cells of tubules (asterisks). C) Gills of an animal exposed to 100 mg L^{-1} of TMP which exhibit a strong CYP1A immunostaining in the epithelial cells lining both primary and secondary lamellae. D) Gills of an animal from control group which exhibit a moderate CYP1A immunostaining in the epithelial cells lining the secondary lamellae (arrows). E) Intestine of an animal exposed to 100 mg L^{-1} of TMP which exhibits a strong CYP1A immunostaining in the brush border of the intestinal epithelium. F) Intestine of an animal from control group which exhibits a faint CYP1A immunostaining in the brush border of the intestinal epithelium. Scale bars: A-F 20 µm.

4. Discussion

Altogether TMP showed varying levels of toxicity in the four tests performed, with NOECs for the various endpoints in the range 3.12 to 25 mg L^{-1}. The NOEC (3.12 mg L^{-1}) in the *D. magna* reproduction test was higher than the calculated EC_{20} (2.25 mg L^{-1}): as already indicated by Landis *et* al. (2011), hypothesis testing using data from currently used toxicity test protocols cannot effectively detect effects at low concentrations, and this is due in part to the lack of statistical power given the number of replicates and the intrinsic laboratory and organismal variability within the experiments. Park and Choi (2008) found no effects on reproduction of *D. magna* even at a TMP concentration of 6 mg L^{-1}. However, as already observed with sulfamethazine (De Liguoro *et al*., 2009), a folic acid supplement was administered to the daphnids during that test and this may have protected the reproduction of the test organisms by compensating for the deficiencies caused by TMP.

The growth inhibition test on *P. subcapitata* gave an EC_{50} of 83.8 mg L^{-1} which is in good agreement with the value of 80.3 mg L^{-1} presented by Eguchi *et al.* (2004). Here again, even at the lowest exposure concentration (6.25 mg L^{-1}) more than 20% inhibition was observed (Fig.1a), suggesting possible effects at concentrations lower than the NOEC (12.5 mg L^{-1}) although not statistically significant.

L. minor, although not included in the VICH guideline (EMEA, 2005), was confirmed to be a good indicator of pharmaceutical toxicity (Cleuvers, 2003) as its sensitivity to TMP was greater than that of both the eukaryote *P. subcapitata* and the prokaryote *Microcystis aeruginosa* (Halling-Sørensen *et al*., 2000). To support the total frond number endpoint, two endpoints based on biomass were included (total frond area and fresh weight). The fresh weight endpoint, with a calculated EC_{50} of 27.4 mg L^{-1}, was more sensitive to TMP exposure than total frond number.

Lethal effects were not observed in the toxicity test on *P. reticulata* after 72h exposure to 100 mg L^{-1} of TMP, which is in accordance with the observations of Halling-Sørensen *et al*. (2000) on *Brachydanio rerio*, or at the end of the test (14 days). Although the effects on length and body weight were not statistically significant, the behavioural endpoint turned out to be of interest. The effect on swimming activity was apparent to the naked eye at the highest concentration tested and, after video tracking and analysis, was also present and statistically significant at 50 mg L^{-1}. While it may be only the consequence of the metabolic disturbance caused by the drug, the possibility that TMP could have some direct effect on the nervous system of fish cannot be excluded. As a matter of fact, some cases of neurotoxicity have been reported after TMP administration in humans (Saidinejad, 2005) and horses (Stack and Schott, 2011). Furthermore, in preliminary tests, we have evaluated

other antifolic agents (sulfamethazine, sulfaguanidine and sulfaquinoxaline) at a concentration of 100 mg L^{-1} for their effects on *P. reticulata* swimming activity and their inhibition was absent or clearly lower than that of TMP (Dalla Bona *et al*., 2011). At concentrations lower than NOEC (25 mg L^{-1}) a non-monotonic response was recorded (Fig.1), probably due to the individual variability and to the limited number of individuals (7 fish is the minimum required by the OECD 203 protocol) in each group. Otherwise, between 25 and 100 mg L^{-1} the steep slope of the curve shows that TMP inhibition of swimming activity may become severe with only a slight increase in concentration. In fish exposed to 100 mg L^{-1} of TMP a lower tendency to swim in the peripheral area of the round tank, where fast swimming is possible, was also evident. Swimming activity, like other behavioural endpoints, is sensitive, non-invasive, and is an expression of integrated, whole animal response. Its alteration may be related to ecologically-relevant issues such as predation avoidance, prey capture, growth, stress resistance, reproduction and longevity (Kane *et al*., 2005). Another interesting and visible effect, though not quantified, was the alteration of skin colour: fish exposed to 100 mg L^{-1} of TMP turned darker in colour. This also may have ecological impacts, as fish colour affects susceptibility to predation and mating success (Labonne and Hendry, 2010). However, it is highly unlikely that TMP can exert such effects in the aquatic environment as field concentrations, even in aquaculture settings, are well below the 50-100 mg L^{-1} range.

