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USE OF BIOASSAYS AND BIOMARKERS IN *DAPHNIA MAGNA* TO ASSESS THE EFFECT OF PHARMACEUTICAL RESIDUALS IN FRESHWATER ECOSYSTEMS

Presentata da: **Valentina Varano**

Coordinatore Dottorato

Prof. Enrico Dinelli

Relatore

Prof.ssa Elena Fabbri

Correlatore

Prof. Andrea Pasteris

Esame finale anno 2014

Dedicated to my family

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Abstract

Widespread occurrence of pharmaceuticals residues (ng/L to µg/L) has been reported in aquatic ecosystems. However, their toxic effects on aquatic biota and environmental risks remain unclear. Generally, the acute toxicity towards non-target organisms has been assessed in laboratory experiments, while chronic toxicity studies have rarely been performed. Of importance appears also the assessment of mixture effects, since pharmaceuticals never occur in waters alone.

The aim of the present work is to evaluate acute and chronic toxic response in the crustacean *Daphnia magna* exposed to single pharmaceuticals and mixtures.

We tested fluoxetine, a selective serotonin reuptake inhibitor widely prescribed as antidepressant, and propranolol, a non selective β-adrenergic receptor-blocking agent used to treat hypertension.

Acute immobilization tests and chronic reproduction tests were performed according to OECD guidelines 202 and 211, respectively. Single chemicals were first tested separately. Toxicity of binary mixtures was then assessed using a fixed ratio experimental design. Five concentrations (0.5, 0.71, 1, 1.41, 2 Toxic Units) and 5 percentages of each substance in the mixture (0, 25, 50, 75 and 100%) were tested for a total of 26 experimental conditions, including the negative control. Six replicates for each treatment were carried out. The conceptual model of Concentration Addition (CA) was adopted in this study, as we assumed that the mixture effect mirrors the sum of the single substances for compounds having similar mode of action. The MixTox model was applied to analyze the experimental results. This tool evaluates if and how observed data deviates from the CA model, and tests if significantly better descriptions of the observed data can be achieved using a set of deviation functions. These functions allow a differentiation between synergism and antagonism, along with deviations based on the dose-level and chemical ratio dependency. Results showed a significant deviation from CA model that indicated antagonism between chemicals in both the acute and the chronic mixture tests.

The study was integrated assessing the effects of these compounds with a battery of biomarkers. We wanted to evaluate the organism biological vulnerability caused by low concentrations of pharmaceutical occurring in the aquatic environment. Indeed, biomarkers are early warning signals able to inform about alteration in health status of organism before life cycle traits are compromised. We evaluated three biomarkers

in response to a sub-chronic exposure of *Daphnia magna* to fluoxetine. We assessed the acetylcholinesterase and glutathione s-transferase enzymatic activities and the malondialdehyde production. No treatment induced significant alteration of biomarkers with respect to the control.

Biological assays and the MixTox model application proved to be useful tools for pharmaceutical risk assessment. Although promising, the application of biomarkers in *Daphnia magna* needs further elucidation.

1. Introduction

1.1 Pharmaceuticals as emerging contaminants

Pharmaceuticals, in addition to their important role in health care (Brun et al., 2006), are addressed to as a new class of widespread environmental pollutants. Pharmaceuticals belong to the group of PPCPs (Pharmaceuticals and Personal care Products) found in the environment. PPCPs comprise a very broad, diverse collection of thousands of chemical substances, including prescription, veterinary, and therapeutic drugs, antibiotics, vitamins, but it includes also cosmetics, fragrances, shampoos, soaps, toothpastes, and sunscreens. The acronym "PPCPs" was coined in the 1999 and then has become an adopted term used in both the technical and popular literature.

Pharmaceuticals enter into aquatic environments via biomedical, veterinary medicine, agricultural, and industrial routes (Jorgensen and Halling-Sørensen, 2000) (Fig.1.1).

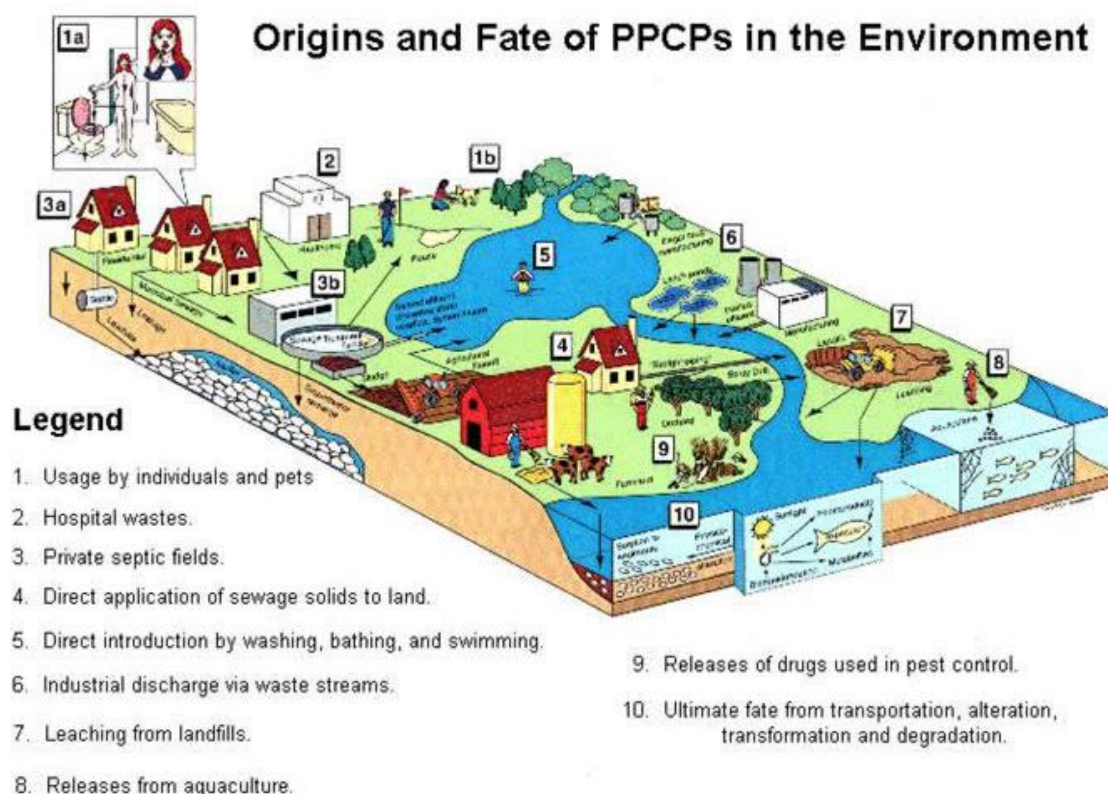


Fig. 1.1. Origins and fate of pharmaceuticals and personal care products (PPCPs) in the environment (Source: EPA)

The biomedical route via human sewage is perhaps the main path that pharmaceuticals follow to enter in the environment. Once pharmaceuticals are ingested, they are partially excreted in a biologically active form, either as the parent substance or as an active metabolite (Calamari et al., 2003). Because of incomplete elimination in wastewater treatment plants, residues of pharmaceuticals and their metabolites occur in surface waters (Fent et al., 2006).

The result is that the waterways of all over the world are exposed to a cocktail of chemicals ranging from β-blockers to steroidal hormones, from analgesics to neuroactive compounds (Fig. 1.2).

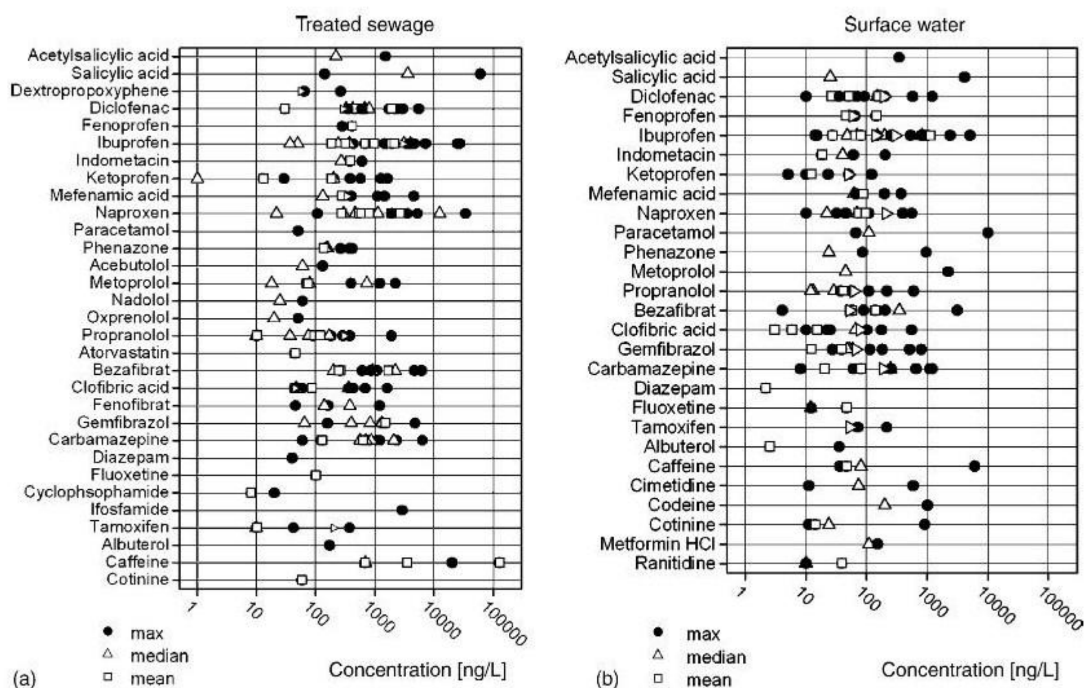


Fig. 1.2. Concentration of pharmaceuticals in treated wastewater (a) and surface water (b) (source: Fent et al., 2006)

Pharmaceuticals are ubiquitously present in rivers, lakes, and in marine coastal waters and occasionally were found in ground and potable waters (Fent et al., 2006).

Since pharmaceuticals are designed to target specific metabolic and molecular pathways in humans or animals (veterinary usage), when introduced into the environment they may affect the same or comparable pathways in vertebrates and invertebrates having identical/similar targets (Christen et al., 2009).

Only little information is available about ecotoxicological effects of pharmaceuticals on aquatic and terrestrial organisms and wildlife. Numerous scientific studies have documented a range of detrimental impacts of pharmaceuticals upon freshwater ecosystems, in particular, adverse effects on fish developing sexual and behavioral abnormalities such as males producing eggs or females disinclined to spawn.

Perhaps the most famous case was detected in the Washington basin where in 2003, during health assessments conducted in response to kills and a high prevalence of skin lesions observed in smallmouth bass *Micropterus dolomieu* in the South Branch of the Potomac River, the presence of immature oocytes within testes was noted (Blazer et al., 2007) and scientists started to indagated on fish intersex and testicular oocytes (TO) usually indicators of exposure to estrogenic compounds. Thus, the scientific consensus is that pharmaceuticals threaten aquatic organisms (www.cicleofblue.org), though the effects on humans drinking contaminated water or eating contaminated food are not as clear.

Standard acute ecotoxicity data have been reported for a number of pharmaceuticals, however, such data alone may not be suitable for specifically addressing the question of environmental effects, and subsequently evaluating the hazard and risk (Fent, 2003). That is due firstly to the normally trace-level environmental concentrations of pharmaceuticals, usually in the range of ng/L, that means that toxicity testing endpoints may not be sensitive enough to characterize adequately the risk associated with these chemicals (Brooks et al., 2003). Although ecotoxicological studies agree that pharmaceuticals residues concentrations in surface water generally do not cause acute toxicity to aquatic organisms, as their environmental concentrations are typically too low, the continuous discharge exposes non-target aquatic organisms such as *Daphnia magna* continuously through the life cycle for multiple generation. Moreover, although they can be degraded in the environment by biotic (Winkler et al., 2001) or abiotic process (Andreozzi et al., 2002), it is assumed that pharmaceuticals could act as persistent compounds simply because of their continual infusion into aquatic media via Sewage Treatment Plant (STP) effluents, which sustain a multigenerational exposure for the resident organisms (Daughton and Ternes, 1999). Even slight, non significant influences on single components which would not result in any acutely discernible effect, with regulatory cascades, might ultimately affect a whole population by their negative consequences on fitness: disturbances in hormonal homeostasis (endocrine disruption), immunological status, signal transduction or gene activation, for example (Seiler, 2002).

Another item to take in consideration is that the majority of ecotoxicological studies in the aquatic and terrestrial environments focus mainly on the toxicity of single compounds in controlled conditions (Barata et al., 2006) and relatively few studies

have examined sublethal or mixture response effects. Studies have demonstrated that a chemical will rarely be found alone in the environment but commonly in combination with others, thus in order to evaluate in a more realistic way the effects of contaminants when they occur in the environment, researches should provide studies on pharmaceuticals mixtures.

Since pharmaceuticals are designed with the intention of performing a biological effect (Henschel et al., 1997), this concept may be very important for these specific substances in terms of fate and effects toward non-target organisms in the environment, even at low concentrations (Ferrari et al., 2003). Unfortunately, until now, there is very little information about the chronic toxicity, and the potential bioamagnification of pharmaceuticals along the food chains. Since pharmaceuticals are present in water bodies throughout the world, although no studies have shown a direct impact on human health and studies are still in progress to determinate the environmental impacts, the lack of information cannot rule out the possibility of adverse outcomes due to interactions or long-term exposures to these substances. Certainly, the scientific evidence merits new safeguards for freshwater ecosystems.

1.1.1 Pharmaceuticals in the environmental legislation

Data from 2008 describes a slight increases in the market for pharmaceutical substances in Europe and due to the rising average age of the population in Europe, consumption of pharmaceuticals is expected to rise again in the next years.

Since it was demonstrated that, although at low concentrations, pharmaceutical residues and their metabolites are present in freshwater worldwide, gain new knowledge on effects of them to the aquatic organisms is necessary. Unfortunately there is a significant lack of knowledge about effects of pharmaceuticals on aquatic organisms.

Although further research is needed, our general understanding on pharmaceutical ecotoxicity has improved in recent years, when governments started to recognize pharmaceuticals resides as a potential problem concerning public health.

Thus, in the last years, different US regulatory agency as The Drug Enforcement Administration (DEA), the Food and Drug Administration (FDA), and the Environmental Protection Agency (EPA) have all taken steps to address pharmaceutical residues in both the community and institutional settings:

- Several regulations proposed by the Drug Enforcement Administration (DEA), a division of the US Department of Justice, hold the potential to affect members of industry, and in particular the pharmaceutical industry. One such proposed rule, *Disposal of Controlled Substances*, proposes to require a system in which manufacturers and users would need to secure leftover or otherwise unused controlled substances (any scheduled substance under the *Controlled Substances Act, or CSA*) for proper disposal. The regulation is required under the terms of the *Secure and Responsible Drug Disposal Act* of 2010, and DEA has already sought opinions from industry in the form of a January 2009 *Federal Register* notice in which it called for "options for the safe and responsible disposal of dispensed controlled substance." An electronic copy of this document is also available at the http://www.regulations.gov website.
- The White House Office of National Drug Control Policy with input of the Food and Drug Administration (FDA) provided guidelines on pharmaceuticals disposal and good manufacturing practice. The guidance is a compilation of pharmaceuticals regulations primarily directed to drugmakers and consumers.
- EPA oversees implementation of the Resource Conservation and Recovery Act (RCRA), which is a federal law controlling the management and disposal of solid and hazardous wastes. EPA proposed in 2008 to add pharmaceuticals to the Universal Waste Program governing waste disposal, but has since declined to finalize that proposal. EPA published a new proposed regulation in summer 2013 intended to govern the disposal of pharmaceutical waste generated by health care facilities.

In Europe, the need to report any potential environmental risk related to the use of medicinal products was addressed for the first time in 1993 with Directive 93/39/CEE. The environmental aspects of the pharmaceuticals were taken in consideration by the European Medicines Agency (EMA), the REACH regulation, and the Water Framework Directive (FWD):

- The EMA introduced the principle of the environmental risk assessment for all new drugs before effecting registration. The European Medicines Agency (formerly known as the European Agency for the Evaluation of Medicinal Products EMEA), is the European Union agency for the Evaluation of Medicinal Products. The foundation of EMA (http://www.ema.europa.eu/ema/) in 1995 with

the financial assistance of the European Union and the pharmaceutical industry, was intended to harmonize (but not replace) the work of national regulatory bodies in terms of pharmaceuticals; in analogy to what was already the Food and Drug Administration. The agency in 1997 published the first guidelines for the environmental risk assessment of veterinary drug, and the first draft of the guidelines of the environmental risk assessment of medicines for human use were more belatedly published in 2001. According to the guidelines, "applications for authorization to the marketing of pharmaceutical products for human use must be accompanied by an Environmental Risk Assessment (ERA). "It determines an obligation on the environmental risk assessment for new medicinal products for which is required marketing authorization in the European Union. If a pharmaceutical is recognized as dangerous for the environment its production will be stopped. If the procedure can not exclude the possibility of a risk to the environment related to the test substance (active substance and/or metabolites), the procedure requires the adoption of a series of precautionary and safety measures, designed to mitigate the exposure of the environment to the new pharmaceutical. In particular, special labeling requirements, with an indication of the potential environmental risks posed by the medicinal product to be described on the label, and that should be reported in the "Summary of Product Characteristics" and the package leaflet of the medicinal product, with an indication of special precautions that must be followed for the storage and administration to patients with the aim to promote the proper disposal and minimize improper disposal of medication in the environment, informing the patient and the medical staff on the potential environmental risks related. The guidelines recommend that the package leaflet or the packaging of drugs, all drugs but especially those for which we can not exclude a potential environmental risk, bring back the following sentence: "to protect the environment and reduce pollution environmental, unused or expired products should not be disposed of with normal garbage or to sewer, but must be returned to the pharmacy”.

The EMA guidelines will govern the environmental risk in relation to new drugs for human use, for now nothing is planned with regard to drugs already on the market to the entry into force of the Regulation, in particular in the field of pollution of water bodies.

- REACH (Registration, Evaluation and Authorization of Chemicals) is based on the principle that industry should manufacture, import, use substances or place them on the market in a way that, under reasonably foreseeable conditions, human health and the environment are not adversely affected. In order to ensure this, manufacturers and importers need to collect or generate data on the substances and assess how risks to human health and the environment can be controlled by applying suitable risk management measures. To prove that they actually meet these obligations, as well as for transparency reasons, REACH requires manufacturers and importers to submit to the European Chemicals Agency (ECHA) a registration dossier containing a technical dossier including data on the substance and, for substances in quantities of 10 tonnes or more per year per registrant, a chemical safety report assessing how risks to human health and the environment can be controlled (http://www.prc.cnrs-gif.fr/reach/en/registration_obligation.html). Generally, they apply to all individual chemical substances on their own or in preparations. Substances falling under the scope of the REACH regulation and not exempted from the registration obligation must be registered before they can be manufactured or imported into the European Union. Some substances are completely excluded from REACH and waste is also excluded as it is not a substance, a preparation or an article within the meaning of REACH. They are considered as causing minimum risk to human health and the environment (substances listed in annexe IV) or their registration is deemed inappropriate or unnecessary (substances or processes listed in annexe V). In base of REACH regulation chemical preparations such as paints (which include such solvents, dyes, and other elements that confer particular characteristics of the product) detergents and cosmetics are included in the regulation, but pharmaceuticals while being of chemical preparations are excluded from the REACH.
- In 2012 a proposal to implement the WFD on pharmaceuticals residues has been put forward. The Water Framework Directive (Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy) is a European Union directive which commits European Union member states to achieve good qualitative and quantitative status of all water bodies (including marine waters up to one nautical mile from shore) by 2015. It is a framework in the sense that it

prescribes steps to reach the common goal rather than adopting the more traditional limit value approach. The ecological and chemical status of surface waters are assessed according to the following criteria:

- Biological quality (fish, benthic invertebrates, aquatic flora);
- Hydromorphological quality such as river bank structure, river continuity or substrate of the river bed;
- Physical-chemical quality such as temperature, oxygenation and nutrient conditions;
- Chemical quality that refers to environmental quality standards for river basin specific pollutants. These standards specify maximum concentrations for specific water pollutants. If even one such concentration is exceeded, the water body will not be classed as having a “good ecological status”.

Article 14 of the directive requires member states "to encourage the active involvement of interested parties" in the implementation of the directive.