In fish, CYP1A evaluated in target tissues of a sentinel species, is a widely accepted environmental biomarker of exposure for several xenobiotic groups (petroleum compounds, dioxins, PCBs, PAHs etc.) present in aquatic environments (Stegeman and Hahn, 1994). Although the liver represents the main site of CYP1A expression in fish (Stegeman and Hahn, 1994), CYP1A presence and induction is often detectable in various extrahepatic tissues, particularly in the epithelia of organs involved in osmoregulation (gills, intestine and kidney). In our study, the CYP1A protein was detected in the cytoplasm of cells of renal tubules and gills and in the brush border of the intestinal epithelium, and the intensity of reactivity showed an apparent correlation to the TMP exposure level. CYP1A immunopositivity was observed also in the endothelial cells of vessels. This result is in accordance with those of Sarasquete and Segner (2000) and of Ortiz-Delgado e*t al*. (2005) who detected CYP1A immunostaining in various organs and particularly in endothelial cells of teleost fish exposed to various chemicals. Since the endothelial cells regulate the exchange between blood and the underlying tissue, they are of critical importance in the maintenance of the internal milieu (Sarasquete and Segner, 2000). Although immunopositivity was found also in liver parenchyma, no differences in terms of intensity were observed among groups suggesting that other organs may be mainly involved in CYP1A induction. Actually, Gagné *et al*. (2006) showed that some pharmaceuticals,

including TMP, failed to induce EROD activity (7-ethoxyresorufin O-dethylase) in rainbow trout hepatocytes.

According to the VICH Guideline (EMEA, 2005), to obtain the PNEC for TMP in freshwater, an AF of 10 should be applied to the NOEC that we obtained in the *D. magna* reproduction test (3.12 mg L^{-1}), as this is the lowest NOEC for TMP produced so far in a chronic toxicity test on a freshwater organisms (see the review of Santos *et al*., 2010). However, an AF of 100 is currently considered more adequate and has recently been strongly recommended for the lowest of three chronic NOECs (von der Ohe *et al*., 2011). Then, the ratio between the TMP concentrations detected in freshwater (<1 µg L^{-1}; Santos *et al*., 2010) and the calculated PNEC (31.2 µg L^{-1}) gives a risk quotient (RQ) of <0.03 which is far higher than the RQ of $9.5x10^{-4}$ obtained for TMP by Halling-Sørensen *et al*. (2000) using a calculated PEC, but is still largely <1 . So, at the concentrations usually found in the aquatic environment, TMP should not harm freshwater organisms. However, TMP is widely used in aquaculture for metaphylactic mass treatments (Hektoen *et al*., 1995; Boxall *et al*., 2003). Treatments used in aquaculture typically have a high potential to impact the aquatic environment, primarily because they are added directly to the environment, and secondly because of the low feed intake by diseased animals and the incomplete absorption of drugs even in healthy fish (Weston, 1996). In aquaculture ponds from Viet Nam, very high concentrations of TMP (up to 2.03 mg L^{-1}) were detected and a TMP average concentration of about 0.2 mg L^{-1}, which is still higher than the calculated PNEC for *D. magna* (31.2 µg L^{-1}), was measured in the surrounding canals (Le and Munekage, 2004). While the reported situation might be a consequence of poorly controlled and irresponsible antibiotic use, it is a good example of how aquaculture usage of antibacterials may contribute to the environmental drug load. It should be noted also that during bacterial infection therapy, the negative effect of TMP on swimming activity may have detrimental consequences on the treated fish by reducing their feed intake, as fish increase swimming speed to increase their search area and improve the chance of finding food (Andrew *et al*., 2002). If this occurs, both the reduced swimming activity and feed intake could be erroneously ascribed to the disease itself. However, it is difficult to say if the usual dosage of 5 mg kg^{-1} applied in aquaculture is enough to trigger such side effects. Anyway, there might be fish species more sensitive than *P. reticulata*, and TMP is administered to fish in combination with other antifolic agents, the additive effects of which (De Liguoro *et al*., 2009) should be taken into account. Another major source of environmental contamination by TMP and other antifolic agents are hospitals. Brenner *et al*. (2011) have recently reported concentrations up to 37.3 µg L^{-1} sulfamethoxazole and 11.3 µg L^{-1} TMP in the final effluent of the treatment system of a hospital. Nevertheless, particularly in the context of sewage effluents, there might be other contaminants that should be considered; these may act in some way to increase toxicity, but not necessarily. Elements

such as nutrients may in fact act to offset some of the effects of the contaminants, and in a complex ecosystem is not necessarily fair to assume that additional chemicals will always have a potentiating effect.

5. Conclusions

TMP was toxic at concentrations <100 mg L^{-1} to all the four organisms tested, confirming the two Risk Phrases 'Harmful to aquatic organisms' and 'May cause long-term adverse effects in the aquatic environment' already reported in the Safety Data Sheet of the drug (Chemwatch, 2009). The drug was able to induce the CYP1A protein in various tissues of *P. reticulata*, but it is not known if this happens through the classical activation cascade of the Ah receptor or through other signalling pathways (Hu *et al*., 2007). Further studies are needed to clarify this point. The sensitivity to TMP of the duckweed *L. minor* was higher than that of the prokaryote *Microcystis aeruginosa* (Halling-Sørensen *et al*., 2000), highlighting the value of extending toxicity tests of pharmaceuticals to aquatic organisms that are not included in the current guidelines for the Environmental Impact Assessments of Veterinary Medicinal Products (EMEA, 2005).

The presence of trace concentrations of TMP in the aquatic environment has been reported by several authors (Kümmerer, 2009; Santos *et al*., 2010). While the concentrations usually detected are below those able to evoke appreciable biological effects in aquatic organisms, TMP concentrations in aquaculture and hospital effluents might be one to three orders of magnitude higher than the concentrations usually found in surface waters (Le and Munekage, 2004; Brenner *et al*., 2011). Finally, the co-occurrence and additive effects of other antifolic agents (De Liguoro *et al*., 2009) should be taken into account for a more cautious risk assessment of the drug.

Acknowledgments

We gratefully acknowledge the collaboration of Dr. Silvia Boaretto. This investigation was funded by grants from the University of Padua (ex60% 2011).

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Chapter 5

Effects of Enrofloxacin, Ciprofloxacin and Trimethoprim on two generations of Daphnia magna

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Submitted

Abstract

Multigenerational tests on *Daphnia magna* were performed exposing two subsequent generation of the crustacean to enrofloxacin (EFX), his metabolite ciprofloxacin (CPX) and trimethoprim (TMP). Mortality rate of 100% and 50% was detected in F0 at concentrations of ≥12.5 mg L^{-1} (EFX) and 50 mg L^{-1} (TMP), respectively. In F1 with respect to F0, both for growth and reproduction, a worsening trend of the response with EFX, a similar response with CPX and an attenuating trend with TMP was observed. Furthermore, the lowest EC_{20} for reproduction inhibition (1.27 mg L^{-1}) was calculated for F1 exposed to EFX. However, other experimentations, longer and more complex, are necessary in order to confirm that EFX is more hazardous to daphnids than CPX and TMP. EC_{50s} measured for the three assayed antibacterials were in the 6.49-36.53 mg L^{-1} range and therefore environmental unrealistic, except in case of exceptional contaminations that may occur in relation to poorly controlled wastewaters from pharmaceutical factories or excessive use of prophylactic treatments in aquaculture.

1 Introduction

Veterinary pharmaceuticals, in particular antibacterials, are pollutants of relatively new concern. Most of them can reach the aquatic environment via different pathways, principally as a consequence of the use in aquaculture or in livestock treatments. Emissions during manufacturing and improper disposals also play a role in the environmental burden of these compounds (Boxall et al., 2004; Halling-Sørensen et al., 1998). Notwithstanding the successful policies adopted by some developed countries in order to reduce their overuse, the global consumption of antibacterials is constantly increasing, with an estimated annual rate of 4% (Hamad, 2010).

In EU Fluoroquinolones (FQs) and trimethoprim (TMP), account for 3.2% of antibacterial agents sold for food producing animals (EMA, 2011). Residues of TMP and FQs have been frequently found in surface waters, the concentrations usually ranging from 10 ng L^{-1} to 10 µg L^{-1} (Santos et al., 2010) with exceptional FQs peaks (mg L^{-1}) detected in an effluent from drug manufactures (Larsson et al., 2007; Le and Munekage, 2004).

FQs mechanism of action is directed toward bacterial DNA-gyrases and topoisomerase IV (Martinez et al., 2006). However, as a consequence of their weak affinity also to eukaryotic topoisomerase FQs can cause DNA damages in non target organisms (Pommier et al., 2010; Thomé et al., 2012). Other crucial aspects of FQs regard their persistence and the toxicity of metabolites. In fact, even though FQs are not particularly stable when exposed to light sources (Knapp et al., 2005; Sturini et al., 2010), their easy sorption to the soil may lead to the accumulation and to the subsequent slow desorption

and contamination of the aquatic environment (Picó and Andreu, 2007). Furthermore, FQs metabolic and photolysis products are active and, in some cases, even more toxic than the parent compounds (Li et al., 2011).

Due to its high efficacy and wide spectrum, the veterinary FQ enrofloxacin (EFX) is used worldwide for livestock diseases and, in some countries, also for aquaculture treatments (Quesada et al., 2013; Rico et al., 2013). In shallow waters EFX photodegrades to its main metabolite Ciprofloxacin (CPX, Knapp et al., 2005; Li et al., 2011), itself a FQ mainly used in human medicine.

Trimethoprim (TMP) disrupt the synthesis of DNA by competitively inhibiting dihydrofolate reductase, which catalyses the conversion of dihydrofolate to tetrahydrofolate (Abou-Eisha et al., 1999). It is largely employed both in veterinary and in human medicine, alone or in association with sulphonamides. In various studies, genotoxic effects of TMP have been reported in non target organisms and justified as an indirect consequence of the disruption of DNA synthesis (Abou-Eisha et al., 1999; Binelli et al., 2009).

Based on their occurrence in the environment and the available toxicity data, Kim and coll. (2008) suggested that EFX, CPX and TMP are likely to be hazardous for the environment and/or for human health, thus representing priority drugs for in depth environmental risk assessment.

Multigenerational tests are time consuming and laborious, and only few research studies have thus far applied this kind of tests in aquatic ecotoxicology (Chen et al., 2014; Dietrich et al., 2010; Kim et al., 2012; Lamichhane et al., 2013; Tsui and Wang, 2005; Vandegehuchte et al., 2010). Nevertheless, when compared to the acute toxicity test and one generation chronic test, they allow to obtain a more representative picture of the population effects of antibacterials; the real pattern of environmental exposition to these drugs being low throughout the entire life cycles for numerous generations (Kim et al., 2012). In this work lethal and sub-lethal (inhibition of reproduction and growth) effects of EFX, CPX and TMP were evaluated throughout two generations of *D.magna.*

2 Materials and methods

2.1 Test chemicals

Tested pharmaceuticals were purchased from Sigma–Aldrich (Milano, Italy), and their purities ranged between 95 and 99%. Their solubilisation in Aachener Daphnien Medium (ADaM:hardness 193 mg $CaCO_3$ L^{-1}; (Klüttgen et al., 1994a,b)) was achieved by bringing back the pH of the medium to the original value (8.0) using 1 M NaOH or HCl. The pH was measured using a BASIC 20 pH-meter (CRISON, Carpi, Italy).