Article 16 of the Water Framework Directive (2000/60/EC) (WFD) sets out "Strategies against pollution of water", outlining the steps to be taken. In 2001 was established a first list of priority substances to become Annex X of the WFD. These substances were selected from amongst those presenting a significant risk to or via the aquatic environment. This first list was replaced by Annex II of the Directive on Environmental Quality Standards (Directive 2008/105/EC) (EQSD), also known as the Priority Substances Directive, which set environmental quality standards (EQS) for the substances in surface waters (river, lake, transitional and coastal) and confirmed their designation as priority or priority hazardous substances, the latter being a subset of particular concern. As required by the WFD and EQSD, the Commission subsequently reviewed the list in 2008 and in 2012 and it put forward a proposal for a Directive amending the WFD and the EQSD as regards priority substances. According to Annex V, point 1.4.3 of the WFD and Article 1 of the EQSD, good chemical status is reached for a water body when it complies with the EQS for all the priority substances and other pollutants listed in Annex I of the EQSD. The Water Framework Directive foresees an obligation to regularly review the list of priority substances, a review of the priority substances provisions has to be done at least every four years. The

list have been established by Decision No 2455/2001/EC and Directive 2008/105/EC and the priority substances were 33 at this point. Following a comprehensive consultation and assessment process, the Commission proposal of 31 January 2012 foresees the inclusion of further 15 priority substances (http://ec.europa.eu/environment/water/water-dangersub/pri_substances.htm), amongst them substances used in pesticides and biocides, combustion by-products and, for the first time, pharmaceutical substances, as well as designation of further priority hazardous substances (unfortunately, as will be explained below, this has remained only a proposal for pharmaceuticals). For six of the 15 new priority substances the classification proposed would require their emissions to water to be phased out within 20 years. The proposal also includes stricter standards for four currently controlled substances, and a requirement to phase out the emissions of two others already on the list. The proposed 15 additional priority substances are:

- Plant protection product substances: Aclonifen, Bifenox, Cypermethrin, Dicofol, Heptachlor, Quinoxyfen;
- Substances used in biocidal products: Cybutryne, Dichlorvos, Terbutryn;
- Industrial chemicals: Perfluorooctane sulfonic acid (PFOS), Hexabromocyclododecane (HBCDD);
- Combustion by-products: Dioxin and Dioxin-Like PCBs;
- Pharmaceutical substances: 17 alpha-ethinylestradiol (EE2), 17 beta-estradiol (E2), Diclofenac; pharmaceuticals are proposed for the first time.

In July 2013 the European Commission (EC) with Directive 2013/39/EU has added 12 new substances to its priority list of 33 known pollutants in the latest priority list of chemicals known to pose a risk to the safety of surface waters. In addition, for the first time, the EC has placed three pharmaceuticals on a ‘watch list’ of emerging aquatic pollutants that may be added eventually to the priority list later (http://www.euractiv.com/health/new-chemicals-pharmaceuticals-ad-news-529073). The three chemicals are diclofenac, a commonly-used generic anti-inflammatory painkiller which is suspected of killing fish, and the hormones 17 alpha-ethinylestradiol (EE2) and 17 beta-estradiol (E2), which the EC claims can disrupt the endocrine system in humans and harm fish reproduction.

Richard Seeber, the Austrian Member of European Parliament (MEP) hailed the addition of pharmaceuticals to the list, predicting that water policy is a long-term policy and it will be a very interesting field in the future because our waters are unfortunately increasingly burdened with pharmaceuticals.
Adding them to the watch list is a partial victory for the pharmaceutical industry, which had lobbied to remove the pharmaceutical ingredients from the WFD altogether, arguing that the data behind the EC's claims was not robust enough to warrant action. This list allows pharmaceutical residues not to be controlled in water until a further revision of the list comes about, meaning that control mechanisms for these substances could be in place as far as in 2027 (year of the extension of the deadline for achieving water quality targets) and from 2021 to 2027 they could be the new additions to the list. That has been a missed opportunity for the European Parliament to send out a clear message that significant negative impacts on freshwater and marine ecosystems and ultimately humans is caused by pharmaceutical substances and other emerging pollutants.

European Federation on National Association of Water Services (EUREAU) has commented the implementation of the WFD considering the need to tackle water pollution by chemical and pharmaceutical substances at the source and not in treatment plants for drinking water or in waste water treatment plants, which should be the last resort. It proposed the inclusion of a new article in the WFD which will seek to develop a strategic approach to pollution of water by pharmaceutical substances aimed at reducing their discharges, emissions, and losses to the aquatic environment. It is true that upgrading wastewater treatment plants with advanced techniques that would remove not only pharmaceuticals but also other micropollutants can be costly, but there are simple and low cost upstream measures already in use in different Member States (MS) such as collection schemes for unused pharmaceuticals that EU legislation already requires MS to implement; encouraging design of green pharmaceuticals that are fully metabolized in the body and rapidly biodegrade in the environment into harmless compounds; and educating healthcare professionals to optimize medicines prescription behavior so that only the right amount of pharmaceuticals needed is prescribed, giving priority to the least environmentally hazardous medicines.

1.2 Mixtures toxicity

Chemicals of anthropogenic origin, arising from an urban, industrial or agricultural use, usually are found in the environment in combination with other substances, therefore we need to study combined effects of mixtures of two or more chemicals to determinate what happens in nature in a more realistic way compared to when toxicants are tested individually. Pharmaceutical residues, as all other chemicals, occur as mixture in the environment, therefore it is important to study the effect of a pharmaceutical in combination with another, both abundantly present in freshwater.

Pharmaceuticals in mixture can produce an interaction effect, i.e. synergistic or antagonistic effect. In toxicology, synergism is when organisms are exposed to two or more chemicals at the same time and the toxic effects are greater than the sum of the effects of the single pharmaceuticals. It can be explained as 1+1 > 2. On the other hand antagonism is the opposite of synergism. It is the situation where the combined effect of two or more compounds is less toxic than the sum of the individual effects. If the toxicity tests on mixtures of pharmaceuticals prove that there is a synergistic effect, we could have a potentiated toxic effect with severe consequences for the organisms in the environment that is why these studies should be taken into account by international laws and regulations combining them to the classic studies on individual substances.

In order to address the effect of a toxic mixture several theoretical models have been developed, which compare toxic effects from the mixtures with a reference model in which it is assumed that there are no interactions between chemicals in expected combined effects. Two different reference models are well established: concentration addition (CA) and independent action (IA), both are used to describe the joint toxicity depending on the mode of action (MoA) of the single chemicals.

Concentration Addition (Loewe and Muischnek, 1926) is based on the assumption that the mixture components have the same mechanism of action, in other words they act on the same biochemical pathway and strictly affect the same molecular target (Martin et al., 2009). This model has concentration-based summation of toxicity of similarly acting chemicals, scaled to reflect their relative toxicities (Loureiro et al., 2010). On the other hand the model of Indipendent Action, also known as Bliss independence, is based on the assumption that the components in a mixture of chemicals or in a combination of a natural and a chemical stressor do not interact

physically, chemically or biologically (Bliss, 1939). The IA model is, therefore, a measure of the joint probability of individual sensitivity to the compounds in the mixture assuming that the chemical mechanisms of action in fully independent (Martin et al., 2009).

Chou (2006) explains that "because of biological systems as well as dose-effect models are exceedingly complex, there have been numerous models, approaches, hypotheses, and theories as well as controversies on drug combination analysis"… "There are many common errors associated with these claims: for instance, additive effect is not a simple arithmetic sum of two (or more) drugs. If A and B each inhibits 30%, then the additive effect is not 60% because if A and B each inhibits 60%, the combined additive effect cannot be 120%"..."It is to be noted that in one review article by Goldin and Mantel (1957) alone, seven different definitions for synergism were given, and none of them supported the others. In a more recent review by Greco et al. (1995), 13 different methods for determining synergism were listed. Again, none of them supported the others". Thus, pharmaceutical combination is a field of biomedical science rich of controversies and confusion.

This ambiguity does not help to predict the response of organisms simultaneously exposed to more than one substance which remains one of the most difficult tasks in risk assessment. In response to the need to predict mixture effects to biota and in particular to aquatic environment, various models including concentration addition and independent action have been evaluated. Setting the combinations of substances concentrations to test is the first step for assessing mixture toxicity and interaction among chemicals; one of the most common method is using the Toxic Unit approach which is described in chapter 3.3.2. Another tool used for characterizing deviation from the reference mixture model is the MixTox model (Jonker et al., 2005), which is a data analyzer who consent to establish if and how observed data, resulting from combinations of pharmaceuticals, deviate from CA or IA reference model leading some interaction effect. How MixTox tool works is described in chapter 3.6.2.

1.3 Tested pharmaceuticals

1.3.1 Fluoxetine (FLX)

Fluoxetine ($C_{17}H_8F_3NO$) is one of the most prescribed antidepressants (RxList, 2000) indicated for the treatment of depression, obsessive-compulsive disorder, bulimia nervosa, and panic disorder (Stanley et al., 2007).

Commonly prescribed to over 40 million people globally (Eli Lilly and Co, 2001), fluoxetine is excreted from the human body primarirly via the urine; approximately 2.0-11.0% of the administered dose is excreted as unchanged parent compound (Altamura et al., 1994). In surface waters, Kolpin et al. (2002) reported that out of 84 streams sampled fluoxetine concentrations did not exceed an estimated 0.012 µg/L, however Weston at al. (2001) indicated that fluoxetine concentrations may reach water concentrations as high as 0.540µg/L.

Fluoxetine acts by blocking serotonin reuptake transporters. Physiologically serotonin released in the synaptic cleft is re-uptaken into the presynaptic terminal by the action of a specific protein pump. Fluoxetine as well as other selective serotonin re-uptake inhibitors (SSRI) inhibit this pump, thus increasing the serotonin level in the synapse space (Fent at al., 2006). Serotonin is involved in several mechanisms, hormonal and neuronal, and it is also important in functions such as food intake and sexual behavior. In invertebrate, serotonin may stimulate juvenile hormone responsible for controlling oogenesis and vitellogenesis (Nation, 2002). Fluoxetine, putatively by increasing the serotonin levels, is also known to increase reproduction in *Daphnia* (Flaherty and Dodson, 2005) at low concentration.

1.3.2 Propranolol (PRP)

Propranolol ($C_{16}H_{21}NO_2$) is a non selective antagonist blocking both β adrenergic receptors of all subtypes. The β-1 receptors are localized in the cardiac tissue. The β-2 are present mainly at the level of cells smooth muscle of peripheral vessels (where they play the role of vasodilators) and bronchi, but also in other types of tissues such as the heart. Overall, the stimulation of both receptors leads to an increase of cardiac contractility. The β-1 receptors are predominantly localized in the synaptic junction, while the β-2 receptors are also present at the presynaptic level, where they facilitate

the release of norepinephrine. The β-1 are stimulated for the more by the neurotransmitter norepinephrine, for which possess high affinity while β-2 are mainly activated by adrenaline in free circulation, which still retains an affinity equivalent to both receptors (Spoladore et al., 2010). The heart contains β adrenergic receptors (predominantly β_1 receptors), which, upon agonist binding cause an increase in heart rate and heart contractility (positive chronotropic and inotropic responses) (Huggett et al., 2002). Viceversa, β-blockers inhibit the action of the endogenous catecholamines epinephrine and norepinephrine, reducing frequency rate and myocardial contractility strength.

Besides its action as β-receptor blocker, propanolol also acts as a serotonin (5-HT) receptor antagonist, with affinity for the 5-HT1 receptor (Alexander and Wood 1987).

DL-propranolol is widely prescripted for treatment of cardiovascular diseases and also is also used as a migraine prophylactic and to control symptoms of anxiety (Stanley et al., 2006). In patients with hypertension and angina, a decrease in heart rate and contractility is therapeutically beneficial (Huggett et al., 2002), it is also prescribed to treat patients after heart attack to prevent further attacks.

β-blockers are one class of environmental pharmaceuticals that has received recent attention because have been detected in effluent discharges worldwide. Ternes (1998) reported the same maximum concetration of propranolol, with level between 0.29 and 0.59 μg/L in effluent of sewage treatment plants as well as in rivers.

1.3.3 Carbamazepine (CBZ)

Carbamazepine ($C_{15}H_{12}N_2O$) is an anticonvulsant and mood stabilizing drug used primarily in the treatment of epilepsy, bipolar disorder, and trigeminal neuralgia (Garcia-Morales et al., 2007). This antiepileptic drug is among the pharmaceuticals most frequently encountered in surface waters (Daughton and Ternes, 1999). Reports indicate that approximately 3% of the therapeutic dose is excreted unchanged through urine and is persistent when released to the environment, its average removal efficiencies by wastewater treatment plants are below 10% (Zhang et al., 2008). No biodegradation of CBZ even at low concentrations has been found. For these properties, carbamazepine is considered a marker for urban discharges. Fent et al. (2006) reported values in the range of 0.1-1 μg/L for surface waters, with levels up to 1.1 and 0.03 μg/L in groundwater and drinking waters, respectively.

1.3.4 Mode of action of fluoxetine and propranolol referred to *Daphnia magna*

The mode of action of a pharmaceutical is evaluated for the treatment of human diseases, however, this desired property in a patient, might possibly provide an unwanted adverse effect in a non-target aquatic species (Lange and Dietrich, 2002). Furthermore, since in the environment pharmaceuticals are found in mixture with others, we should consider the interaction effects between substances in order to correctly estimate the risk posed to the aquatic organisms by pharmaceuticals.

The two pharmaceuticals used to a larger extent in this study are propranolol and fluoxetine and we need to know their mode of action to understand how they could possibly act on *Daphnia* and better explain their interaction in binary mixture.

Both of these pharmaceuticals have an effect on the action of serotonin, although with different mechanisms.

Based on biochemical and pharmacological criteria, serotonin receptors are classified into seven main receptor subtypes, 5-HT1–7. Of major pharmacotherapeutic importance are those designated 5-HT1, 5-HT2, 5-HT4, and 5-HT7, all of which are G-protein-coupled. 5-HT1 receptors are subdivided into 5-HT1A, 5-HT1B, and 5-HT1D receptors; while 5-HT2 subtypes include 5-HT2A, 5-HT2B, and 5-HT2C.

With the exception of the 5-HT_3 receptor, a ligand-gated ion channel, all other serotonin receptors are G protein-coupled receptors that activate an intracellular second messenger cascade to produce an excitatory or inhibitory response. 5-HT1 receptor is coupled to the inhibition of cAMP signaling cascade in vertebrates; on the other hand, β-receptors are coupled to the enhancement of cAMP concentration. Franzellitti et al. (2010) found that propranolol increased cAMP levels in the mantle/gonads of the mussel, *Mytilus galloprovincialis*, possibly by blocking 5HT1 receptors thus reducing the effects of physiological inhibitors of the cAMP pathway, such as serotonin.

Cyclic adenosine monophosphate (cAMP, cyclic AMP or 3'-5'-cyclic adenosine monophosphate) is a second messenger important in many biological processes as intracellular signal transduction, transferring into cells the effects of hormones like glucagon and adrenaline, which cannot pass through the cell membrane; it is also involved in the activation of protein kinase A (PKA) which is normally inactive. A protein kinase is a kinase enzyme that modifies other proteins by chemically adding phosphate groups to them (phosphorylation). Phosphorylation usually results in a

functional change of the target protein (substrate) by changing enzyme activity, cellular location, or association with other proteins. Kinases are known to regulate the majority of cellular pathways, especially those involved in signal transduction. cAMP is synthesised from adenosine triphosphate (ATP) by adenylyl cyclase (AC) located on the inner side of the plasma membrane. Adenylyl cyclase is activated by a range of signaling molecules through the activation of adenylyl cyclase stimulatory G (Gs)-protein coupled receptors and inhibited by agonists of adenylyl cyclase inhibitory G (Gi)-protein-coupled receptors.

For our research it is interesting to understand how the previously described physiological mechanisms work in the crustacean *Daphnia magna.*

The mechanisms of action of SSRIs affecting phenotypic responses of exposed *D. magna* were analyzed by studying effects on level of lipids, carbohydrate, proteins, oxygen consumption rates, survival, and offspring production (Campos et al., 2012). The results from this study showed that SSRIs act following a mechanism of action similar or identical to their intended pharmacological effects in humans by increasing serotoninergic activity, but in doing so they alter physiological process as increasing glycogen and aerobic metabolism. Furthermore, the recent sequencing and public deposition of the genome of *Daphnia pulex* provide an alternative approach to protein discovery in crustaceans. Despite the genome of its close relative *Daphnia magna* is still incomplete, we may benefit from the former. McCoole et al. (2012) using *D. pulex* genomic information and *Drosophila melanogaster* proteins as queries (in *D. melanogaster* three serotonin receptors subgroups has been identified and characterized: 5HT1R, 5HT2R and 5HT7R, which are the homologs of those present in the human) identified putative serotonin receptor subgroups in *Daphnia pulex*: type 1 (5HT1R) and the type 7 (5HT7R). No Daphnia 5HT2R homologs were identified.

As regards the presence of the adrenergic receptors (α, β) in *Daphnia magna*, Huggett et al. (2002) referred that the presence have never been reported in crustaceans; however in genomic studies on *Daphnia pulex*, McCoole et al. (2012), on the basis of *D. melanogaster* sequences, identified *D. pulex* genes encoding putative members of the OctαR and OctβR octopamine receptors classes. Octopamine is the crustacean adrenergic agonist equivalent to dopamine in vertebrate, and its receptors are similar to adrenergic receptors (Dzialowski et al., 2006).

1.4 *Daphnia magna* Straus, 1820

1.4.1 *Daphnia magna* phylogeny and characteristics

Table 1.1. Scientific classifcation of *Daphnia magna*

Kingdom	Animalia
Phylum	Arthropoda
Subphylum	Crustacea
Class	Brachiopoda
Order	Cladocera
Family	Daphniidae
Genus	*Daphnia*
Species	*magna*

The gender *Daphnia* was given its name by O.F. Mueller in 1785, but it had been known to scientists for more than a century before that (Edmonson, 1987). Swammerdam (1669) used the term water flea, a term taken up in 1675 by his acquaintance Leeuwenhoek (Dobell 1932).

The planktonic species *Daphnia magna* belongs to the phylum Arthropoda (Table 1.1) and shares with all other members of this phylum an exoskeleton, jointed limbs and a hemocoel as primary internal cavity. The hemocoel accommodates their internal organs and has an open circulatory blood or hemolymph system (Ruppert et al., 2004).

The exoskeleton forces members of the phylum Arthropoda to moult, shedding the old exoskeleton in favor of a new one, in order to grow (Ruppert et al., 2004). *D. magna* belongs to the subphylum Crustacea. Crustaceans main differences to other Arthropods are the presence of a nauplius larva, although often suppressed in favor of a more advanced larva at the hatching stage (Ruppert et al., 2004), and biramous (splitted) limbs (Hejnol and Scholtz, 2004). *Daphnia* belongs to the class Branchiopoda and to the subclass Cladocera with whom it shares a two-valved carapace covering most of the body except the antennae. All Cladocerans have an unpaired compound eye which is the result of a fusion of two eyes in the late embryonic development (Ebert, 2005). An also unpaired nauplius eye is located between the compound eye and the mouth (Fig. 1.3).

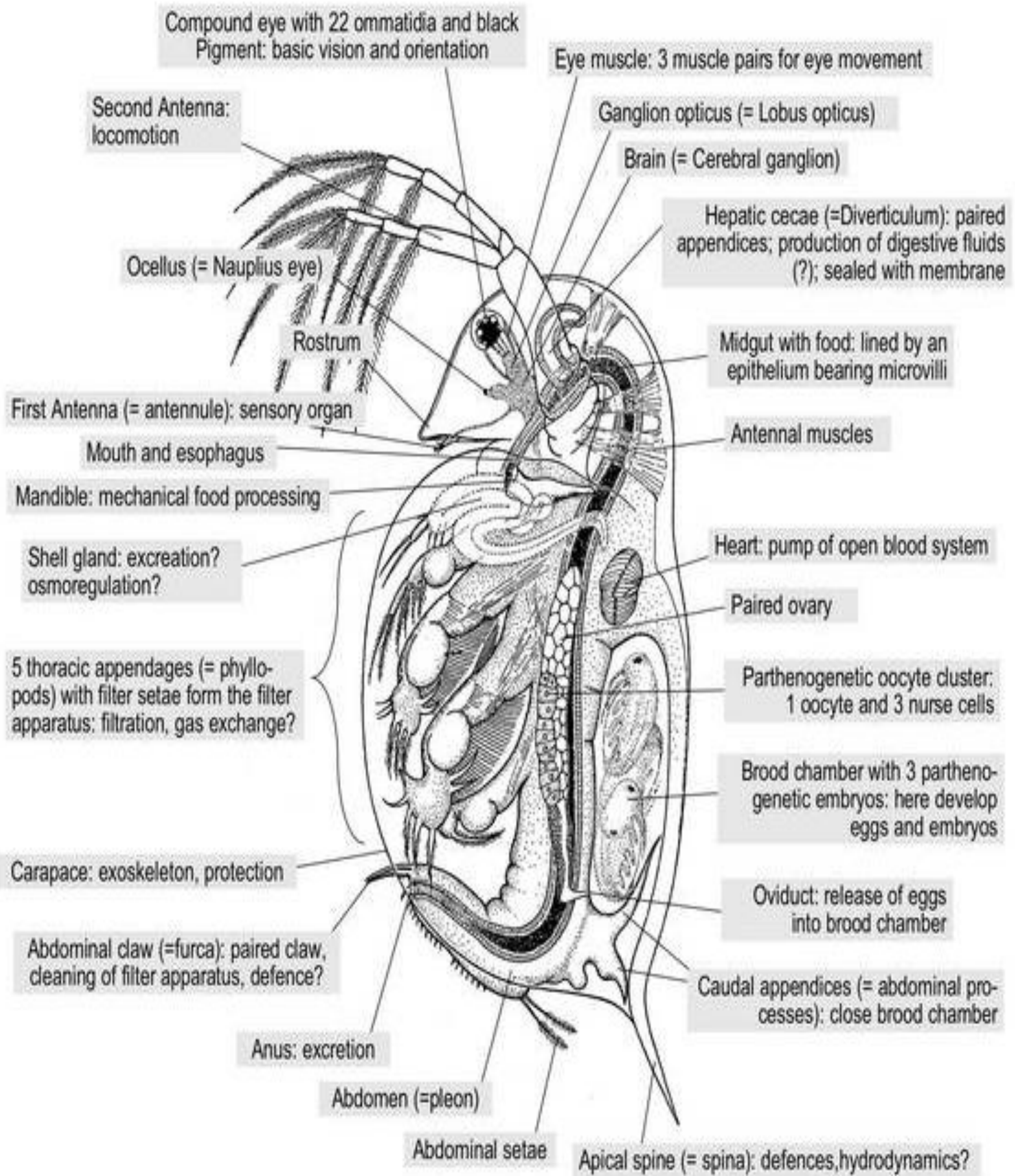


Fig. 1.3. The functional anatomy of Daphnia (source: http://www.evolution.unibas.ch/ebert/publications/parasitismdaphnia/ch2f1.htm)

Like other Crustaceans, *D. magna* has two pairs of antennae. The first pair of antennae is located beneath the rostrum and acts as a sensory organ (Ebert, 2005). In female *D. magna* the first pair of antennae is rudimentary and does not protrude over the rostrum, in male *D. magna,* however it is elongated and movable (Mitchell, 2001). The second pair of antennae is used for locomotion.

The average number of eggs per instar is approximately six to ten.

D. magna plays a very important ecological role in freshwater habitats, this includes a role as a very efficient filter feeder, in fact *Daphnia magna* has been classified as fine mesh filter feeders (Geller and Muller, 1981), being able to filter particles suspended in the water column with a minimum size as small as 200 nm. This capacity makes *Daphnia* sensitive to environmental conditions and to a whole range of contaminants, in addition to a short life cycle observable in laboratory, it is not surprising that several studies have focused on *D. magna* as test species when assessing the effects of compounds in the aquatic environment as explained in sections 1.4.3 and 1.4.4.

1.4.2 Life cycle

Under ideal environmental conditions *D. magna* reproduce parthenogenetically producing clonal offspring. Development of eggs is direct (immediate). At 20ºC, the embryos hatch from the eggs after about 1 day but remain in the brood chamber for further development. After about 3 days in the brood chamber, the young *Daphnia* are released by the mother through ventral flexion of the post-abdomen. The newborn look more or less like the adult *Daphnia*, except that the brood chamber is not yet developed (Ebert, 2005). Usually, only females develop from the parthenogenetic eggs, but under certain environmental conditions which can be stressful conditions, as a change in e.g. temperature or food levels, males can also born with subsequent sexual reproduction. Fertilized, amphigonic eggs are extruded into the brood chamber which is modified to form the ephippium. The development of these eggs is arrested early and the ephippium, containing the dormant embryos, is shed at the maternal molt (Zaffagnini, 1987). After a resting period, only females develop from amphigonic eggs. The two sexual and asexual cycles are illustrated in Fig. 1.4.

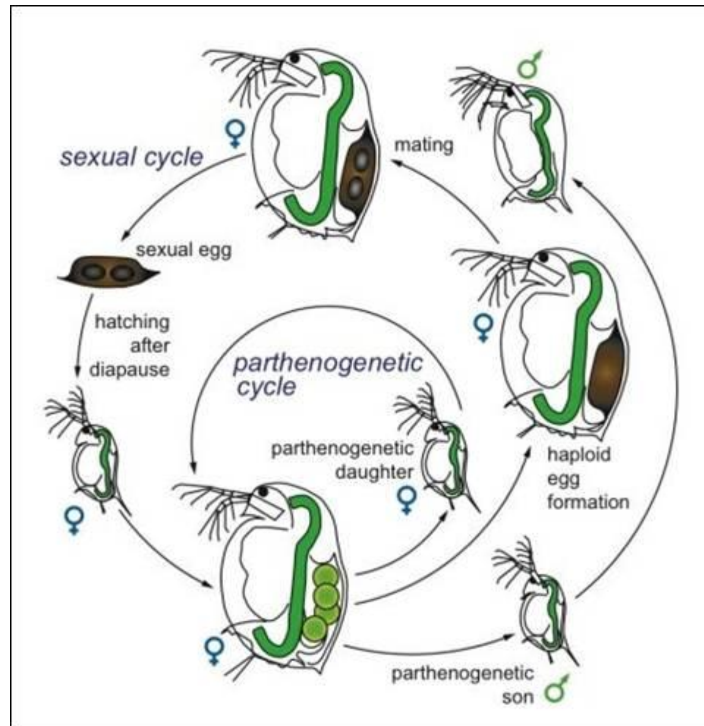


Fig. 1.4. Sexual and the asexual (parthenogenetic) life cycle of a *Daphnia.* Drawing by Dita B. Vizoso, Fribourg University

Males should not be present in a laboratory culture reared under a regime of constant light, temperature and food. At 20ºC *D. magna* reach sexual maturity in 6 - 8 days releasing their eggs into a brood chamber. The embryos complete their development inside the brood chamber and hatch as free – swimming neonates at day 8 - 10. In the following 2 - 4 days the mature females release a 2nd brood of neonates with reproduction peaking around the 3rd brood (day 12 -14) or 4th brood (day 14 - 17). As the adults become older the time between broods will increase and the size of the brood will decrease.

D. magna has three to five juvenile instars (developmental stage between moults), followed by a single adolescent instar and 6-22 adult instars. Each instar is terminated by a molt. Under favorable conditions an instar lasts two days but can last up to a week under unfavorable conditions. The average number of eggs per instar is approximately six to ten.

The life span of a single *D. magna* depends on environmental conditions like temperature or food supply and is roughly 40 days at 25°C, and about 56 days at 20°C (U.S. EPA, 2002).

1.4.3 *Daphnia* as a model organism

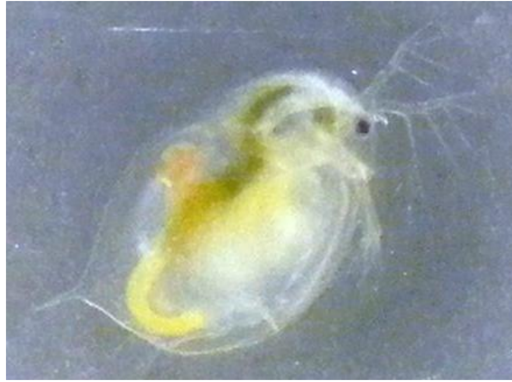


Fig. 1.5. A *Daphnia magna* specimen coming from laboratory cultures at CIRSA

The crustaceans of the genus *Daphnia* (Fig. 1.5), have been subject to intense biological investigations for over a century. *Daphnia* species have a rich literature and are sensitive test organisms which respond to environmental stressors that are important factors affecting freshwater ecosystems integrity. *Daphnia* is increasingly used as a model organism, extensively studied to understand particular biological phenomena, it is highly sensitive to pollution and it is used for evaluating environmental toxicity of chemicals on aquatic invertebrates. The characteristics that make *Daphnia* a model organism for ecotoxicological laboratory studies are:

- relatively easy to grow and maintain in a restricted space;
- relatively easy to provide necessary nutrients for growth;
- parthenogenetic mode of reproduction and short life cycle (egg to adult in less than 10 days);
- relatively well understood growth and development;

Moreover, *Daphnia* species, as a whole, have a wide, nearly cosmopolitan, distribution. They occur in a highly diverse set of habitats ranging from freshwater to lakes.

1.4.4 Use of *Daphnia magna* in aquatic toxicity tests

A toxicity test, also referred to as bioassay, is an experimental determination of the effects of a material on a selected group of organisms under defined conditions. An environmental toxicity test measures effect after exposure to specific concentrations of chemical, effluent, elutriate, receiving water, sediment or soil.

Current risk assessment methodologies require the deployment of standard assays and species for the detection of hazard in relation to specific scenarios (U.S. EPA, 2002). Multiple methods have been standardized by multiple organizations, e.g.:

- American Society for Testing and Materials (ASTM);
- Organization for Economic Cooperation and Materials (OECD);
- International Organization for Standardization (ISO);
- National Toxicology Program (NTP).

This approach brings many advantages:

- Test are uniform and comparable to previous results within the same or other laboratories;
- Can be replicated (confirmed) by other laboratories;
- Makes it easier for decision makers to accept test results;
- Logistics are simplified, developmental work already done;
- Methods establish baseline from which modifications can be made if necessary;
- Data generated can be combined with those from other laboratories for use in ERA's;
- Detailed listing of apparatus, dilution water, test material, test organisms, etc;
- Experimental, analytical and documentation procedures are detailed.

But there are also some disadvantages:

- Often very specific and, as such, hard to apply to other situations or answer other questions;
- Tend to be used in inappropriate situations;
- May not be applicable to natural environment.

Several standard methods have been developed for a range of test species (cladocerans, freshwater fishes, insects, oligochaete, marine and freshwater amphipods and green algae) depending on their life cycle and habitat, for different types of toxicity test. There are different protocols for testing pollutants in the water column and attached to the sediment, for freshwater as well as for saltwater.
Standard aquatic toxicity tests can be divided into: acute and chronic tests.

- **Acute tests** are short term tests and mostly have mortality as the endpoint. They can be divided into static, non renewable tests, static renewable and flow through tests. The *Daphnia* acute test is a static non-renewable tests, which is the simplest one; the tests water is not renewed over the test period. They are very cost effective, but are the least realistic and sensitive tests.
- **Chronic tests** simulate several, or a continuous, application and cover a substantial part of the organisms' life cycle. They can have different endpoints that are affected by sublethal concentrations of the contaminant. The most accurate method to ensure that the concentration of a pollutant, or effluent, is kept at the required level is the flow-through method. Here the test chambers are provided continuously with the solution or suspension to be tested to keep the concentrations stable. The downside of the flow-through method is that it is very labor intensive and requires large amounts of test solution. The *Daphnia* chronic test is a static renewal test where organisms are fed during the all test to keep them alive and able to reproduce. Test organisms are exposed to a fresh solution of the same concentration of test sample at a defined time interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers. The risk of volatilization and degradation is reduced in this method but it is still existent. The renewal method provides an acceptable tradeoff between labor intensity, feasibility in laboratory work and simulating possible natural occurrences.

1.5 Selected battery of biomarkers

Utility of biochemical approaches in environmental pollution monitoring is based on the assumption that low concentration of a toxicant will cause biochemical responses within individual organisms before these effect are observed at higher levels of biological organization (Sarkar et al., 2006).

The EU Water Framework Directive (WFD, Directive 2000/60/EC), requiring to assess the achievement of good chemical and ecological status of water bodies, pointed out the importance of biological monitoring for the determination of water quality. Biomarkers, although not incorporated in the WFD, are among the emerging biological monitoring tools considered for use in monitoring programs necessary for the implementation of the WFD. Allan et al. (2006) proposed the use of biomarkers in the WFD in order to "provide more realistic assessment of impacts and exposure of aquatic organisms to specific contaminants present in water".

When a toxic compound penetrates into an ecosystem, it can cause a variety of damages at different levels of hierarchical organization, from the primary level of toxicity at the molecular level, determining changes in enzyme activities, alterations in DNA, to the higher levels of biological organization as cells, tissues, organs and organisms until it reaches the levels of populations and communities. Biomarkers are indices of stress, they can be defined as cellular, biochemical or molecular alterations which can be detected and quantified in a tissue or cell of an organism exposed to a contaminant before the effect is observed on the survival or reproduction of the organism itself. In particular, the responses at the primary level often represent cytoprotective responses since they tend to decrease the toxic effect of the pollutant through the activation of multienzymatic systems. These enzymatic systems can detoxify all or part of the body.

One of the early biomarkers characterizing the environmental exposure is represented by the inhibition of the enzyme acetylcholinesterase (AChE), biomarker indicating effects on nervous system.

Other biomarkers assess which level of oxidative stress is reached by the organism. Oxidative stress is caused by an imbalance between the production of highly reactive chemical species, free radicals, and the physiological defense capabilities, antioxidants. The cellular damage can begin at the level of the lipid membrane (and malondialdehyde is an intermediate product of lipid peroxidation) until to DNA

damages. Reactive oxygen species (ROS) play a key role in mediating of cellular/tissue damage because the oxygen, in addition to being essential to life, it is also toxic. Among the most important ROS there are:

- HO• hydroxyl radical;
- $\bullet O_2^-$ superoxide anion;
- H_2O_2 hydrogen peroxide;
- O_2 singlet oxygen.

The free radical produced in higher quantities is the superoxide anion $\bullet O_2^-$. It reacts with the hydrogen peroxide H_2O_2 and form the dangerous hydroxyl radical HO•. Organisms are equipped to defend themselves from the presence of these free radicals with molecules anti-radicals, which belong to antioxidant system..

Antioxidants can be divided into enzymatic and non-enzymatic subtypes. Several antioxidant enzymes are produced by the body, with the three major classes being the superoxide dismutase (SOD), which converts superoxide anion into hydrogen peroxide, catalase (CAT), which metabolizes H_2O_2 to molecular oxygen and water and the reduced glutathione (GSH) peroxidases which is described in paragraph 2.4.3. Non-enzymatic antioxidants include vitamins obtained through the diet, such as α-tocopherol (vitamin E), ascorbic acid (vitamin C), and β-carotene.

If, however, the quantity of free radicals produced is higher than the physiological response and the antioxidant system is not able to neutralize this excess, free radicals will cause damages. As an overall, this phenomenon is called oxidative stress.

Daphnia magna is widely used as test organism in acute 48-h inhibition and chronic 21-d reproduction tests in the aquatic toxicology field., but also a number of biochemical biomarkers have been studied in *Daphnia magna* for assessing the potential impact of chemicals on the aquatic environment. Most biomarkers have been evaluated on *Daphnia* after acute exposure to pesticides or metals, only few studies have been conducted on pharmaceuticals after a chronic or sub-chronic exposition.

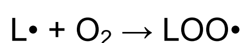
The use of biomarkers does not give a quantitative assessment of the toxicity level in a organism, but determinates its “state of health" from the state of homeostasis to that of disease, and can predict the "level of risk" to which the organism is subjected.

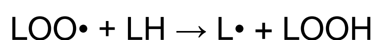
1.5.1 Acetylcholinesterase (AChE)

AChE activity serves to terminate synaptic transmission, preventing continuous nerve firings at nerve endings. Therefore, it is essential for the normal functioning of the central and peripheral nervous system. AChE hydrolyzes acetylcholine into choline and acetate after activation of acetylcholine receptors at the postsynaptic membrane. Inhibition of AChE provokes an absence of such hydrolysis resulting in overaccumulation of acetylcholine and prolonged electrical activity at nerve endings occurs. The inhibition of AChE is usually an indicator of exposure to organophosphates and carbamates pesticides, but also metals can affect this enzyme.

1.5.2 Malondialdehyde (MDA)

Polyunsaturated fatty acids, contained in the membranes, are particularly susceptible to attack by free radicals. The removal of a hydrogen atom by a $-CH_2-$ group from a radical (usually HO•) leads to the formation of a lipid radical, which may react with oxygen to give a radical perossilipidic (LOO•). This can extract a hydrogen atom from a chain of fatty acids adjacent and initiate a cascade of reactions, which convert other membrane lipids in lipid hydroperoxides (L- OOH).





This process is called lipid peroxidation and is a major cause of the loss of cell function under conditions of oxidative stress. Indeed, the presence of L- OOH in a membrane can compromise its functionality by altering the fluidity and allowing ions to freely pass through. This can result also in a rupture of the membrane itself. This process of deterioration O_2-dependent which leads to an alteration of the integrity of biological membranes, has as product the lipid peroxides and their by-products such as aldehydes. Among these, the malondialdehyde (MDA) is the main product of the lipidic peroxidation.

MDA assess is used as a non-enzimatic marker of oxidation of phospholipidic membrane and an increment in MDA level in organisms it has been considered a relevant index of chemical damage induced by toxicants.

1.5.3 Glutathion S-trasferase (GST)

The antioxidants include glutathione (GSH) which is a tripeptide extremely important for the health of the cell and performs several functions: it is the substrate of some antioxidant enzymes, plays an action against scavenger of hydroxyl radicals and singlet oxygen, and also has a role in the regeneration of vitamin E (Reddy et al., 1983). The defense system has evolved a battery of enzymes to combat the formation of oxygen radicals, GST belongs to the class of phase II detoxifying enzymes that catalyse the conjugation of glutathione with xenobiotics, including organophosphates. Induction of GST activity is an indication of a detoxification process. GST also catalyses the conjugation of glutathione with cytotoxic aldehydes produced during lipid peroxidation (Halliwell and Gutteridge, 1999). The activity of antioxidant enzymes is potentially a useful biomarker in the assessment of the quality of aquatic environments.

2. Aim of the work

The increased pharmaceuticals consumption together with the recent development of more sensitive analytical techniques has lead to identification of pharmaceuticals as emerging contaminants in the aquatic environment (Quinn et al., 2008). As pharmaceuticals are continuously added and not efficiently removed by wastewater treatment plants, certain pharmaceuticals exhibit pseudopersistence, giving rise to concerns for the health of aquatic wildlife and a theoretical risk to humans who consume these organisms or drink the water in which they live (Franzellitti et al., 2011). Pharmaceuticals are present in surface waters at low concentrations of ng-µg/L, and standard acute ecotoxicity data reported for a number of pharmaceuticals may not be sensitive enough to adeguately characterize the risk associated with these chemicals (Brooks et al., 2003). To the other hand there is very little information about chronic toxicity. To fill this gap of information we tested pharmaceuticals known to be widespread in freshwaters combining ecotoxicological 48-h acute inhibition test with 21-d chronic reproduction tests on the crustacean *Daphnia magna*, a representative freshwater zooplankton species.

Regulatory risk assessment of chemicals and the majority of ecotoxicological studies in the aquatic and terrestrial environments focus mainly on the toxicity of single compounds (Barata et al., 2006). However, organisms in the environment are constantly exposed to complex mixtures or combination of stressors (Pavlaki et al., 2011). Since a chemical will be rarely found alone in the environment but commonly in combination with others (Pavlaki et al, 2011) a further aim of this work was to understand the ecotoxicological effect of the binary mixtures of the pharmaceuticals propranolol and fluoxetine. Since the possible antagonistic or synergistic interactions that can occur between chemicals (Loureiro et al., 2010) makes difficult the assessment of environmental hazards of complex mixtures to evaluate the possible interaction effects between pharmaceuticals we used the MixTox model (Jonker et al., 2005), a data analysis tool used in the ecological risk assessment to predict mixture effects from concentrations of the component substances. The MixTox tool highlights a possible antagonistic or synergistic effect between chemicals and indicates if this effect depends upon the composition or the concentration of the compounds in the mixture.

The research has been completed assessing the response of *Daphnia magna* to fluoxetine at the physiological level: production of malondialdehyde, and acetylcholinesterase and glutathione s-transferase enzymatic activities were measured.
The study intends to bring new knowledge on evaluation of toxicity of pharmaceutical residuals in the environment, and provide a useful method to be applied in the ecological risk assessment.

3. Materials and methods

3.1 Culturing of *Daphnia magna*

Daphnia magna has been successfully cultured in the Laboratory of Ecotoxicology of CIRSA for more than ten years. Cultures of *D. magna* were maintained in 8 L capacity plastic vessels (Fig. 3.1), and were fed three times a week with a mixture of around 10^8 cells of the green alga *Pseudokirchneriella subcapitata* (for counting see paragraph 3.4) and 40 mg of brewer's yeast. The cultures were kept in a controlled temperature chamber at 20°C at 16:8 light:dark photoperiod through neon tubes and culture medium was renewed once a week.