2.2 Chemical Analysis

To check for the stability of the compounds, samples of freshly prepared and old test solutions, were collected, from the highest and lowest concentration tested, during the tests. As the ADaM medium did not interfere with the analysis, extraction and purification were unnecessary. Samples were simply filtered through Phenex-PTFE 0,45 m (Phenomenex, Castel Maggiore, Italy) and then analyzed by means of HPLC. The HPLC system (Jasco, Tokyo, Japan) consisted of a PU-980 HPLC pump equipped with an LG-980-02 ternary gradient unit, a UV-975 detector and an AS-950 autosampler. The analytical columns used were a Zorbax XDB C18 (Agilent Technologies, USA) for TMP and a *Sinergy Fusion* RP (Phenomenex, Castel Maggiore, Italy) for FQs. The analytical conditions for TMP were the following: injection volume 10 μL, mobile phase in isocratic elution composed by 85% of a 25 mM solution of orthophosphoric acid (pH 3) and 15% acetonitrile, with a 0.5 mL/min flux and the detector UV-975 (Jasco, Tokyo, Japan) set at 268 nm. The analytical conditions for FQs were the following: injection volume 10 μL, mobile phase consisting of (A) 25 mM solution of orthophosphoric acid (pH 3) and (B) acetonitrile in gradient elution,
fluorescence detector FP-920 (Jasco, Tokyo, Japan) set at 280 nm excitation and 450 nm emission. Mobile phase composition (A/B, v/v) was 80:20 from 0 to 4 min, 20:80 from 8 min to 10 min, 80:20 from 12 to 14 min. For each compound, the linearity of response was verified in the 0.050 - 6.25 mgL^{-1} range. In order to match this concentration range, samples were accordingly diluted in HPLC mobile phase before injection.

2.3 Culture conditions

Resting eggs of *Daphnia magna* were originally provided by ECOTOX (Milano, Italy). A single clone culture was selected based on itsadequate sensitivity to potassium dichromate (ISO, 1996). The sensitivity was then checked periodically (every 4 months). The organisms were maintained in ADaM at 20±1°C, with a 16-h light (2.6 mE m^{-2} s^{-1}): 8-h dark photoperiod. They were fed three times per week with *Scenedesmus dimorphus* ($8x10^5$ cells mL^{-1}). Further details about the culturing method have already been reported (De Liguoro et al., 2012).

2.4 Tests

The OECD 221 test guideline was applied both to the first and to the second generation of daphnids. The first generation (F0) was composed of neonates produced by the monoclonal culture between second and fifth broods, while the second generation (F1) was composed of neonates produced by F0 during the last day (21^{th}) of exposition to each pharmaceutical.

For each exposure level (control included) 10 daphnids were used, each allocated in a 150 mL beaker containing 50 mL of test solution. F0 daphnids were exposed for 21 days to 5 scaled concentrations of the three drugs in the following ranges: 25-1.6 mg L^{-1} (EFX), 30-1.88 mg L^{-1} (CPX) and 50-3.13 mg L^{-1}. During the following 21 days, F1 daphnids were either exposed to the same concentration as their parents (F1e) or returned to a clean medium (F1n) (Figure 1); this in order to assess both the cumulative effects of the drugs after two generations exposition and the capacity of the crustaceans to recover from a parental and prenatal exposure. However, where the number of neonates was not sufficient, due to excessive mortality and/or excessive reproduction inhibition in F0, the test on F1 was not carried out.

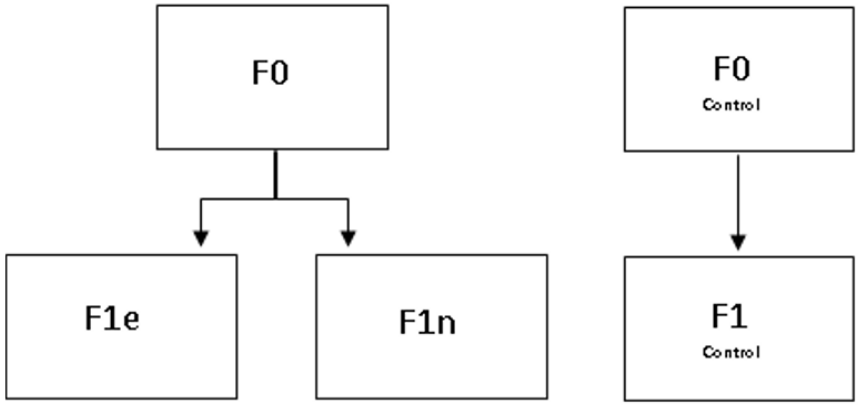

Figure 1. Experimental design of two generation test on *D.magna.* F0, exposure group; F1e offspring re exposed at the parental conditions; F1n offspring reported in clean medium; $F0_{control}$ controls of first generation, $F1_{control}$ controls of second generation.

2.5 Endpoints

The evaluated endpoints were: mortality, growth inhibition and reproductive inhibition. Before the beginning of each experiment, in order to measure growth inhibition, 30 offspring not intended for the test were isolated, fixed in ethanol 70% and measured under a microscope. Length was defined as the distance from the upper edge of the compound eye to the base of the tail spine. At the end of each experiment all the adults were collected and measured as above, and the daily growth rates calculated.

2.6 Water quality parameter

In all tests the recorded values were always within the following ranges: pH 7.6 - 8.4, O_2 6.54 - 8.40 mg L^{-1}, Conductivity 1005 - 1120 μS cm^{-1}.