Fig. 3.1. ***Daphnia*** **cultures maintained in 8 liter plastic vessels at constant environmental conditions**

This allowed to maintain the *Daphnia* cultures in good conditions ready to be used for the tests. Periodically organisms condition were checked by a 24-h acute immobilization test in according with ISO (1996) with potassium dichromate ($K_2Cr_2O_7$ CAS number 7778-50-9) in order to evaluate if the sensitivity of *Daphnia* was in the range expected from the guideline.

3.1.1 Daphnia Medium

Two different kinds of water were used as culture and test medium, both maintained and aerated in 20 L tanks (Fig. 3.2).

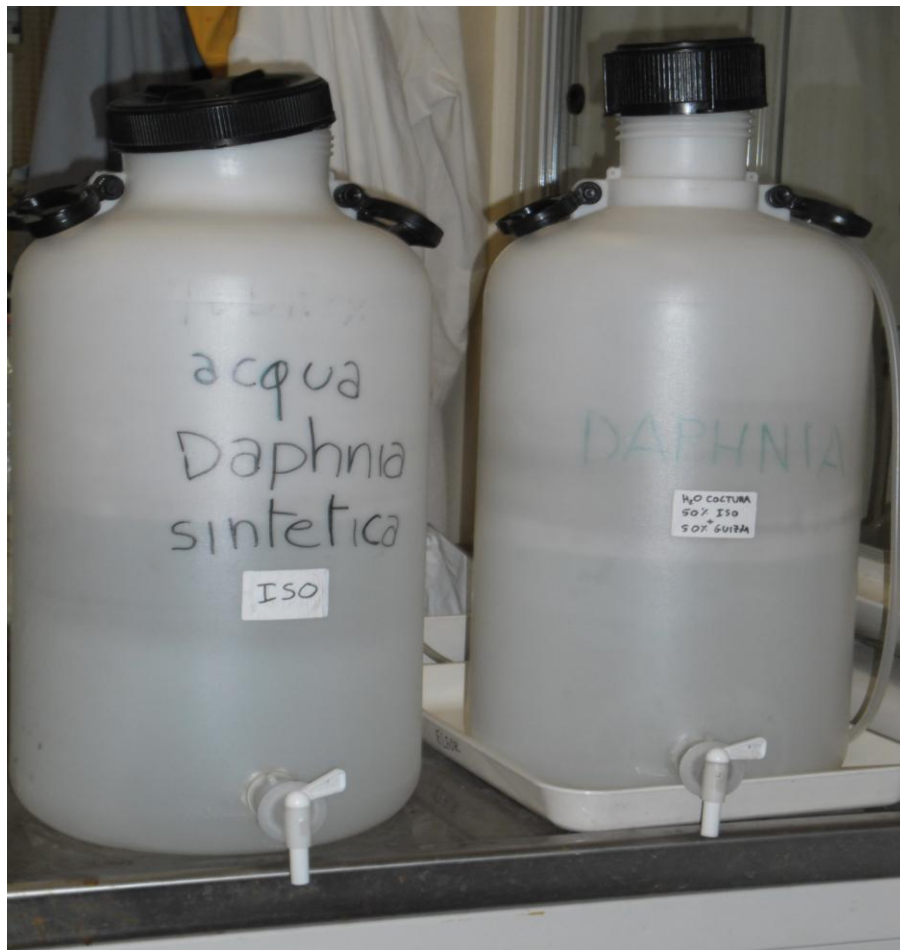


Fig. 3.2. Two 20 liters tanks containing the re-constituted water

- Acute and sub-chronic test medium (hereafter ISO water) was made in accordance with ISO (1996) protocol. Before starting the test the water was oxygenated for 24 h. After aeration The water had a pH of 7.8 ± 0.2, a hardness of 250 mg/l ± 25 mg/l (expressed as $CaCO_3$), a molar Ca/Mg ratio close to 4:1 and a dissolved oxygen concentration above 7 mg/l. This medium was prepared by mixing 250 ml of each of the four solutions reported below and by bringing the total volume to 10 liters with distilled water. The four solutions were made by dissolving four salts in distilled water:

1) Calcium chloride solution: 11.76 g/L of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$);
2) Magnesium sulfate solution: 4.93 g/L of magnesium sulfate heptahydrat ($MgSO_4 \cdot 7H_2O$);
3) Sodium bicarbonate solution: 2.59 g/L of sodium bicarbonate ($NaHCO_3$);
4) Potassium chloride solution: 0.23 g/L of potassium chloride (KCl);

Due to the paucity of salts in the ISO water, this kind of water has not been used to maintain *Daphnia* cultures or for the reproduction test.

- The culture and chronic test medium (hereafter culture water) was made by mixing in the same proprortion the ISO water and a natural spring water, commercialized as bottled mineral water (Acqua Guizza, Acqua minerale San Benedetto S.p.A., Scorzè, Italia). The choice of the Guizza (was determined by its salt composition (Table 3.1), in fact the sum of the salts which compose the two waters is very close to the salt concentration required by the chronic test protocol ISO 10706:2000 annex B (ASTM water). The only big difference between our culture water and water required by the protocol is for the paucity of sulphates in our culture water respect to ASTM water.

Table 3.1 Composition of Guizza and the ISO water

	Water composition								
	Mg^{++}	NO^{3+}	SO_4^{--}	K+	Ca^{2+}	SiO_2	Na+	Cl-	F-
Guizza (mg/L)	28.7	8.7	4.0	1.1	49.0	17.5	6.5	2.4	<0.15
ISO 6342 (mg/L)	12.0		0.05	3.0	80.5		17.7	0.003	

3.1.2 Algae culturing

The green algae have been cultured in 1 liter flasks (Fig. 3.3) under non-aseptic, but controlled conditions of temperature (20°C), photoperiod (16:8 light:dark) and aeration. Each culture was grown from a 10 ml inoculum derived from an older culture. The culture medium was in accordance with Gorbi (1987) and was composed by 8 solutions dissolved in distilled water. A concentrated medium was prepared by mixing 20 ml of solutions 1 to 7 and 2 ml of solution 8, and then bringing the volume to 1liter with distilled water. This concentrated medium was diluted tenfold with distilled water to obtain the culture medium.

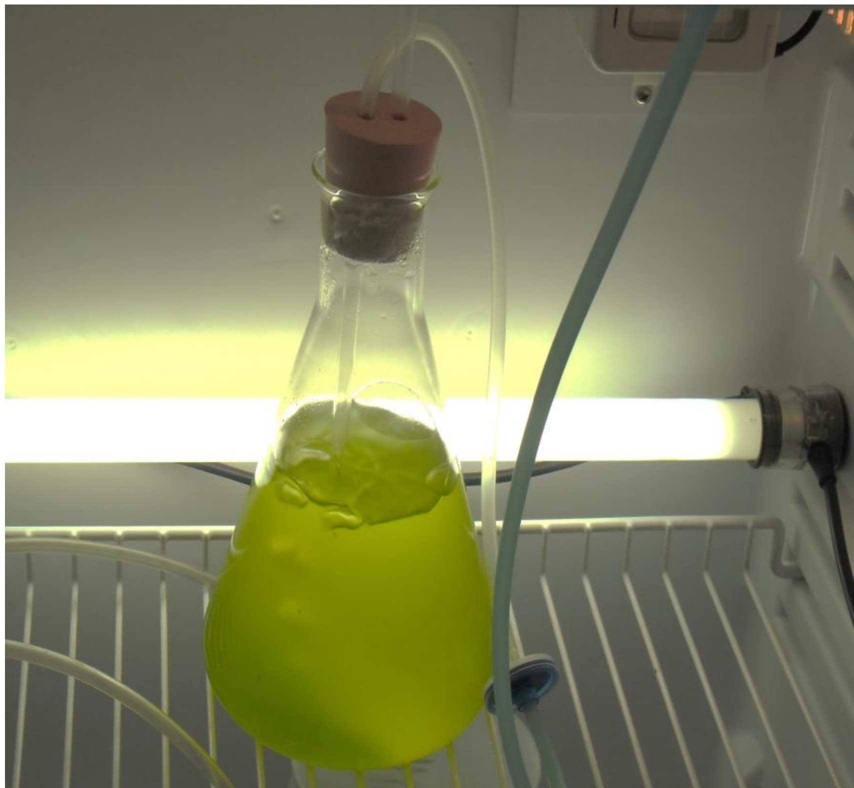


Fig. 3.3. Flask of algal culture maintained under controlled conditions.

The salts solutions are:

1. 2.55 g $NaNO_3$ in 100 ml distilled water
2. 0.104 g K2PO4 in 100 ml distilled water
3. 1.22 g $MgCl_26H_2O$ in 100 ml distilled water
4. 1.47 g $MgSO_47H_2O$ in 100 ml distilled water
5. 0.441 g $CaCl_2*2H_2O$ in 100 ml distilled water
6. 1.50 g $NaHCO_3$ 100 ml distilled water
7. Fe EDTA (0.096 g FeCl3 + 0.300 g Na EDTA $2H_2O$ in 1 liter distilled water)
8. 10 ml 8A solution (0,0327 g/l $ZnCl*6H_2O$), 1 ml 8B solution (0,0726 g/l $NaMoO_4$ $2H_2O$), 1 ml 8C solution (0.0143 g/l $CoCl_2$ $6H_2$ O), 0,1 ml 8D solution (0.141 g/l $CuCl_2*2H_2O$), 0.186 g H_3BO_4, 0.416 g $MnCl_2*4H_2O$, diluited to 1 liter distilled water

The normal growth period of *Pseudokirchneriella subcapitata* cultures was about 6 days. At the end of the growth period the algae culture was sedimented in a beaker for about a week and then resuspended in culture water.
During the reproduction tests, algae were often controlled under microscope to make sure that within the culture there was always just the type of algae indicated in the protocol.

3.2 Tested substances

3.2.1 Fluoxetine

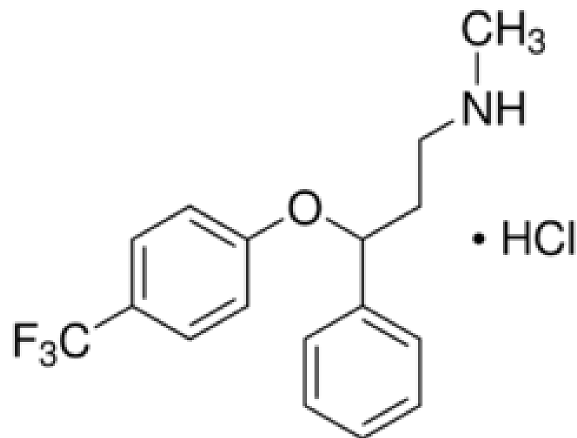


Fig. 3.4. Structure and configuration of fluxetine hydrochloride used for testing.

Fluoxetine, currently marketed as fluoxetine hydrochloride, is a chiral molecule (Fig. 3.4). In the present study the racemic mixture of fluoxetine hydrochloride (*(±)-N-Methyl-γ-[4-(trifluoromethyl)phenoxy]benzenepropanamine hydrochloride), CAS number* 56296-78-7, *which contains equal parts of the two enantiomers R- and S-* has been used. The substance was purchased from Sigma Aldrich (Milan, Italy). FLX hydrochloride is readily soluble, so no solvents were necessary for preparation of stock solutions, which were therefore prepared in ISO or culture water for acute/sub-chronic and chronic test exposure respectively. Nominal concentrations were not analytically verified for this study.

3.2.2 Propranolol

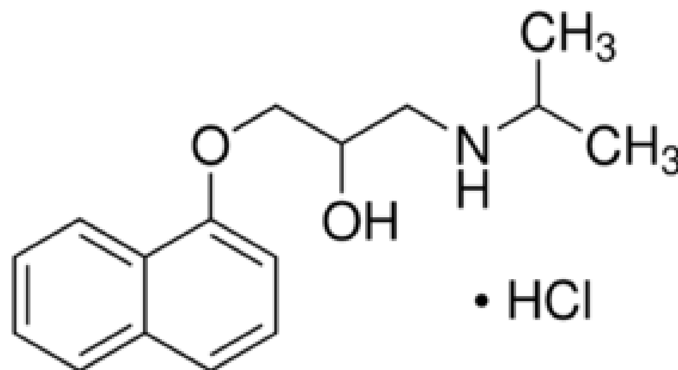


Fig. 3.5. DL-Propranolol hydrochloride used for testing

Propranolol, commercially available as propranolol hydrochloride, is a chiral molecule (Fig. 3.5) which exists in the form of two enantiomers with different spatial configuration: (S) - (-) - propranolol and (R) - (+) – propranolol. In this study we used the racemic mixture DL-Propranolol hydrochloride ((±)-1-Isopropylamino-3-(1-naphthyloxy)-2-propanol hydrochloride), CAS number 318-98-9. The substance was purchased from Sigma Aldrich (Milan, Italy). PRP hydrochloride is readily soluble, so no solvents were necessary for preparation of stock solutions, which were therefore prepared in ISO or Culture water for acute/sub-chronic and chronic test exposure respectively. Nominal concentrations were not analytically verified for this study.

3.2.3 Carbamazepine

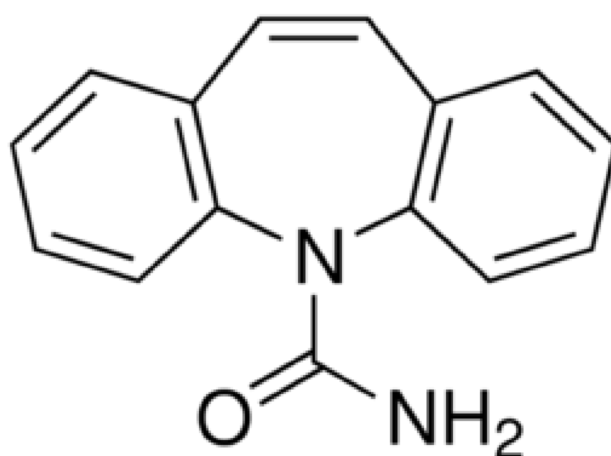


Fig. 3.6. Structure of Carbamazepine

Carbamazepine (Fig. 3.6) (5*H*-Dibenz[*b*,*f*]azepine-5-carboxamide), CAS number 298-46-4, was purchased by Sigma (Milan, Italy). Due to its low solubility it was not possible to dissolve CBZ in water; for this reason a stock solution was prepared dissolving CBZ in ethanol. This solution was further diluted with ISO water in order to reach in the highest CBZ concentration an ethanol concentration of 1% (v/v) in order to avoid a toxic effect due to this solvent.

3.3 Acute Immobilization Toxicity Test

All acute experiments were conducted in accordance with the ISO 6341 standard and the OECD 202 guideline. The same procedure was used for both single chemical and mixture exposures, only the experimental designs were different.
One day before the start of the exposure to the chemicals, adult females containing parthenogenetic eggs (no first brood progeny) were isolated by eliminating the juveniles from the culture vessels and then kept under the same culture conditions. They derived from healthy stocks with no signs of stress such as high mortality, presence of male or ephippia. The next day, newborns (less than 24 h old) present in the culture vessels were collected and exposed to different concentrations of pharmaceuticals. Groups of five daphnids for beaker were placed in a 25ml glass jar containing 20ml of test solution. The acute test involves at least five concentrations arranged in a geometric series with a separation factor not greater than 2.2, of which the highest concentration must determine the 100 % of immobilization and the lowest no observable effect. Test solutions of the chosen concentrations are prepared by dilution with ISO water of a stock solution prepared before. Beakers were randomly placed into trays at the same condition of light and temperature of the cultures. Daphnids were not fed during the test. After 24 and 48 h daphnids were observed and the number of mobile individuals in each container was reported. Those which were not able to swim after gentle agitation of the liquid for 15 s have been considered immobilized. Results of experiments were considered as valid if the percentage immobilization of the controls was less than or equal to 10 %.

3.3.1 Acute test on reference toxicant (potassium dichromate)

The acute test with potassium dichromate ($K_2Cr_2O_7$) has been carried out periodically, with the aim to verify that the sensitivity of *Daphnia magna* were maintained within a range of acceptable variability. Seven increasing concentrations were prepared, with two replicates per concentration, in a geometric series from 0.80 to 3.00 mg/L (0.80, 1.00, 1.24, 1.55, 1.93, 2.41, 3.00 mg/L) of potassium dichromate obtained by diluting a stock solution of 100 mg/L and a control treatment with water as only dilution. After 24 hours from the beginning of the test in each beaker the number of individuals still alive was counted and were reported.

3.3.2 Acute single pharmaceuticals exposure

Preliminary tests were performed in order to determinate the range of concentrations to be tested in the final toxicity tests. For each chemical, the highest concentration tested in the final tests was the lowest among the concentrations that caused 100% immobilization in the range finding tests. The lowest concentration tested in the final tests was the highest among the concentrations that did not cause any observable effect. Within this range, seven test concentrations, arranged in a geometric series were used. The seven nominal concentrations were from 3 to 12 mg/L for propranolol, from 2 to 20 mg/L for fluoxetine and from 60 to 130 mg/L for carbamazepine.

The experimental design consisted in 8 treatments (7 concentrations plus control), two replicate beakers for each treatment. Experiments with propranolol and fluoxetine have been repeated three times, with carbamazepine twice.

3.3.3 Acute mixture pharmaceuticals exposure: Toxic Unit approach.

Toxicity tests on mixtures were carried out using the same procedures as the respective single-compound tests, the only difference being the adoption of a fixed-ratio ray experimental design (Meadows et al., 2002).

Treatments consisted in a control (test medium only) and 25 mixtures of propranolol and fluoxetine, identified using two criteria: total concentration and proportion of the two chemicals in the mixture.

Concentration of each chemical was expressed as TU50, i.e. the concentration of the chemical (mg/L) divided by its EC50 (the EC50 values estimated from the single pharmaceutical experiments were used). Total concentration of each mixture was computed as ∑TU50, i.e. as the sum of the TU50 of the chemicals making up the mixture. Five total concentrations were tested: 0.5, 0.71, 1, 1.41 and 2 ∑TU50.

For each total concentration, five mixtures were tested that differed in the proportion of ∑TU50 contributed by each of the two substances: 100:0, 75:25, 50:50, 25:75 and 0:100% (Table 3.2). The resulting treatment concentrations of propranolol and fluoxetine, as mg/L, were calculated using the EC_{50} estimated in the single chemical experiments (Fig. 3.7). This concentration decision system allows to have a standardized method since 1 TU of a substance has the same effect of 1 TU of

another substance (in this case the loss of 50 % of survival or reproduction of *Daphnia*).

Two replicate beakers for treatment were used in each experiment; three independent experiments were carried out.

Table 3.2. Concentrations assayed expressed in TU. In each column the total concentration in the mixtures is constant increasing from the left to the right part of the table; in the lines the percentage of the pharmaceuticals in the mixtures is constant (propranolol decreasing and fluoxetine increasing top-down).

	TU in the mixture				
	0.50	**0.71**	**1.00**	**1.41**	**2.00**
100 % PRP	0.50	0.71	1.00	1.41	2.00
0 % FLX	0.00	0.00	0.00	0.00	0.00
Tot MIX	0.50	0.71	1.00	1.41	2.00
75 % PRP	0.38	0.53	0.75	1.06	1.50
25 % FLX	0.13	0.18	0.25	0.35	0.50
Tot MIX	0.50	0.71	1.00	1.41	2.00
50 % PRP	0.25	0.35	0.50	0.71	1.00
50% FLX	0.25	0.35	0.50	0.71	1.00
Tot MIX	0.50	0.71	1.00	1.41	2.00
25 % PRP	0.13	0.18	0.25	0.35	0.50
75 % FLX	0.38	0.53	0.75	1.06	1.50
Tot MIX	0.50	0.71	1.00	1.41	2.00
0 % PRP	0.00	0.00	0.00	0.00	0.00
100 % FLX	0.50	0.71	1.00	1.41	2.00
Tot MIX	0.50	0.71	1.00	1.41	2.00

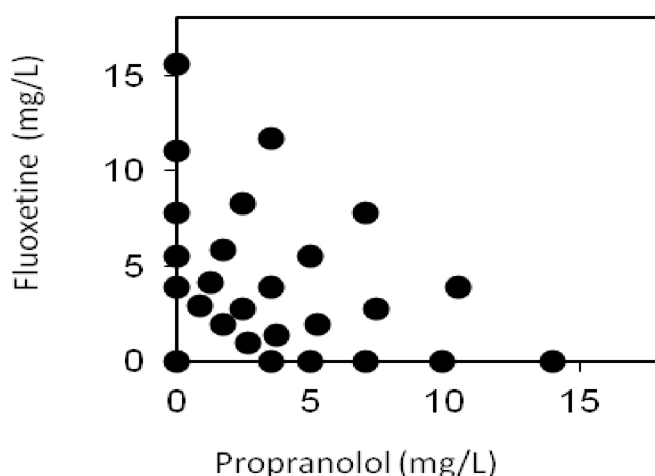


Fig. 3.7. Fixed-ratio ray design adopted in the acute mixture exposure experiments. Each point indicates the propranolol and fluoxetine concentration (mg/L) of an actually tested mixture. Along each ray, the proportion of $\sum TU_{50}$ contributed by each of the two substances is constant, while ΣTU_{50} increases moving away from the origin of the axes.