2.7 Statistical Analysis

Data were analyzed using GraphPad Prism® 5.02 (La Jolla, California, USA). EC_{20} and EC_{50} for reproduction inhibition and average daily growth were obtained using dose response curves (Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*Hill Slope)). Since data were normally distributed and variances homogeneous LOEC and NOEC were determined using the analysis of variance (ANOVA) followed by the Bonferroni post hoc test.

3 Results

3.1 Mortality

Survival rates in the control groups were always ≥80%. The F0 generation exposed to 12.5 mg L^{-1} and 25 mg L^{-1} of EFX showed 100% mortality while 50% mortality was recorded in F0 exposed to 50 mg L^{-1} of TMP and 30% mortality in F0 exposed to 30 mg L^{-1} of CPX. ≥(for EFX,. Some mortality was recorded also in F1e and F1n born to the EFX exposed F0. In F1n a negative correlation with parental exposurewas seemingly present (Table 1).

Table 1. Survival rate, offspring production and average daily growth for the different compounds and generations

Copmpound	Generation	Level of Exposure (mg L^{-1})	Survival Rate %	Total Number of Neonates	Neonates per Female (mean ± SD)	Average daily growth (length, µm ± SD)
EFX	F0	Control	90	1038	115.3 ± 26.09	154.26 ± 7.571
		C5 (1.57)	100	962	96.2 ± 22.14	148.81 ± 6.640
		C4 (3.13)	100	957	95.7 ± 25.10	142.61 ± 4.419 **
		C3 (6.25)	80	447	55.9 ± 18.72 ***	131.05 ± 7.776 ***
		C2 (12.5)	0	-	-	-
		C1 (25)	0	-	-	-
	F1e	Control	80	770	96.6 ± 37.82	149.15 ± 16.45
		C5 (1.57)	90	620	68.9 ± 19.67	142.95 ± 7.689
		C4 (3.13)	100	596	59.6 ± 25.39 *	142.66 ± 7.416
		C3 (6.25)	50	143	28.6 ± 28.65 ***	100.53 ± 26.99 ***
	F1n	C5 (0)	40	320	80 ± 38.08	147.28 ± 15.69
		C4 (0)	60	394	65.7 ± 31.07	152.25 ± 7.590
		C3 (0)	70	107	15.3 ± 14.13 ***	96.75 ± 22.12 ***
CPX	F0	Control	100	1183	118.3 ± 23.83	154.3 ± 5.741
		C5 (1.88)	100	1261	126.1 ± 43.52	156.1 ± 6.902
		C4 (3.75)	100	1362	136.2 ± 34.06	156.0 ± 6.788
		C3 (7.5)	100	817	81.7 ± 40.38	146.1 ± 25.36
		C2 (15)	90	866	96.2 ± 21.12	128.1 ± 5.301
		C1 (30)	70	314	44.86 ± 16.95 ***	127.4 ± 5.417 ***
	F1e	Control	100	858	85.8 ± 29.37	152.2 ± 6.568
		C5 (1.88)	100	1016	101.6 ± 32.58	160.9 ± 5.730
		C4 (3.75)	100	1396	139.6 ± 45.08	160.2 ± 10.11
		C3 (7.5)	80	989	123.63 ± 24.15	160.9 ± 5.829
		C2 (15)	80	879	109.88 ± 26.42	156.0 ± 7.123
		C1 (30)	100	775	77.5 ± 17.58 *	143.3 ± 7.141 *
	F1n	C5 (0)	100	1138	113.8 ± 9.76	155.1 ± 4.037
		C4 (0)	100	1267	126.7 ± 18.48	158.0 ± 5.234
		C3 (0)	100	1121	112.1 ± 43.52	156.0 ± 6.811
		C2 (0)	100	1173	117.3 ± 20.53	156.4 ± 4.215
		C1 (0)	100	1050	105 ± 25.02	153.5 ± 5.515
TMP	F0	Control	100	1298	129.8 ± 16.36	168.3 ± 7.514
		C5 (3.13)	80	962	120.3 ± 34.31	165.3 ± 13.65
		C4 (6.25)	90	1119	124.3 ± 12.31	158.6 ± 4.536
		C3 (12.5)	100	1040	104 ± 10.11 *	156.1 ± 6.903 *
		C2 (25)	80	34	4.25 ± 7.41 ***	120.2 ± 5.068 ***
		C1 (50)	50	0	0 ***	60.28 ± 9.518 ***
	F1e	Control	100	1506	150.6 ± 31.80	166.2 ± 8.410
		C5 (3.13)	100	1411	141.1 ± 17.03	158.5 ± 5.582
		C4 (6.25)	90	1198	133.1 ± 8.22	159.7 ± 5.584
		C3 (12.5)	100	1334	133.4 ± 32.46	162.1 ± 8.890
	F1n	C5 (0)	100	1308	130.8 ± 8.22	161.4 ± 6.589
		C4 (0)	100	1351	135.1 ± 11.37	165.2 ± 4.843
		C3 (0)	90	966	129.1 ± 39.69	157.7 ± 6.713

*significantly different from the controls ($p<0.05$), ** $p<0.01$, ***$p<0.001$

3.2 Inhibition of Reproduction

In F0 a significant inhibition of reproduction was observed with 6.25 mg L^{-1} of EFX, 30 mg L^{-1} of CPX and ≥12.5 mg L^{-1} of TMP (Table 1). In F1e significant inhibitory effects were observed at concentrations of ≥3.13 mg L^{-1} EFX and 30 mg L^{-1} CPX (Table 1). In F1n only the group exposed to 6.25 mg L^{-1} of EFX showed a significant decrease of reproduction. The EC_{20} and EC_{50} values for

reproduction inhibition are reported in Table 2 while Figure 2 shows the reproduction inhibition curves for EFX, CPX and TMP.

3.3 Average Daily Growth

Significant reduction in daily growth was observed in F0 when exposed to 3.13 mg L^{-1} and 6.25 mg L^{-1} of EFX, 30 mg L^{-1} of CPX and ≥ 12.5 mg L^{-1} of TMP (Table 1). The F1e daphnids were inhibited when exposed to 6.25 mg L^{-1} of EFX or to 30 mg L^{-1} of CPX. Only F1n generated by mothers exposed to 6.25 mg L^{-1} of EFX showed a significant decrease in average daily growth (Table 1). Since the inhibition of growth was generally weak, EC_{50} and EC_{20} were calculated only for the F0 groups exposed to TMP (Table 2).

4 Discussion

For obvious reasons, both high mortality and strong reproduction inhibition represent an obstacle to the performing of multigenerational tests, particularly when they already occur in F0. In this experimentation, only with CPX was possible to carry on the test on F1 at all five dose levels, while only three concentrations were assayed with EFX and TMP, due to the excessive mortality or reproduction inhibition observed in F0. Consequently, EC50s measured in F1 for EFX and TMP were obtained from curves fitted on only three points and were rather approximate.

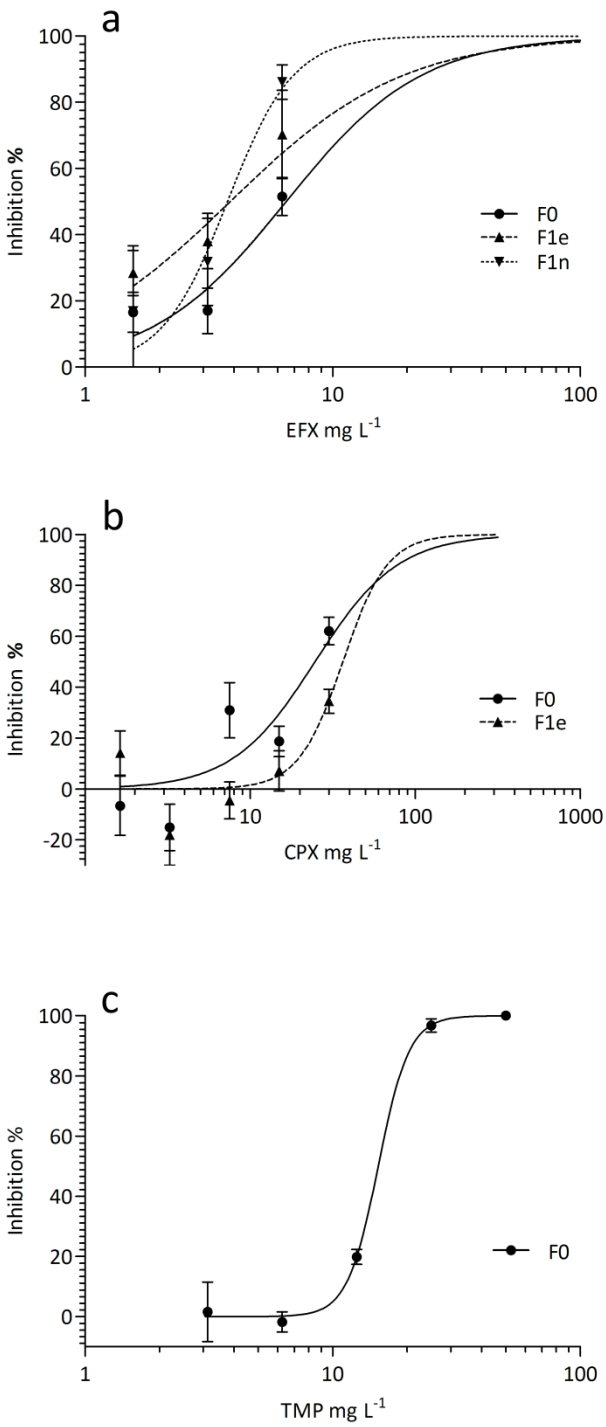

Figure 2 Dose response curves for inhibition of reproduction. **a,** enrofloxacin; **b**, ciprofloxacin; **c**, trimethoprim.

4.1 Mortality

In order to avoid lethal effects, tested concentrations of the three compounds were chosen taking into account available data on their acute toxicity in *D.magna.* However a 100% mortality rate was recorded when exposing F0 daphnids to 12.5 or 25 mg L^{-1} of EFX. During the chronic test, these lethal effects were already evident after 48h of exposition; this is in disagreement with Robinson et al. (***) claiming absence of lethal effects in *D.magna* exposed for 48h to 10 mg L^{-1} of EFX. But more recent data (Dalla Bona et al. 2013) indicate for EFX an acute EC_{50} of 16.34 mg L^{-1} in *D.magna* which may explain the 100% mortality observed in the chronic test and allows the calculation of an ACR (EC_{50} acute/EC_{50} chronic ratio) of 2.5. A similar ACR (3.5) can be calculated for CPX (EC_{50} 87.14 mg L^{-1}, Dalla Bona et al., 2013) and an ACR of 5.6 was reported for ofloxacin in *Ceriodaphnia dubia* (Isidori et al., 2005). These values are quite low when compared with those obtained by various authors testing other classes of antibacterials on *D.magna* (De Liguoro et al., 2009; Wollenberger et al., 2000; Zounková et al., 2011) and underline the difficulty of obtaining complete data from chronic tests on daphnids with fluoroquinolones. As a consequence, while the chronic test with EFX showed limitations due to the excessive mortality, the one with CPX was able to show only slight effects on reproduction.