3.4 21-d Chronic Reproduction Toxicity Test

21 days *Daphnia magna* chronic toxicity test was carried out according to 211 OECD procedure and ISO 10706 standard. The same procedures were used for both single and mixture exposures, only the experimental designs were different. To initiate the test daphnids younger than 24 h were collected as described at paragraph 3.2. They were placed in a 100 ml glass jar containing 50 ml of test solution. One daphnid was deployed in each beaker. Concentrations were made by diluition of stock solution in culture water (aerated for 24 h before test initiation and every medium renewal). Containers were marked and placed randomly under a light source and incubated at the same environmental condition of cultures. This was a static renewal test, which means that water and nutrients were renewed three times a week in order to maintain good conditions for daphnids which must grow up and reproduce. Every time the medium was renewed newborns were counted and removed from the vessels. After 21 days we counted the number of neonates per surviving organism. In accordance with ISO 10706, the results of a test were considered valid when the following criteria were respected:

a) the total number of control replicates exhibiting adult mortality and male development was ≤ 20 % at the end of the test;

b) the mean number of living offspring per living parent in the controls was ≥ 60 (the control animals have to produce their first brood within 11 days from the start of the test otherwise criterion may not be met);

Organisms were fed with living algal cells of *Pseudokirchneriella subcapitata* and a suspension of brewer's yeast. Diet was constant during the test, but a lower rate was used at the beginning and then increased during the test to take account of growth of the parent animals.

A series of preliminary experiments was carried out in order to set up the method and to identify the feeding regimen that enabled to meet the above criteria.

Pseudokirchneriella subcapitata cells were counted with a microscope and a Bürker counting chamber (Fig. 3.8). The chamber consists of a thick glass microscope slide with indentation that creates a grid. The grid is divided in squares and rectangles of different sizes. The device is carefully crafted so that the area bounded by the lines is known, and the depth of the chamber is also known. This allows an easy counting of cells:

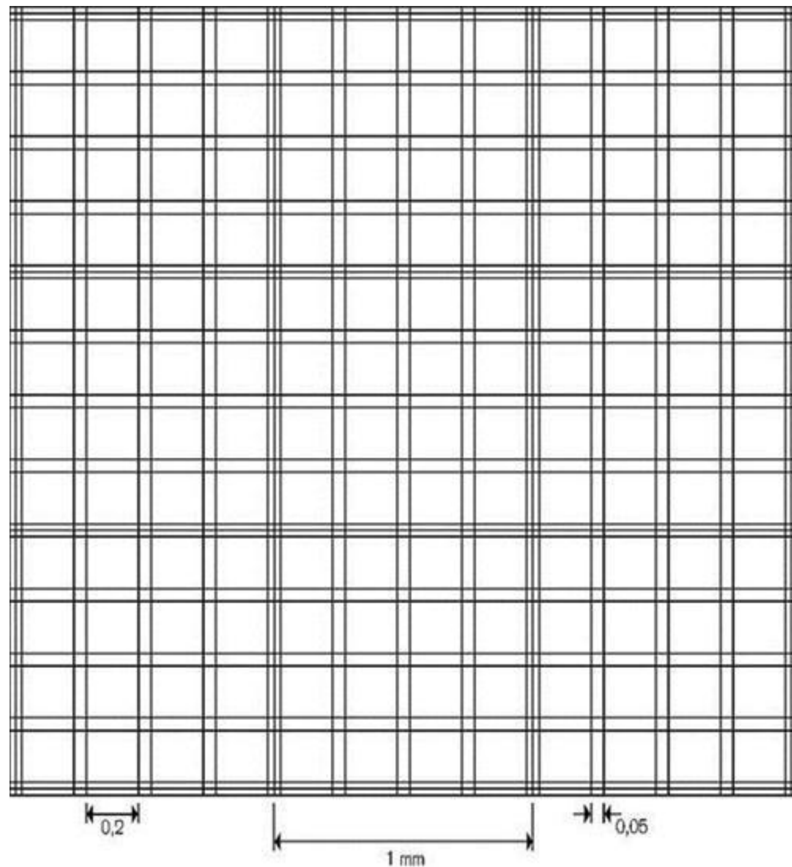


Fig. 3.8. Bürker chamber is divided in 9 counting grids. Large squares (1 mm² each) are divided into 16 group squares by double lines - 0,05 mm apart. The double lines form small squares, each with an area of 0,0025 mm². The chamber depth is 0,1 mm.

The number of cells in a specific volume of the chamber was counted, and then we calculated the concentration of cells in the fluid. The mean of the counting of three replicates was the basis for a series of dilutions to reach the required cell density in the test medium.

In the final tests *Daphnia magna* were fed with $1.25*10^8$ cell/l/d and 80 mg/L/d of brewer's yeast. We fed only 75% of the daily dose of nutrients during the first week. The total organic carbon amount of the food ration was assessed combining dry weight and CHN analysis data from algal cells and brewer's yeast. The total organic carbon feeding amount was 0.123 mg per animal per day, within the interval prescribed by ISO 10706 (0.1 to 0.2 mg of carbon per animal per day).

3.4.1 Chronic single pharmaceutical exposure

A range-finding test was conducted to determine the range of concentrations for the definitive test. The final experimental design consisted of one daphnid per test chamber and 5 test chambers per treatment level. 6 treatments plus control and 7 treatments plus control for propranolol and fluoxetine were performed, respectively. Concentrations were 0.25, 0.42, 0.71, 1.19, 2.00 mg/L for propranol and 0.025, 0.05, 0.10, 0.20, 0.40, 0.80 mg/L for fluoxetine. Each experiment was repeated twice.

3.4.2 Chronic mixture pharmaceutical exposure

Chronic mixture compounds tests were carried out using the same experimental design described at point 3.3.2, using the propanolol 21-d EC_{50} and fluoxetine 21-d EC_{50} calculated from the single exposure study. Concentrations tested are illustrated in Fig. 3.9. Chronic mixture test design consisted in two replicates vessels for treatment (10 replicates for the control), one organism per glass container, and experiments repeated three times.

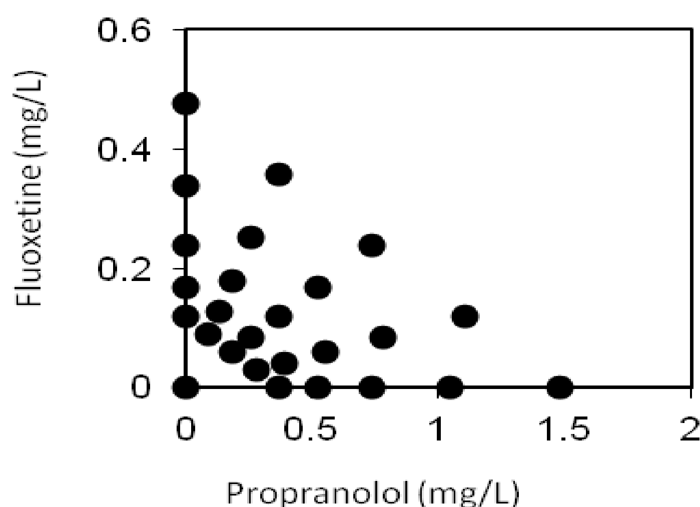


Fig. 3.9. Fixed-ratio ray design adopted in the chronic mixture exposure experiments. Each point indicates the propranolol and fluoxetine concentration (mg/L) of an actually tested mixture. Along each ray, the proportion of $\sum TU_{50}$ contributed by each of the two substances is constant, while ΣTU_{50} increases moving away from the origin of the axes.

3.5 Battery of Biomarkers

Enzymatic activity of acetylcholinesterase and gluthatione s-transferase, and malondialdehyde production were evaluated. For each biomarker *Daphnia magna* response to a sub-chronic exposure to fluoxetine has been assessed.

3.5.1 Experimental set up and 72-h *Daphnia* exposure

To start the test we needed four-five days old daphnids, for this reason we isolated the adult *Daphnia* on Thursday, and the next day we transferred the newborns in a new tank. We fed them with half adult dose on Friday and the next Monday, and on Tuesday they were ready for test starting.
Groups of 40 daphnids were exposed to 200 ml of test medium with different concentrations of fluoxetine in 500 ml beakers. Stock solution and diluitions were prepared using ISO water. Organisms were not fed during the test.
After 72-h still mobile individuals were collected. Individuals from similar treatments were combined in eppendorf tubes and stored at -80°C for future enzyme analysis.

3.5.2 Acetylcholinesterase (AChE) activity

Daphnids were exposed to 0.2, 1.0 and 5.0 mg/L fluoxetine. Two replicates for each treatment level were performed.
AChE activity has been assessed in accordance with the method described by Jemec et al. (2007) for *Daphnia* assays.
Animals contained in one sample were manually homogenized for 3 min in 0.4 ml of 50 mM homogenization phosphate pH 7.0 buffer, using a glass mini homogenizer. The homogenates were centrifuged for 15 min at 15000 xg at 4°C.
Enzyme activities in each sample were measured in quadruplicate using a microplate reader. The reaction mixture was prepared in 100 mM of phosphate pH 7.4 buffer containing acetylthiocholine chloride and 5,5' dithiobis-2-nitrobenzoic acid (DTNB) at a final concentrations of 1 mM and 0.5 mM, respectively. 100 µl of protein surnatant were added to start the reaction, which was followed spectrophotometrically at 412 nm at 25°C for 15 min. Total protein content in the supernatant fraction was measured according to Lowry et al. (1951). AChE activity was expressed in nmol min^{-1} mg^{-1} protein.

3.5.3 Malondialdehyde (MDA) assay

Daphnids were exposed to 1.0 and 5.0 mg/L fluoxetine with four and three replicates for treatment level respectively, six replicates for control.

In order to verify the applicability of this test on *Daphnia magna* we performed also a positive control with chromium. We tested 0.040 and 0.200 mg/L concentrations of chromium in the form of potassium dichromate. Four replicates for each treatment level.

MDA content was used to assess lipid peroxidation and was carried out following the test described by Banni et al. (2007) with slight modifications for *Daphnia*.

Animals contained in one sample were treated with two series of 30 s ultrasonication (using an XL Heat System Ultrasonic Processor) in 0.250 ml of 20 mM Tris–HCl pH 7.4 containing 0.1% β-mercaptoethanol and then centrifuged at 10000 xg for 20 min at 4°C. The supernatants obtained were used for the determination of MDA content: samples were incubated for 40 min in 5 mM methanol/1-methyl-2-phenylindoleinacetonitrile and 6% HCl at 45°C, and subsequently centrifuged at 15000 xg for 10 min at room temperature. Enzyme activities in each sample were measured in triplicate using a microplate reader. Absorbance was read spectrophotometrically at 586 nm against a standard curve of 1,1,3,3-tetramethoxypropan (TMOP) treated in an identical manner. Total protein content in the supernatant fraction was measured according to Lowry et al. (1951) and the final results were expressed as nmol mg^{-1} protein.

3.5.4 Glutathione S-transferase (GST) activity

Daphnids were exposed to 1.0 and 5.0 mg/L fluoxetine. Two replicates for each treatment level were performed, and the test was repeated twice.

Animals contained in one sample were processed with two series of 30 s ultrasonication in 0.2 ml of 50 mM potassium–phosphate buffer (KPB), pH 7.0 containing 0.5 mM Na_2EDTA following the protocol reported by Mimeault et al. (2006). The samples were then centrifuged for 15 min at 15000 xg at 4°C.

Surnatant was withdrawn and used for the determination of content of GST activity: enzyme activities in each sample were measured in quadruplicate using a microplate reader. 10 μl of protein supernatant was added to start the reaction. Glutathione S-

transferase (GST) activity was determined by measuring the increase in absorbance at 340 nm due to the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). The reaction was followed for 10 min and the total protein content in the supernatant fraction was measured according to Lowry et al. (1951). GST activity was expressed as nmol min^{-1} mg^{-1} protein.

3.6 Statistical Analysis

3.6.1 Single compounds exposure

Data from single exposures (pharmaceuticals and potassium dichromate) were analyzed using non-linear regression (Levenberg-Marquardt estimation method) as implemented in the commercial software Statistica (Statsoft, Tulsa, OK, USA). The EC50 and their confidence intervals were estimated by fitting the data from each experiment to the logistic model:

$$y = \frac{max}{1 + \left(\frac{c}{\mathrm{EC50}}\right)^{\beta}} \qquad (1)$$

where *y* is the endpoint value (number of active individuals for the acute test, number of newborns/adult for the chronic test), *max* is the maximum expected endpoint value, when the concentration of the pharmaceutical is zero, C is the exposure concentration of the pharmaceutical, EC50 is the pharmaceutical concentration at which $y = 0.5 \cdot max$ and β is a slope parameter.
Regression was calculated in logarithmic scale and reconverted in mg/L.

3.6.2 Mixture pharmaceutical exposure: MixTox tool

Data from binary mixtures exposures were analyzed using the MixTox approach (Jonker at al., 2005), as implemented for binary mixtures in a Microsoft® Excel spreadsheet made available online by the Centre for Ecology & Hydrology of the UK Natural Environment Research Council at:
http://www.ceh.ac.uk/products/stats/mixturetoxicity-analysistools.html.
Here just a few key points, essential to understand the present results, are reported. See Jonker et al. (2005) and the documentation available at the above web page for a complete discussion.
As already explained concentration addition (CA) was chosen as the reference model here. Concentration addition occurs for a mixture if the following relationship holds:

$$\sum_i \frac{c_i}{\mathrm{EC}x_i} = 1 \qquad (2)$$

Where c_i denotes the concentration of chemical i in the mixture, and ECx_i is the concentration of chemical i solely that results in the same effect (x%) as the mixture. The quotient c_i/ECx_i is the toxic unit (TUx_i) and quantifies the relative contribution to toxicity of the individual chemical i in the mixture.
In particular, for a binary mixture, equation 2 becomes:

$$\frac{c_1}{\mathrm{EC}x_1} + \frac{c_2}{\mathrm{EC}x_2} = 1 \quad (3)$$

Assuming a logistic concentration-response relationship (equation 1), ECx_i can be rewritten as the inversed function:

$$\mathrm{EC}x_i = \mathrm{EC50}_i \cdot \left(\frac{max - y}{y} \right)^{\frac{1}{\beta_i}} \quad (4)$$

Were y is the actual endpoint value (e.g. number of active individuals, number of newborns) that corresponds to a x% effect.
Parameters of equation 3, i.e. $EC50_1$, $EC50_2$, β_1, β_2 and max, were estimated from the data of the mixture experiments, by iterative methods, using the "solver" facility of Microsoft Excel (Newton algorithm).
Equation 3 represents the reference CA model in the absence of interaction between the chemicals and can be generalized to take interaction into account:

$$\frac{c_1}{\mathrm{EC}x_1} + \frac{c_2}{\mathrm{EC}x_2} = exp(G) \quad (5)$$

The quantity G is referred to as the deviation function and describe if and how interaction between chemicals causes a deviation from the base reference model.
If $G = 0$, equation 5 reduces to equation 3, i.e. to the reference CA model.
Synergism or antagonism (S/A) can be described by the following deviation function:

$$G = az_1z_2 \quad (6)$$

were z_i is the relative amount of TU50 of chemical *i* in the mixture. i.e.:

$$z_i = \frac{TU50_i}{TU50_1 + TU50_2} \qquad (7)$$

This deviation function describes antagonism when parameter *a* is positive and synergism when *a* is negative, see table 3.3. If a = 0, then also G = 0 and there is no interaction between the two chemicals.

As previously done for the reference CA model, parameters for the S/A model, i.e. $EC50_1$, $EC50_2$, β_1, β_2 *max* and *a*, were estimated from the same data of the mixture experiments, using the “solver” facility of Microsoft Excel.

To verify if the S/A model provided a significantly better fit to the data than the reference CA model a likehood ratio test was performed. The test statistic was:

$$X^2 = n \cdot \ln\left(\frac{SS_{CA}}{SS_{S/A}}\right) \qquad (8)$$

Were *n* is the number of observations, SS_{CA} the sum of squared residuals from the reference CA model and $SS_{S/A}$ the sum of squared residuals from the S/A model. The statistic was compared to the χ^2 distribution with one degree of freedom, which is the distribution of X^2 if H_0: a = 0 is true (the number of degrees of freedoms is the difference in the number of parameters between the two models). Antagonistic or synergistic effect has been considered significant if $P < 0.05$.

The overall antagonistic or synergistic deviation can be made ratio dependent (DR) by including a second parameter in the deviation function, as follows:

$$G = (a + b_1 z_1) z_1 z_2 \qquad (9)$$

In this equation, the overall antagonistic or synergistic deviation changes with the proportion of chemical 1 in the mixture, where b_1 determines the magnitude of change.

Parameters for the DR model, i.e. $EC50_1$, $EC50_2$, β_1, β_2 *max a* and b_1, were estimated. The DR model was then compared to both the CA and the S/A model, by

two likelihood ratio tests, to verify if provided a significantly better fit to the data, similarly to the already described comparison between CA and S/A models.
To describe synergism and antagonism depending on the dose level, the basic deviation function (equation 6) is extended as:

$$G = a(1 - b_{DL}(TU50_1 + TU50_2))z_1 z_2 \quad (10)$$

In this equation, the value of *a* indicates the deviation at low total concentrations, while the value of b_{DL} indicates at what dose level the deviation changes (i.e., from antagonism to synergism or viceversa).
Similarly to the DR model, parameters of the DL model, including b_{DL} were estimated and the model was compared to the CA and S/A models by likelihood ratio tests. DR and DL models are not directly comparable to each other in this way, since they are not nested (i.e. neither is a special case of the other).

Table 3.3. Interpretation of additional parameters substituited into the Concentration Addition (CA) and Indipendent Action (IA) reference models that define the functional form of the deviation pattern.

Parameter	Value: CA	Value: IA	Meaning
		Synergism/antagonism	
a	>0	>0	Antagonism
	<0	<0	Synergism
		Dose ratio dependence	
a	>0	>0	Antagonism, except for those mixture ratios where significant negative b_is indicate synergism
	<0	<0	Synergism, except for those mixture ratios where significant positive b_is indicate antagonism
b_i	>0	>0	Antagonism where the toxicity of the mixture is caused mainly by toxicant *i*
	<0	<0	Synergism where the toxicity of the mixture is caused mainly by toxicant *i*
		Dose level dependence	
a	>0	>0	Antagonism low dose level and synergism high dose level
	<0	<0	Synergism low dose level and antagonism high dose level
b_{DL}	>1	>2	Change at lower dose level than the EC50
	=1	=2	Change at the EC50 level
	$0 < b_{DL} < 1$	$1 < b_{DL} < 2$	Change at higher dose level than the EC50
	<0	<1	No change, but the magnitude of synergism/antagonism is dose level (CA) or effect level (IA) dependent

[a] EC50 = median effect concentration.

This procedure is summary into the following diagram (Fig. 3.10) :

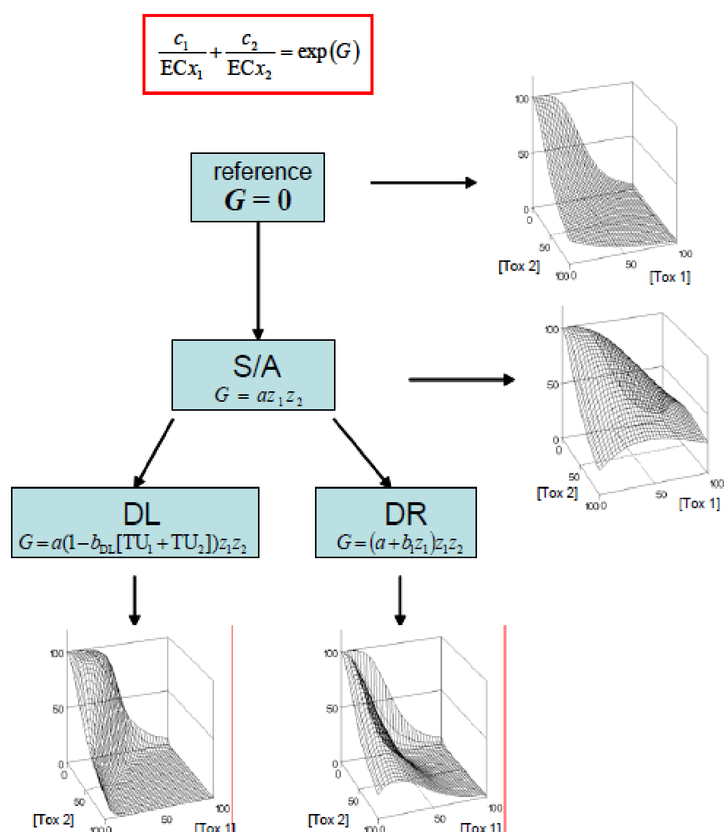


Fig. 3.10. Flowchart of binary mixture development from the CA model.