4.2 Reproduction and average daily growth

Previous studies have already considered the chronic effects of TMP and EFX on *D.magna* (Park and Choi, 2008; De Liguoro et al., 2012) outlining toxicity effects at concentrations similar to those obtained in this work with F0. In particular, Park and Choi reported no effects on reproduction in daphnids exposed to 5 mg L-1 of EFX and to 6 mg L^{-1} of TMP, while De Liguoro et al. indicated an EC_{50} for TMP reproduction inhibition (8.21 mg L^{-1}) which is slightly lower than the 15.28 mg L^{-1} obtained in this study (Table 2). However, those studies did not consider the exposure of subsequent generations, which better represents the real environmental conditions. No data were previously available regarding chronic effects of CPX on *D.magna* and the EC_{50} calculated in F0 (Table 2) shows that this compound is less toxic than EFX. Considering that the two compound have an identical mechanism of action, a possible explanation may be that CPX being more polar and hydrophilic than EFX is less absorbed by daphnids. Thus, the EFX metabolisation and/or degradation to CPX may represent a favourable event for daphnids. However, the effective concentrations determined in the current study are far to be environmentally realistic (Santos et al., 2010), except in exceptional cases (Larsson et al., 2007; Le and Munekage, 2004).

Interestingly, effect levels for reproduction inhibition in F1, when compared to those obtained in F0, showed a different trend for each of the three compounds. With EFX there was correspondence to F0 both in F1e and F1n. With CPX there was correspondence to F0 only in F1e. With TMP there was no correspondence, as no effects were observed in the F1 generation. Analogous considerations may be made with the three compounds also for growth inhibition effects. The latter are frequently observed after exposition of daphnids to toxicants and may be explained with the 'principle of allocation' (Kim et al., 2012)

The effects observed with EFX in F1n, notwhitstanding the return of the offspring to clean medium, have been already observed with a number of other contaminants (Alonzo et al., 2008; Brennan et al., 2006; Jacobasch et al., 2013; Kim et al., 2012; Massarin et al., 2010; Pane et al., 2004); and may be the consequence of the perinatal exposition of daphnids to the antibacterial. As suggested by Abe et al.(2001), a direct exposure of embryos in the brood chamber to environmental pollutants is possible because daphnids actively exchange the fluid in the brood chamber for environmental water, in order to support embryonic oxygen demand. Furthermore, the short time between birth and collection of the daphnids should be taken into account. Another hypothesis could be that the exposed mothers, due to the stress caused by the toxicant, produced an offspring weaker than that produced by the controls. However, at least the body length of F1 newborns was not significantly different from that of controls. Finally, considering that EFX may exert genotoxic (Thomé et al., 2012) or epigenetic (Csoka and Szyf, 2009) effects in eucaryota, the possibility of a transmission of genetic alterations to the offspring cannot be excluded.

The absence of effects observed in F1e with TMP points to a sort of adaptation throughout generations. This phenomenon has been already observed in *D.magna* with other compounds (Dietrich et al., 2010; Ortiz-Rodríguez et al., 2012; Tsui and Wang, 2005). More specifically, Dietrich et al. (2010) studied effects on reproduction of various pharmaceuticals along 6 generations of *D.magna* and showed that, with some compounds (carbamazepine, metoprolol, diclofenac, ethinylestradiol) after an initial adaptation in F1 and F2, the effects can reappear in the subsequent generations. This event, which the authors explained with the difficulty of the organisms to sustain the resistance energetic cost, might also be possible with TMP. So, the absence of effects in the F1 generation does not necessarily imply the possibility of the population of tolerating the assayed levels of TMP in the long run.

Less interesting were the results obtained with CPX where, as one can normally expect, the F1e presented a sensitivity comparable to that of FO while no effects were observed in F1n.

Conclusions

EC_{50s} measured for the three assayed antibacterials were in the 6.49-36.53 mg L^{-1} range and therefore environmental unrealistic, except in case of exceptional contaminations that may occur in relation to poorly controlled wastewaters from pharmaceutical factories or excessive use of prophylactic treatments in aquaculture.

The two generation study has evidenced some aspects that may be critical for the toxicity of the three compounds at population level. In F1 with respect to F0, both for growth and reproduction, a worsening trend of the response with EFX, a similar response with CPX and an attenuating trend with TMP was detected. This could lead to the conclusion that, at least for *D.magna* population, EFX is more hazardous than the other two compounds. However, other experimentations, longer and more complex, are necessary in order to confirm this conclusion because previous works from other authors, performed on several generations of the crustacean, have shown that the trend exhibited by F1 is not necessarily maintained throughout the subsequent generations.