We used a Microsoft® Excel (Redmond, WA, USA) spreadsheet delineated by Jonker et al., distribuited in the NoMiracle-Project (version of spreadsheet setup, a manual, and an example data set are available at http://www.ceh.ac.uk/sections/er/csvendsen.html and the Solver Function (Newton Algorithm) in Microsoft® Excel which allowed to estimate the parameters in the different models. This phase consists of the application of the logistic model to the concentration-response data for the single pharmaceuticals (100% PRP – 0% FLX and 0% PRP – 100% FLX). As initial values of the parameters than those obtained from previous experiments on acute single pharmaceuticals exposure (paragraph 4.1.2) were used. Starting from these initial values, new estimates based on experimental data were calculated. These results are independent of the nature of the interaction between the two substances, since they are obtained separately for each of the two substances, in the absence of the other. The statistical significance of any improvement compared to the basic CA model was assessed using chi-square (λ^2) test as described by Jonker et al. (2005).

3.6.3 Biomarkers

Results from analysis were analyzed using the R statistical package (free resource at www.r-project.org). Significant differences between exposed and control samples were determined using a one-way ANOVA, statistical difference was accepted when $p < 0.05$. For malondialdehyde content the results firstly were log-transformed and, after ANOVA analysis, Dunnet test was carried out to understand which treatments are different from the control.

4. Results

4.1 Acute Immobilization Toxicity Test

4.1.1 Acute test on reference toxicant (potassium dichromate)

EC50 values obtained during the present study are 1.68, 1.67, 1.05, 1.42, 1.66, 1.43 and 1.66 mg/L, which indicate that the sensitivity of the organisms used, even with a certain degree of variability, remains within the range considered acceptable by the protocol. Taking as example the experiment with 1.42 mg/L EC50, the number of still active organisms in each beaker after 24 h of exposure as a function of the concentration of potassium dichromate is shown in Fig. 4.1. In the same Fig. the logistic curve parameterized on experimental data is also shown. Data are well fitted by the model and the experimental points are distributed over all regions of the fitted curve. In correspondence with the two lower tested concentrations no immobilized individuals were observed; from 1.93 mg/L the number of immobilized increases rapidly to reach 100% immobilization. The EC50 is estimated at 1.42 mg/L (95% confidence interval: 1.39 - 1.46). This value is within the range 0.60 to 1.70 mg/L defined as a validity criterion by ISO 6341 (1996).

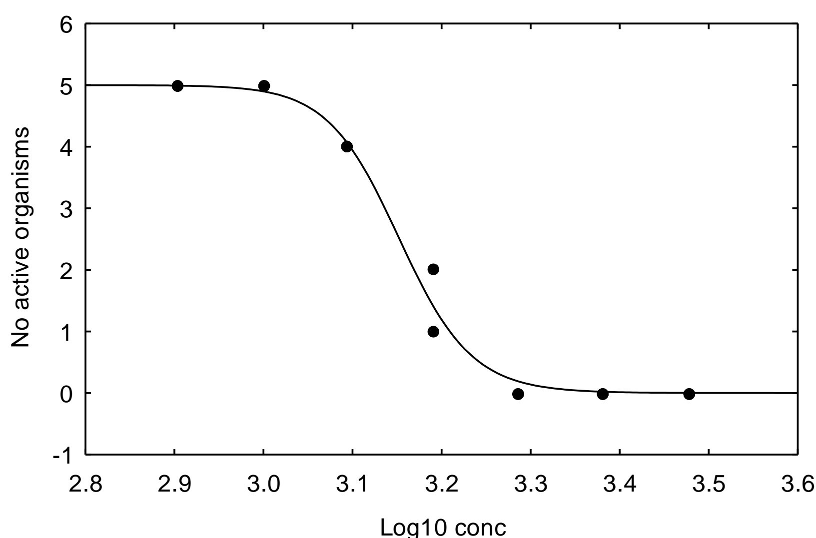
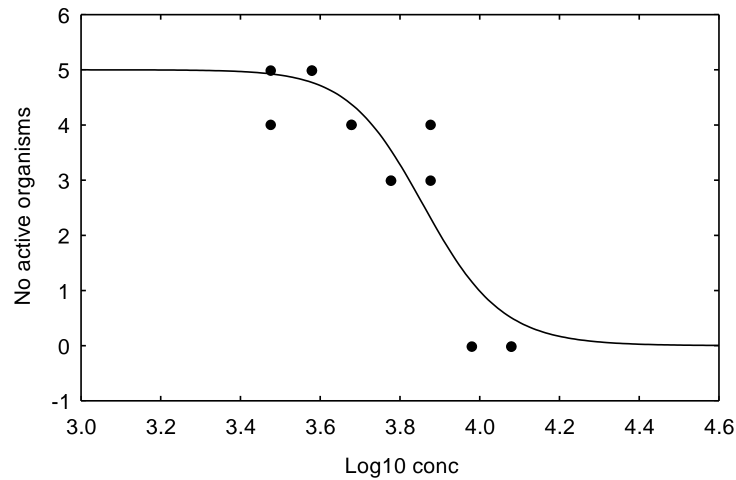


Fig. 4.1. Acute test with potassium dichromate: relationship between the number of active organisms in each beaker after a 24 hour exposure and the $\log_{10}$ of the concentration of the tested substance in µg/L. The dots represent the experimental data, while the continuous line represents the fitted logistic curve used to estimate the EC50.

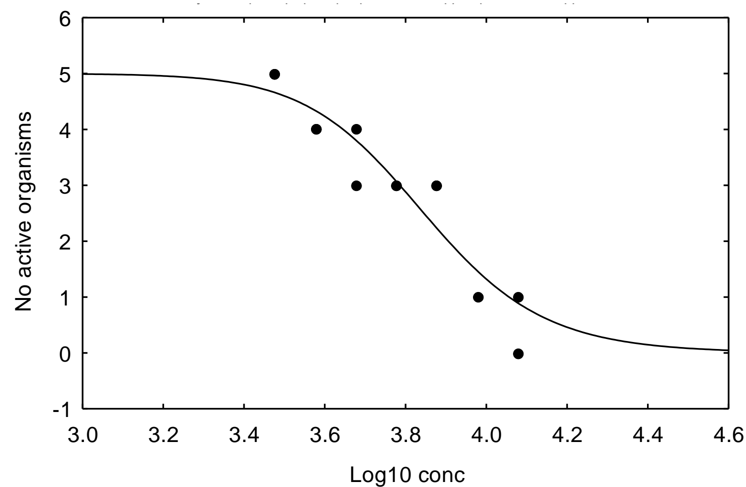
4.1.2 Acute single pharmaceutical exposure

Fig.s 4.2a, b, c; 4.3a, b, c; and 4.4a, b shown here below report the concentration-effect curves for the three substances in the three esperiments:

a)



b)



c)

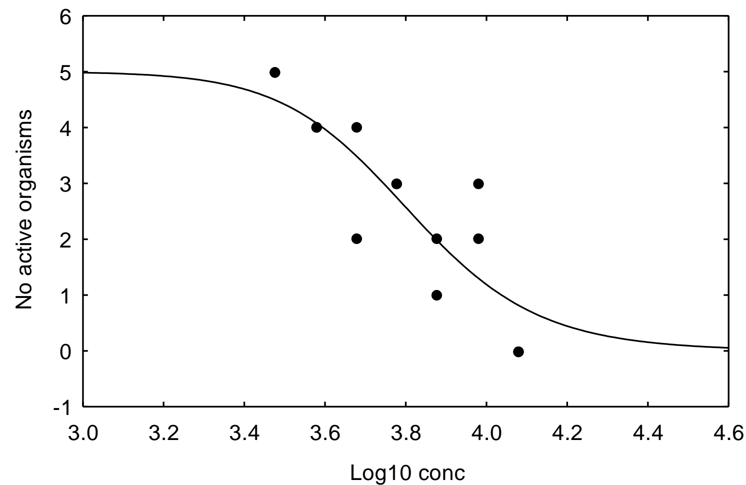
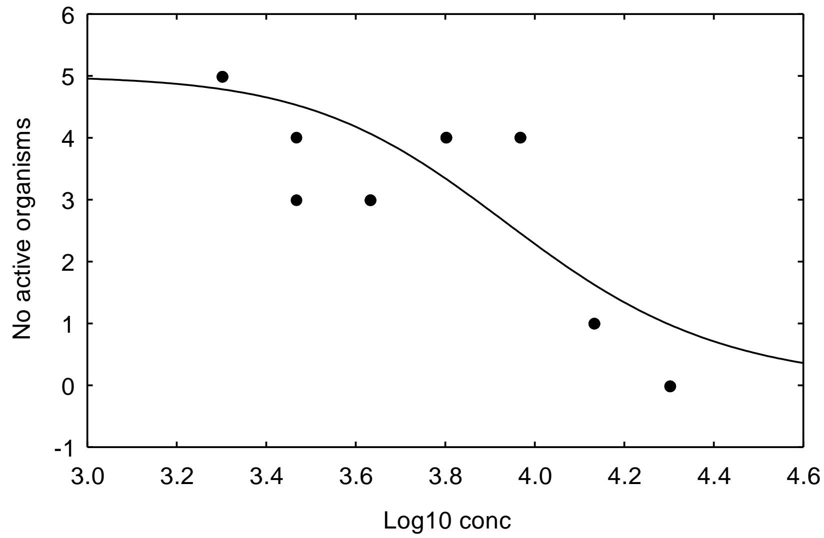
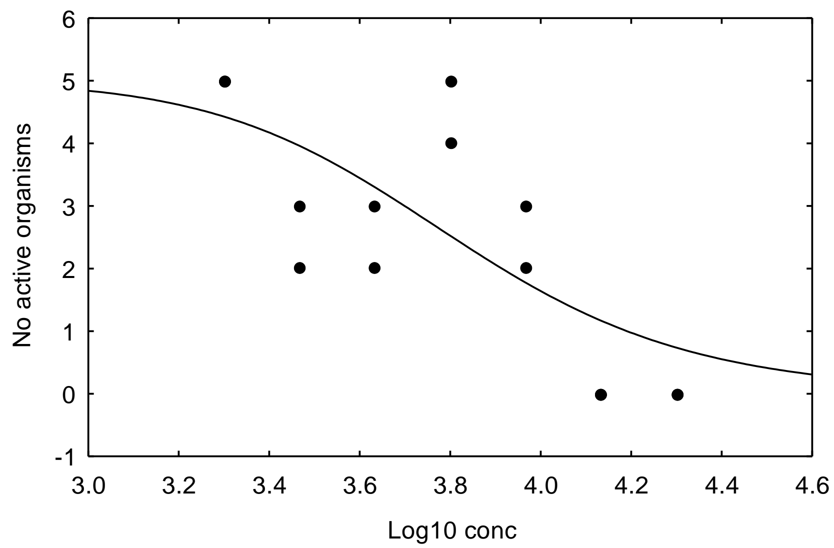


Fig. 4.2. First (a), second (b) and third (c) acute test with propranolol: relationship between the number of active organisms in each beaker after a 48 hour exposure and the log_{10} of the concentrations of the test substance in µg/L. The dots represent the experimental data while the continuous line represents the fitted logistic curve used to estimate the EC50.

a)



b)



c)

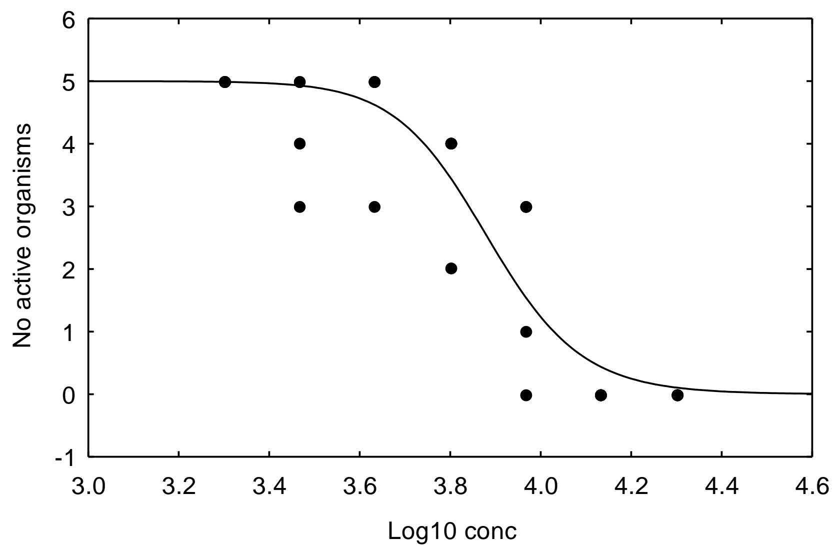
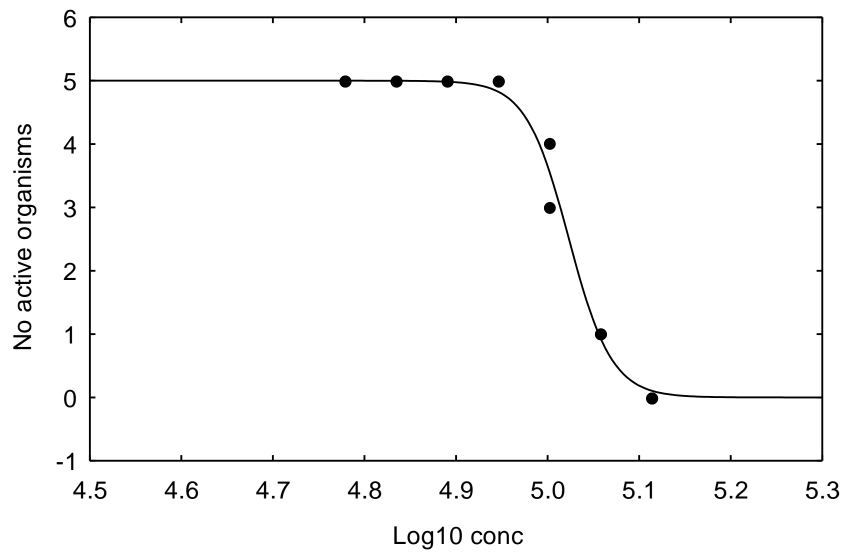


Fig. 4.3. First (a), second (b) and third (c) acute test with fluoxetine: relationship between the number of active organisms in each beaker after a 48 hour exposure and the $\log_{10}$ of the concentrations of the test substance in μg/L. The dots represent the experimental data while the continuous line represents the fitted logistic curve used to estimate the EC50.

a)



b)

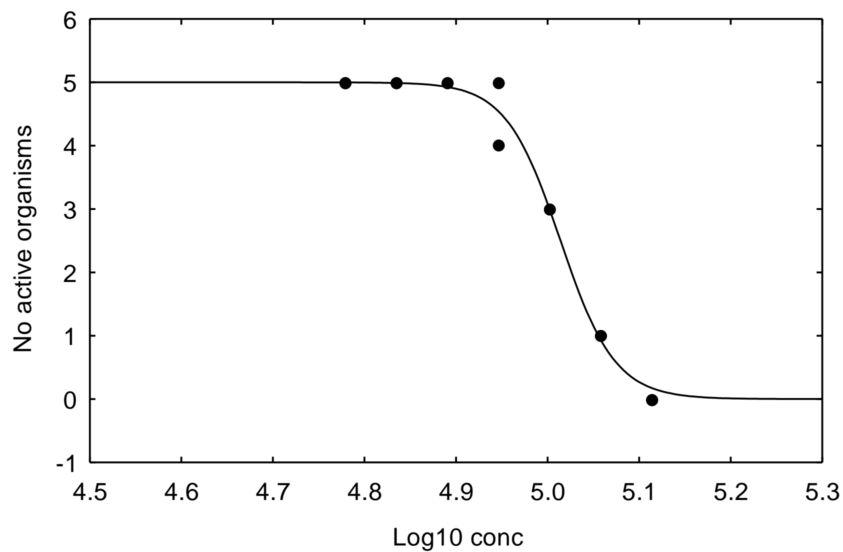


Fig. 4.4. First (a) and second (b) acute test with carbamazepine: relationship between the number of active organisms in each beaker after a 48 hour exposure and the $\log_{10}$ of the concentrations of the test substance in μg/L. The dots represent the experimental data while the continuous line represents the fitted logistic curve used to estimate the EC50.

Clear dose-response relationships were observed in all single-chemical experiments: the number of active (non immobilized) organisms decreased with the increse of chemical concentration. EC50 values obtained from each single-chemical experiment are reported in Table 4.1.

Table 4.1. Median effective concentration (EC50) values calculated for the single pharmaceuticals in the single-chemical acute exposure toxicity tests with *D. magna*. Confidence Limits 95% (CL) are shown in parentheses.

	Substances		
	Propranolol (mg/L) EC50 (CL)	**Fluoxetine (mg/L) EC50 (CL)**	**Carbamazepine (mg/L) EC50 (CL)**
1st experiment	7.3 (6.4 - 8.3)	9.1 (6.3 - 13.2)	105.5 (104.0 - 107.1)
2nd experiment	7.0 (6.1 - 8.1)	6.4 (3.9 - 10.5)	103.3 (101.7 – 105.0)
3rd experiment	6.5 (5.3 - 7.8)	7.6 (6.7 - 8.7)	

4.1.3 Acute mixture exposure

The number of active organisms after the 48 h exposure as a function of total toxic units (∑TU50), and of the percentage of propranolol and fluoxetine in the mixtures, are presented in Fig. 4.5.

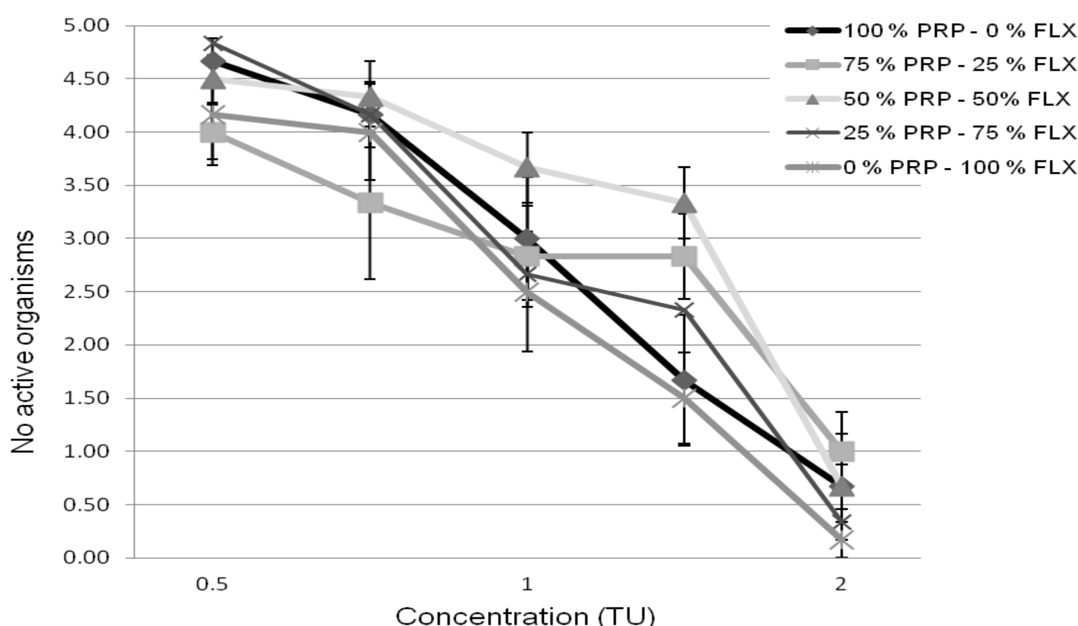


Fig. 4.5. Number of active organisms as a function of the concentration of the mixtures expressed as total toxic units (∑TU50). Each point represents the average of six experimental replicates. The different lines represent different percentages of propranolol and fluoxetine in the mixtures.

This graph shows how the lines representing the concentration-response relationship for the single pharmaceuticals (100% and 0% propranolol) are below the lines that represent the concentration-response relationship for the mixtures. This means that, for a given concentration in terms of ∑TU50, the number of active organisms is higher for mixtures than for the two single substances. In other words, for the same ∑TU50, the mixtures appear to be less toxic than the single pharmaceuticals.

Table 4.2 shows the results of the first phase of the data analysis performed using the implementation of MixTox in Excel and parameter of the logistic models are shown. The value of SSres for fluoxetine is much lower than that for propanolol, which means that the experimental data for fluoxetine are better adapted to the logistic model.

Table 0.2. Application of MixTox to the chronic test of mixtures of propranolol and fluoxetine. Results for the two single substances: the parameters of the logistic model estimated using a nonlinear regression, implemented using the Solver function in Excel. The max parameter corresponds to the total number of organisms exposed at each concentration and to the number of mobile organisms in the control treatment.

	max	β	EC50 (mg/L)	SS_{res}
Propranolol	30	3.59	8.19	25.98
Fluoxetine	30	3.37	6.10	2.22

Afterwards, using the experimental data relative to the single substances and to all the mixtures, parameters for the concentration addition (CA) reference model,, synergism or antagonism (S/A), dose ratio dependent (DR) and dose level dependent (DL), have been estimated (Table 4.3). The parameters values for the single substances (Table 4.2) were used as initial values for the iterative estimation of the CA model. For the other models, the values obtained for the immediately simpler model were used as initial values: parameters from the CA model were used for the S/A model and, on their turn, parameters from the S/A model were used for the DR and the DL models.

Table 4.3. MixTox output for the effects of mixtures of propranolol plus fluoxetine on the number of active organisms after a 48 h exposure. The parameters for the alternative models and the test for detecting of the model which best fits the experimental data are reported

	models					
	CA	**S/A**	**DR**		**DL**	
Max	30	30	30		30	
β PRP	2.82	3.05	3.06		2.95	
β FLX	2.90	3.20	3.20		3.07	
EC50 PRP	9.47	7.81	8.14		7.79	
EC50 FLX	7.73	6.51	6.17		6.49	
a		1.55	2.72		1.99	
b			-2.44		0.14	
SS_{res}	379	213	193		212	
χ^2 test			**CA vs DR**	**S/A vs DR**	**CA vs DL**	**S/A vs DL**
N= 26						
df		1	2	1	2	1
χ^2		14.99	17.56	2.57	15.13	0.14
P		0.0001	0.0002	0.1092	0.0005	0.7062
Significance		***	***	N.S.	***	N.S.

Table 4.3 shows the values returned by the application of MixTox for each model investigated. The SS_{res} value decreases from the CA to the S/A model. SS_{res} is further reduced also from S/A to DR and slightly from S/A to DL. Furthermore, the χ^2 test indicates that the S/A model fits the data significantly better than the CA model. Moreover the χ^2 test indicates that, for both DR and DL that, while the improvement compared to the CA model is significant, the improvement with respect to S/A is not significant. Therefore, the application of MixTox to experimental data indicates that the model that best represents the data is the synergism/antagonism (S/A). Since the estimate for the *a* parameter is positive (*a* > 0), fluoxetine and propranolol act as antagonistic substances on *D. magna* immobilization (see Table 3.3).

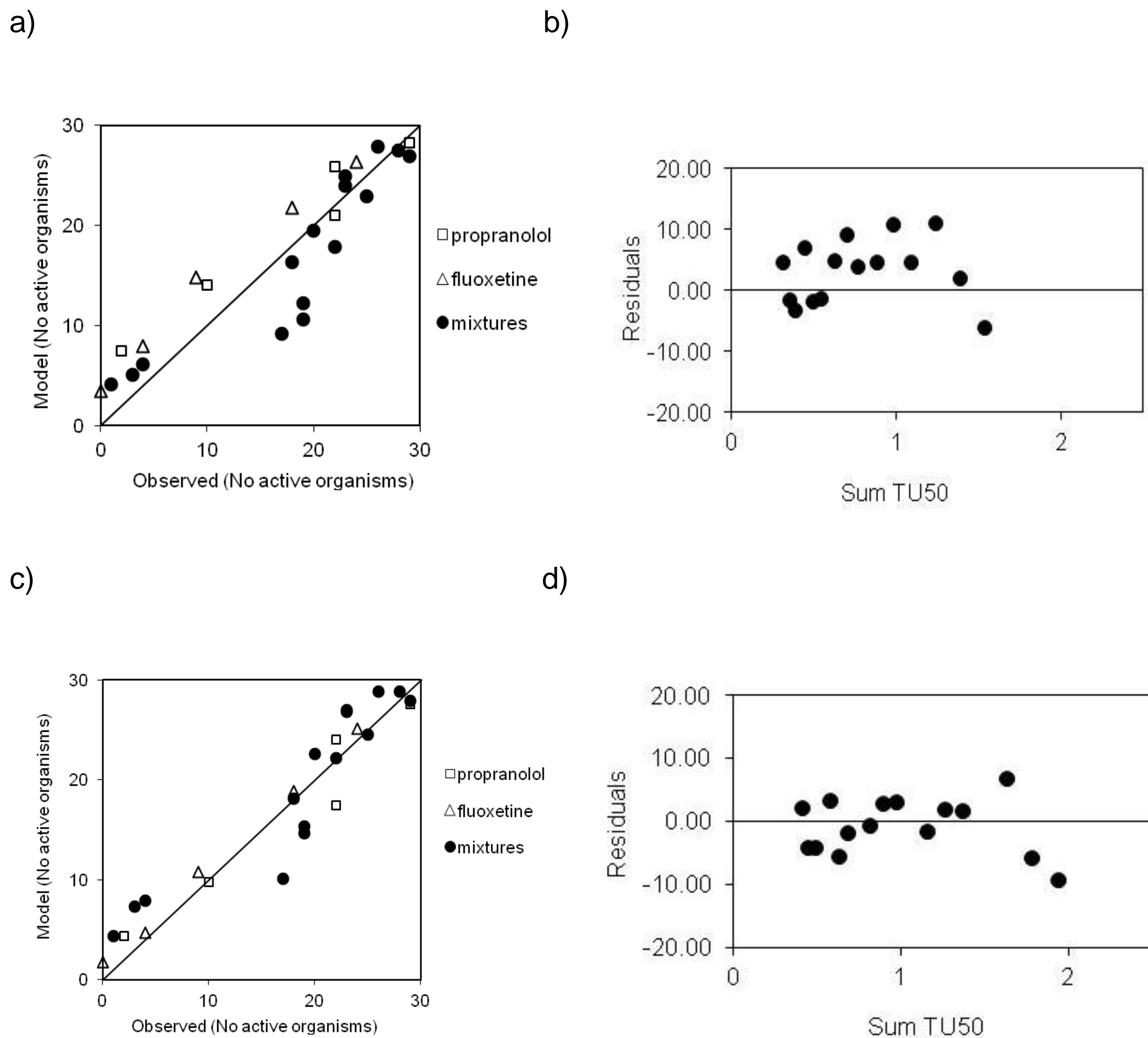


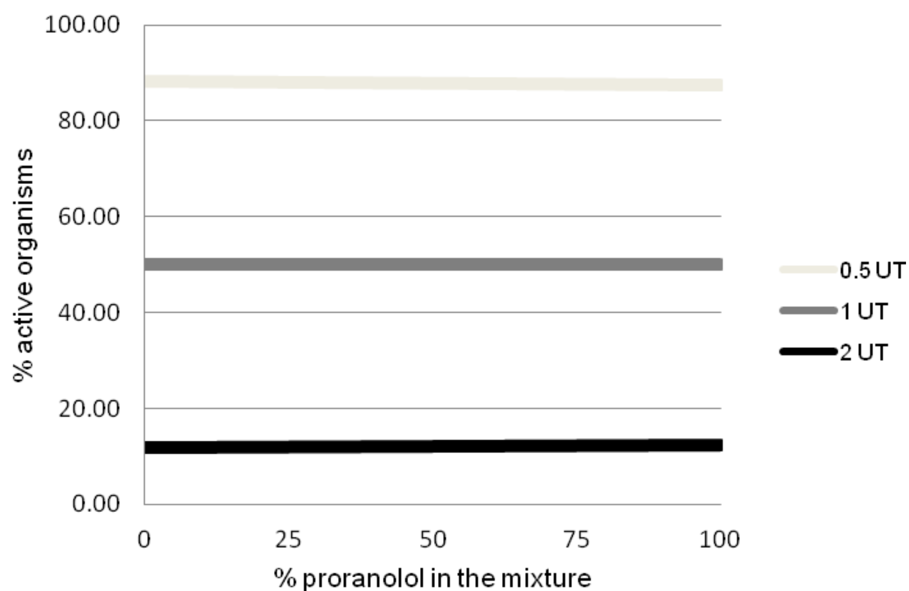
Fig. 4.5. Predicted number of active organisms as a function of the observed number of active organisms for: a) CA model, c) S/A model. The line that cuts diagonally across the graphs indicates perfect agreement between model predictions and experimental observations. Residuals, i.e. differences beween observed and predicted numbers of newborns, for: b) CA model, d) S/A model.

Fig. 4.5 is a graphical comparison of the performances of the reference CA model and the S/A model. Fig. 4.5a shows the number of active organisms predicted by CA, as a function of the observed number of active organisms. Fig. 4.5c shows the same for S/A. In Fig. 4.5c most of the points representing the model's predictions for individual pharmaceuticals are above the line, which means that the number of newborns is overestimated for single pharmaceuticals and underestimated for mixtures. In the S/A model, there is still this systematic aspect, but individual substances and mixture points are better distributed. In fact in general, all points and especially those related to the single substances tend to get closer to the line which

represents the perfect agreement between experimental observations and model predictions. This means that there is a clear improvement in the fit to the experimental data when the S/A model is used, in comparison to the CA model, as also indicated by the decrease in the SS_{res} values. This latter aspect is clear from Fig. 4.10b, d, were residuals, i.e. differences between observed and predicted active organisms, are represented as a function of ∑TU50 (mixtures data only).

To illustrate the differences between the two models, Fig.s 4.6a and 4.11b show, respectively for C/A and SA, the predicted percentage of active organisms as a function of the percentage of propranolol in the mixture and of ∑TU50, for CA and S/A respectively. In particular, the lines representing the number of newborns as a function of the percentage of propranolol are concave curves, for the S/A model. Concavity is more pronounced for mixtures with ∑TU50 = 1 and more flattened for mixtures with higher or lower concentrations. This indicates that, for the same value of ∑TU50, mixtures with different percentages of the two pharmaceuticals have different effects. The single substances cause a greater reduction in the number of newborns compared to mixtures in which both pharmaceuticals are present. In particular for mixtures 1 UT, the maximum of the curve is exactly at 50 % propranolol (i.e. 50% fluoxetine), indicating for this combination, the lowest reduction in the number of newborns compared to the control. For ∑TU50 = 1, the number of active organisms at 100% propranolol and at 0% propranolol (i.e. 100% fluoxetine) is about 50 % both for CA and S/A. This value is exactly half the value of the max parameter in percentage, which means 15 out of 30 active organisms. This was expected, since, independently from the interactions between two chemicals, 1 TU of one of them, in the absence of the other corresponds to the EC50 and, as such, causes a 50% effect, in this case a 50% immobilization.

a)



b)

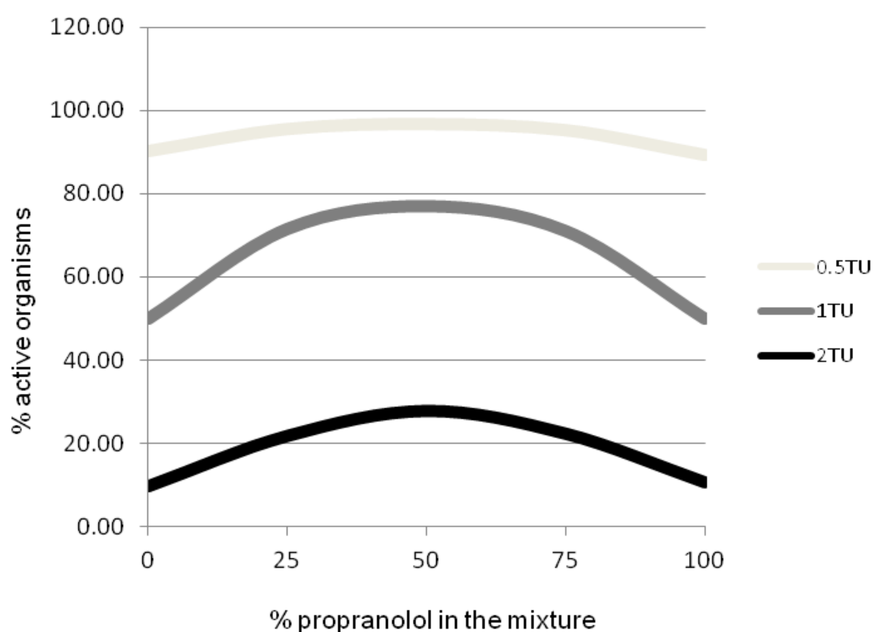


Fig. 4.6. Prediction of the CA (a) and S/A (b) models fitted to the experimental data: percentage of active organisms as a function of the percentage of propranolol present in the mixture (the percentage of fluoxetine is the complement to 100 %). The lines of different colors represent different total concentrations of the mixture (∑TU50).

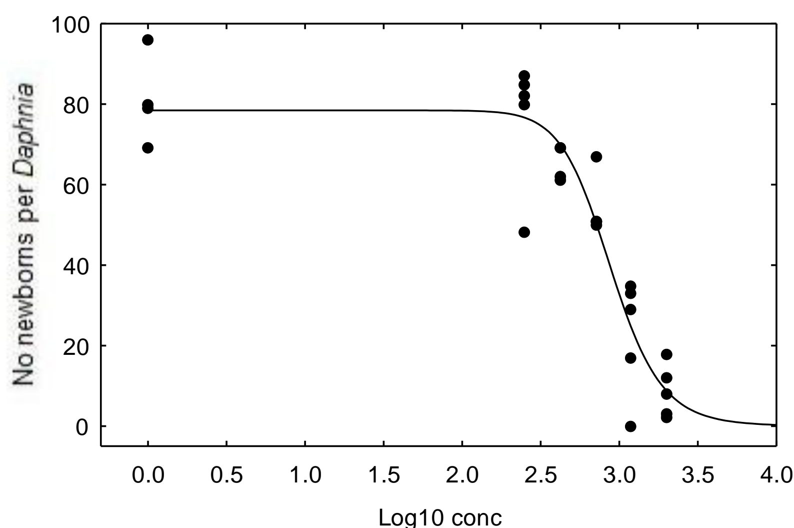
4.2 21-d Chronic Reproduction Toxicity Test

4.2.1 Chronic single pharmaceuticals exposure

The number of newborns produced by each parent organism over 21 days of exposure as a function of the concentration of propranolol for the two experiments are shown in Fig. 4.7a, b. In the same Fig.s the logistic curves fitted to the experimental data are also shown. Although in the first test the slope of the curve is higher, in both tests here is a similar reduction of the average number of births for *Daphnia* while pharmaceutical concentration increase.

Both tests are considered valid as fulfilling the conditions defined by the OECD guidelines 211 (2008) and ISO 10706 (2000) (chapter 3.4).

a)



b)

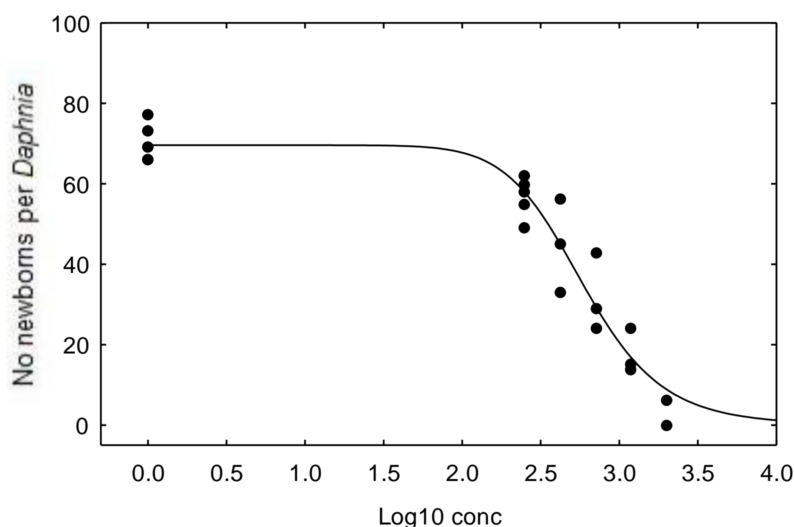
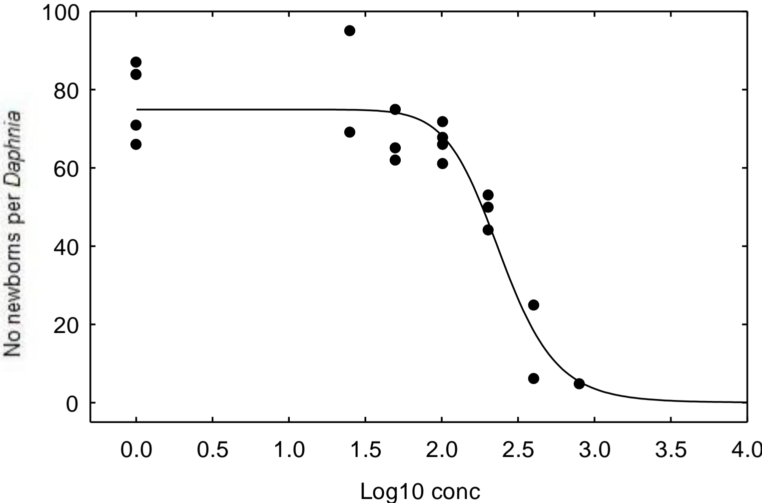


Fig. 4.7. First (a) and second (b) chronic test with propranolol: relationship between the number of newborns produced by each parent organisms over 21 d exposure and the $\log_{10}$ of the concentrations of the test substance in µg/L. The dots represent the experimental data while the continuous line represents the fitted logistic curve used to estimate the EC50.

The number of newborns produced by each parent organism over 21 days of exposure as a function of the concentration of fluoxetine is shown in Fig. 4.8a, b. In the same figure the logistic curve fitted on experimental data is also shown.

As observed from the figures, the number of newborns, differently from the test with propranolol, decreases with increasing concentration of fluoxetine only at the highest tested concentrations. A slightly higher number of newborns was observed in the presence of a low concentration of fluoxetine (0.03 mg/L) compared to the control. These differences, however, are not statistically significant when evaluated using the Dunnet's test. In both tests, the EC50 calculated shows similar values and in general, the curves of the two experiments are similar with a slightly different slope.

a)



b)

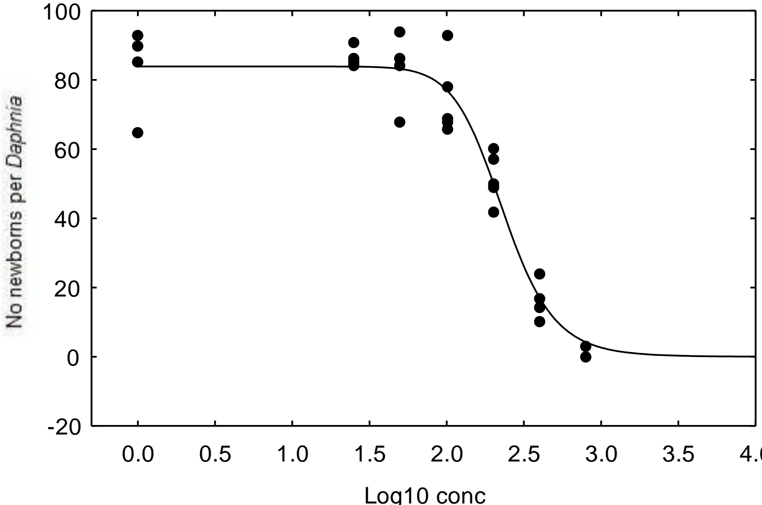


Fig. 4.8. First (a) and second (b) chronic test with fluoxetine: relationship between the number of newborns produced by each parent organisms over 21 d exposure and the log_{10} of the concentrations of the test substance in μg/L. The dots represent the experimental data while the continuous line represents the fitted logistic curve used to estimate the EC50.

The EC50 values for the number of newborns produced by each parent organism are shown in Table 4.4. EC50 of fluoxetine is four times lower than that of propranolol, i.e. 0.74 and 0.24 mg/L for propranolol and fluoxetine, respectively. The average EC50 have been used for the calculation of concentrations expressed as total units toxic for the mixture tests, while the values of the slope of the curve and the highest value observed (top) have been used as starting values for the MixTox analysis.

Table 4.4. Median effective concentration (EC50) values calculated for the single pharmaceuticals in the single chronic exposures toxicity tests with *D. magna*. Confidence Limits 95% (CL) are shown in parentheses.