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General Conclusions

Altogether, results obtained from assays with the selected veterinary antibacterials, paint a rather reassuring picture of their impact on the aquatic environment. Indeed, while the concentrations usually detected in surface water are in the ppt-ppb range, the observed toxic effects were generally elicited after exposing the organisms to ppm of the pharmaceuticals. Furthermore, binary mixture of the compounds did generally show, at least in *D.magna*, less than additive interaction, indicating that simple additivity can be considered as the worst case scenario. Anyway, the increasing amplitude of sensitivity variation along different species, genera, families, orders and classes should be taken into account and we should not forget that in the real aquatic environment the co-presence of a multitude of different contaminants is the rule and not the exception.
D.curvirostris was shown to represent a valid alternative to *D.magna*, at least for acute tests, being as sensitive as the classic model organism and more represented in the local environment.
Some pharmaceuticals as EFX, CPX and SGD, showed a surprisingly high acute toxicity toward non-target organisms , with EC_{50}s in the few ppm range. This may represent a problem in situations of very high pharmaceutical contamination as those detected in some Asian countries nearby pharmaceutical factories or aquaculture ponds.
The prolonged toxicity test on daphnids, settled up as a possible midway between the official acute immobilization test (OECD 202, 2004) and reproduction inhibition test (OECD 211, 1998), was able to detect, in reasonable time, effects that would not be observed within 48h and to anticipate, in some cases, the results of the reproduction test. The particular endpoint considered (time to bear the first egg), not explicitly covered in the official reproduction inhibition test, was confirmed to be sensible enough and to represent an useful complement to the more common ones.
Thanks to the evolution in hardware and software dedicated to video-analysis, swimming activity can now be studied with relative easiness. In the pioneristic study on *P.reticulata* the only parameter considered was the traveled distance. However, using more complex experimental designs, more refined components of swimming such as acceleration, freezing, angles of turn, horizontal and vertical distribution may be evaluated as well and can increase the sensitivity of the tests.
Notwithstanding its limitations, the test showed that some VP, at high concentrations, may have a negative impact on swimming activity, not only in fish but also in crustaceans. Among the studied compounds, TMP was the more effective, causing significant inhibition of swimming activity in *P.reticulata* at 50 mg L^{-1}. Considering that TMP is licensed for use in aquaculture, its negative effect on swimming activity may have an impact during treatments by reducing the feeding activity of the fish.
The two generation study with *D.magna* did evidence some aspects that may be critical for the toxicity of the three compounds (EFX, CPX and TMP) at population level. In F1 with respect to F0, both for growth and reproduction, a worsening trend of the response with EFX, a similar response with CPX and an attenuating trend with TMP were detected. This could lead to the conclusion that, at least for *D.magna* population, EFX is more hazardous than the other two compounds. However, to confirm this conclusion other experimentations, longer and more complex, are necessary, because previous works from various authors, performed on several generations of the crustacean, have shown that the trend exhibited by F1 is not necessarily maintained throughout the subsequent generations.
In conclusion, these studies in the field of sub-lethal effects of veterinary drugs on aquatic organisms represent a starting point for further research that can be accomplished in the near future and may consider: other possible model organisms and endpoints not covered in official tests, effects of more complex mixtures of drugs, finer alterations of swimming activity and effects on reproduction over the course of several generations.

List of Publications

M. Dalla Bona, R. Zunková, R. Merlanti, L. Blahá, M. De Liguoro. (2013) Effects of Enrofloxacin, ciprofloxacin and trimethoprim on two generations of *Daphnia magna.*

M. Dalla Bona, V. Di Leva, M. De Liguoro. (2013) The sensitivity of *Daphnia magna* and *Daphnia curvirostris* to 10 veterinary antibacterials and to some of their binary mixtures. (Submitted)

M. De Liguoro, **M. Dalla Bona**, G. Gallina, F. Capolongo, F. Gallocchio, G. Binato, V. Di Leva. (2013) A monitoring of chemical contaminants in waters used for field irrigation and livestock watering in the Veneto region (Italy), using bioassays as a screening tool. Environmental Science and Pollution Research. Available on line. DOI: 10.1007/s11356-013-2357-7

M. Dalla Bona, K. Hilscherová, A. Jonáš, V. Mlčáková, M. De Liguoro (2013). Teratogenic and Developmental effects of Enrofloxacin and Ciprofloxacin in *Xenopus laevis* and *Brachydanio rerio* embryos. Acta of LXVII National Conference S.I.S. Vet, Brescia 17-19 September 2013.

G. Ribaudo , **M. Dalla Bona**, M. Drigo, C. Montesissa, G. Zagotto (2013).
Chemical and Physical Quality of Well Water of Pig Farms; one Year of Surveillance. Acta of LXVII National Conference S.I.S. Vet, Brescia 17-19 September 2013.

M. Dalla Bona, V. Di Leva, M. De Liguoro (2013). The sensitivity of *Daphnia magna* and *Daphnia curvirostris* to 10 antibacterials and to some of their binary mixtures: a comparison. Presented at the International Conference “Pharmaceutical Products in the Environment: is there a problem?”, Nimes 3-4 June 2013.

M. Dalla Bona, V. Di Leva, M. De Liguoro, Effects of Veterinary Drugs on Swimming Activity in two Freshwater Organisms, in: C. Boiti, A. Ferlazzo, A. Gaiti, A. Pugliese (Eds.), Trends in Veterinary Sciences, Springer Berlin Heidelberg, Berlin, Heidelberg, 2013: pp. 97 - 101.

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M. De Liguoro, V. Di Leva, **M. Dalla Bona**, G. Gallina (2011). An Overall Toxicity Screening of Waters Used for Field Irrigation and Livestock Watering in the Veneto Region, Using Pseudokirchneriella subcapitata and Daphnia magna as Test Organisms. Presented at the Hydroeco 2011, Vienna 2-5 May 2011.

V. Di Leva, **M. Dalla Bona**, F. Gottardo, M. De Liguoro (2010). Toxicity Screening, Using Daphnia magna as a Test Organism, of Groundwaters for Livestock Watering in the Veneto Region. Acta of LXIV National Conference S.I.S. Vet, Asti 8-10 September 2010.