	Propranolol (mg/L) EC50 (CL)	**Fluoxetine (mg/L) EC50 (CL)**
1st experiment	0.883 (0.731-1.067)	0.245 (0.193-0.309)
2nd experiment	0.592 (0.480-0.729)	0.232 (0.203-0.266)

4.2.2 Chronic mixture exposure

In the chronic test with mixtures of propranolol plus fluoxetine *Daphnia* are exposed for 21 days to different concentration proportions expressed in toxic unit of the two pharmaceuticals. In Fig. 4.9 are presented the results of experimental tests in terms of average number of newborns produced by a single *Daphnia* as a function of the concentration of mixtures expressed in total toxic units, and the percentage of propranolol in the mixtures.

This graph shows how the lines representing the relationship concentration-response for the two single pharmaceuticals (100% and 0% propranolol) are below the lines that represent the relationship concentration-response of the mixtures. This means that, for a given concentration in terms of toxic units, the number of newborns is higher for mixtures than for the two single substances. In other words, always at the same concentration, the mixtures appear to be less toxic than the pharmaceuticals alone.

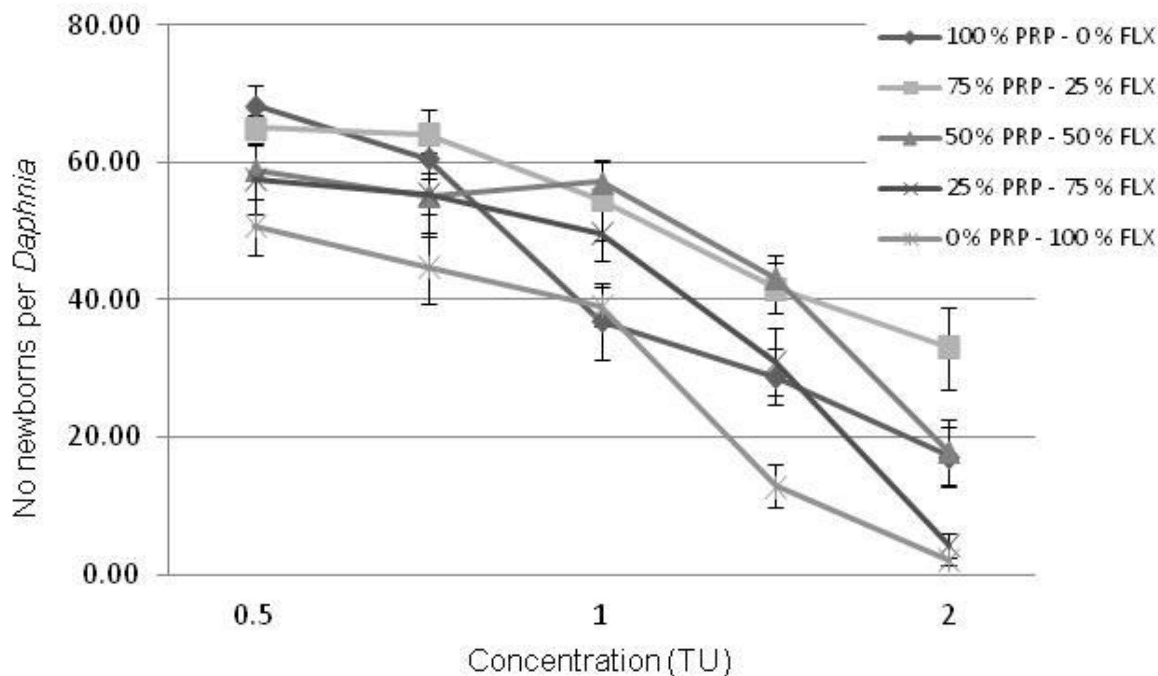


Fig. 4.9. Number of offspring per individual *Daphnia* as a function of the concentration of the mixtures expressed in total toxic units (∑TU50). Each point represents the average of six experimental replicates. The different lines represent different percentages of propranolol and fluoxetine in the mixtures.

Table 4.5 shows the results of data analysis through the implementation of MixTox in Excel. This phase consists of the application of the logistic model to data related only to a single pharmaceutical, either propranolol or fluoxetine (100% PRP and 100% FLX). As initial values of the parameters were used those obtained from previous experiments on chronic single pharmaceuticals exposure (section 4.2.1). Starting from these initial values, new estimates based on experimental data were calculated by the Solver function with iterative methods. These results are independent of the nature of the interaction between the two substances, since they are obtained separately for each of the two substances, in the absence of the other one.

Table 4.5. Application of MixTox to the chronic test of mixtures of propranolol plus fluoxetine. Results for the two single substances: the parameters of the logistic model estimated using nonlinear regression, implemented using the Solver function in Excel.

	Max (No newborns per *Daphnia*)	β	EC50 (mg/L)	SS_{res}
Propranolol	68.74	2.78	0.909	100.04
Fluoxetine	68.74	2.60	0.216	163.74

Afterwards, using the experimental data relative to individual substances and to mixtures, parameters (explained in section 3.6.2) for the CA reference model, S/A, DR and DL models have been estimated. For the CA model the parameters related to individual substances were used as initial values for the iterative process of estimation (Table 4.6). For further models, values previously obtained for the model immediately easier were used as initial values, as previously explained (section 4.1.3).

Table 4.6. MixTox output for the effects of mixtures of propranolol plus fluoxetine on the reproduction of *Daphnia magna* (number of neonates produced by a female after 21 d exposure). The parameters for the alternative models and the test for the detection of the model that best fit the experimental data are reported.

	models					
	CA	**S/A**	**DR**		**DL**	
Max	62.51	63.96	63.61		63.43	
β PRP	2.44	3.07	3.10		2.84	
β FLX	4.25	3.40	3.51		3.15	
EC50 PRP	1.354	1.002	0.978		1.016	
EC50 FLX	0.283	0.228	0.238		0.228	
a		1.70	0.91		3.03	
b			1.59		0.31	
SS_{res}	1443	607	570		575	
χ^2 Test		**CA vs S/A**	**CA vs DR**	**S/A vs DR**	**CA vs DL**	**S/A vs DL**
N= 26						
df		1	2	1	2	1
χ^2 value		22.52	24.15	1.64	23.91	1.39
P		0.000	0.000	0.200	0.000	0.238
Significance		***	***	N.S.	***	N.S.

Table 4.6 shows the values returned by the application of MixTox for each model investigated. A further decrease in the value of SSres from CA to S/A application can be observed. The value of SSres is reduced also from S/A to both DR and DL application. Furthermore, the λ^2 test indicates that the model S/A fits the data significantly better than the CA model, for both DR and for DL the λ^2 test indicates

that while the improvement compared to the CA model is significant, the improvement with respect to S/A is not significant. Therefore, the application of MixTox to experimental data indicates that the model that best represents the data is the synergism/antagonism (S/A). Compared to Table 3.3 of section 3.6.2 it is possible to determine the value of the parameters a, which is greater than zero, indicating that in the mixture the two pharmaceuticals have an antagonism behavior.

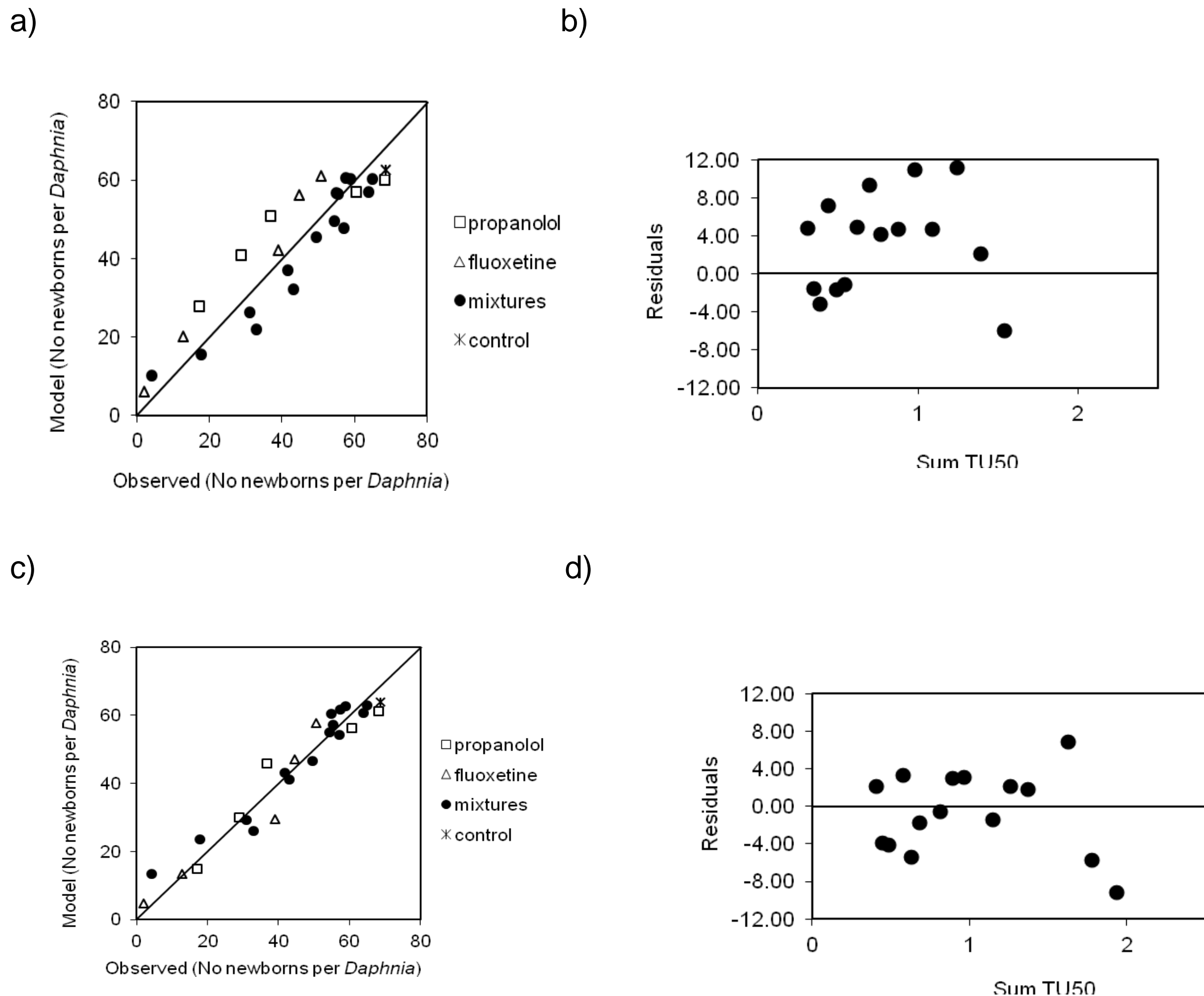


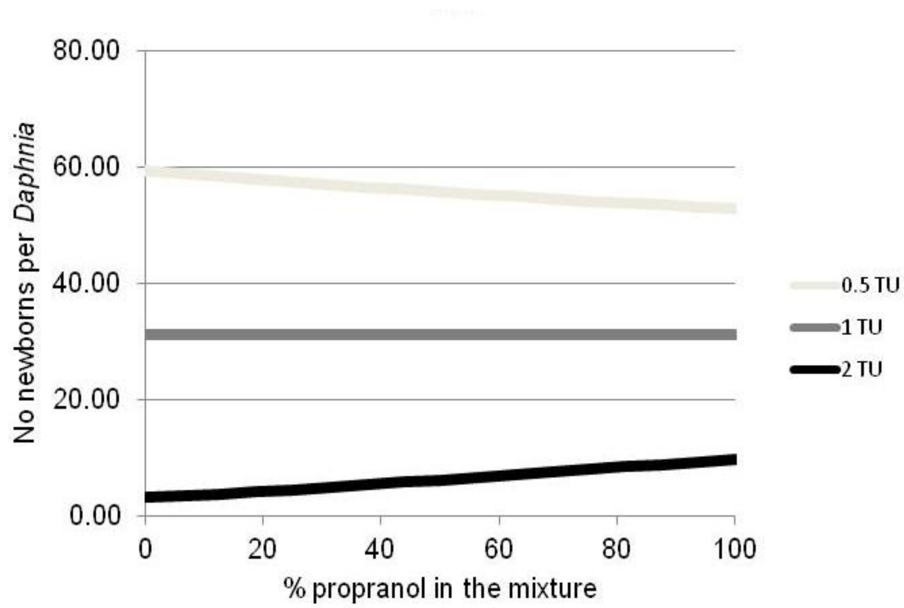
Fig. 4.10. Predicted number of newborns per *Daphnia* as a function of the observed number of newborns for: a) CA model, c) S/A model. The line that cuts diagonally across the graphs indicates perfect agreement between model predictions and experimental observations. Residuals, i.e. differences beween observed and predicted numbers of newborns, for: b) CA model, d) S/A model.

Fig. 4.10 is a graphical comparison of the performances of the reference CA model and the S/A model. Fig. 4.10a shows the number of newborns predicted by CA, as a function of the observed number of newborns. Fig. 4.10c shows the same for S/A. There is a clear improvement in the fit to the experimental data when the S/A model is used, in comparison to the CA model: the points tend generally to get closer to the

line which represents the perfect agreement between experimental observations and model predictions, as already indicated by the decrease in the SS_{res} values. Furthermore, in figure a, most of the points that represent the model's predictions for mixtures are below the line that indicates the perfect agreement, while most of the points representing the model's predictions for individual pharmaceuticals are above the line. So it is clear that the number of newborns is overestimated for single pharmaceuticals and underestimated for mixtures. In the S/A model, this systematic aspect is absent and individual substances and mixture points are better distributed around the line. This aspect is clear also from Fig. 4.10b, d, were residuals, i.e. differences between observed and predicted number of newborns, are represented as a function of ∑TU50 (mixtures data only).

To illustrate the differences between the two models, Fig. 4.11a and 4.11b show, respectively for C/A and SA, the predicted number of newborns per parent organism as a function of the percentage of propranolol in the mixture and of ∑TU50, for CA and S/A, respectively. In particular, the lines representing the number of newborns as a function of the percentage of propranolol are concave curves, for the S/A model. Concavity is more pronounced for mixtures with ∑TU50 = 1and more flattened for mixtures with higher or lower concentrations of propranolol. This indicates that, for the same value of ∑TU50, mixtures with different percentages of the two pharmaceuticals have different effects. The single substances cause a greater reduction in the number of newborns compared to mixtures in which both pharmaceuticals are present. In particular for mixtures 1 UT, the maximum of the curve is exactly at 50 % propranolol, indicating for this combination, the lowest reduction in the number of newborns compared to the control For ∑TU50 = 1, the number of newborns for parent organism at 100% propranolol and at 0% propranolol (i.e. 100% fluoxetine) is about 30 both for CA and S/A. This value is exactly half the value of the max parameter. This was expected, since, independently from the interactions between two chemicals, 1 TU of one of them, in the absence of the other corresponds to the EC50 and, as such, causes a 50% effect, in this case a 50% inhibition of reproduction.

a)



b)

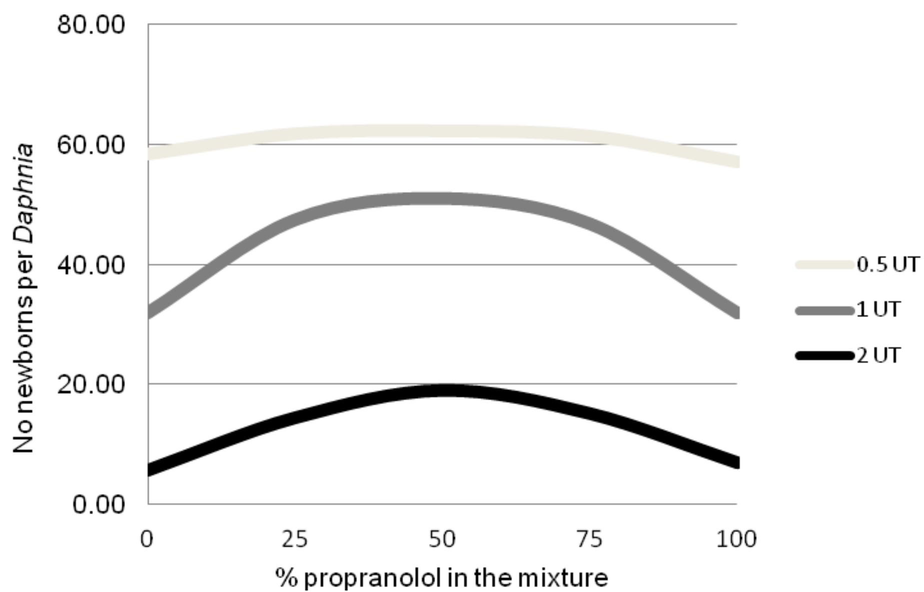


Fig. 4.11. Prediction of the CA (a) and S/A (b) models fitted to the experimental data: average number of newborns per individual as a function of the percentage of propranolol present in the mixture (the percentage of fluoxetine is the complement to 100 %). The lines of different colors represent different total concentrations of the mixture (∑TU50).

4.3 Battery of biomarkers

4.3.1 Acetylcholinesterase activity

Daphnia AChE activitiy in control animals was 1.26 nmol/min/mg protein. The enzyme activity, in *Daphnia* exposed to 0.2, 1.0 and 5.0 mg/L fluoxetine was not significantly different from controls (p>0.5) (Fig. 4.12), suggesting no neuroactive effects.

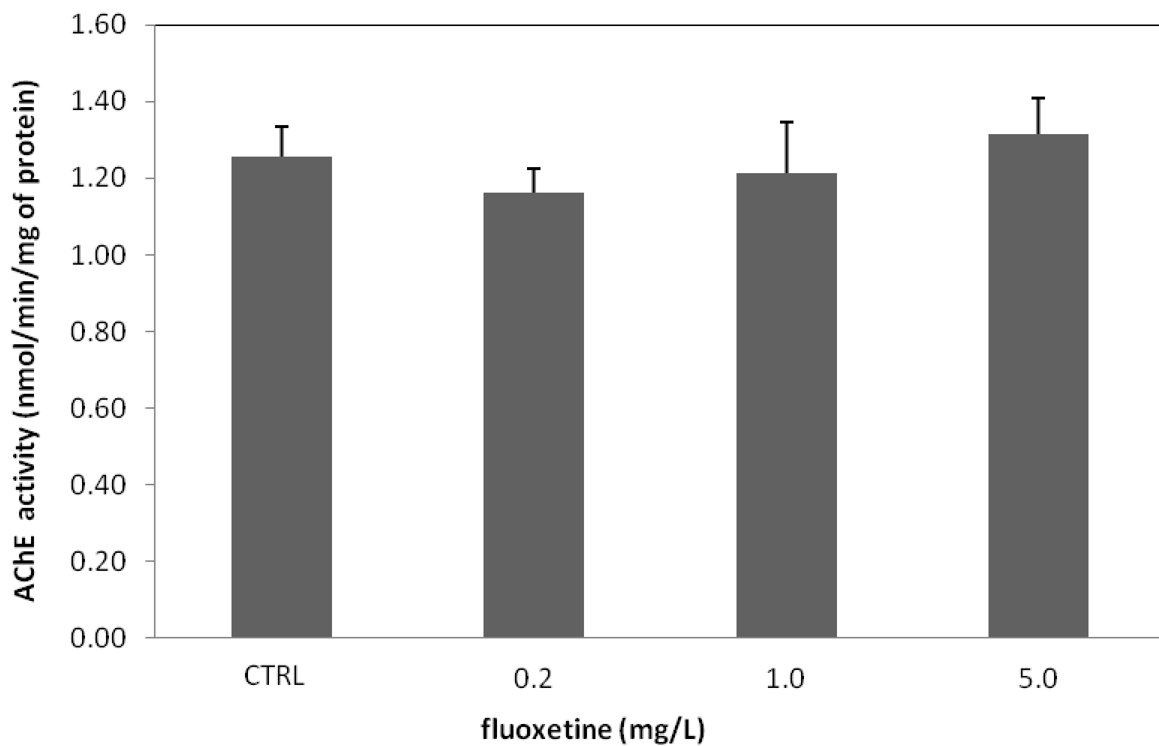


Fig. 4.12. AChE activity in *Daphnia* exposed to fluoxetine.

4.3.2 Malondialdehyde assay

The effect of fluoxetine on *Daphnia* MDA content is illustrated in Fig. 4.13. The MDA content was about 0.530 nmol/mg of protein for control organisms; ANOVA analysis indicates that there are significant differences between treatments ($p < 0.01$). Dunnet's test indicates that 0.04 mg/L of chromium(VI) is the only treatment significantly different from control with $p < 0.01$. MDA content, reflecting oxidative stress, was not change in *Daphnia* exposed to fluoxetine, while a three-fold increase in MDA was found in *organisms* exposed to chromium(VI) which is a known oxidative chemical.

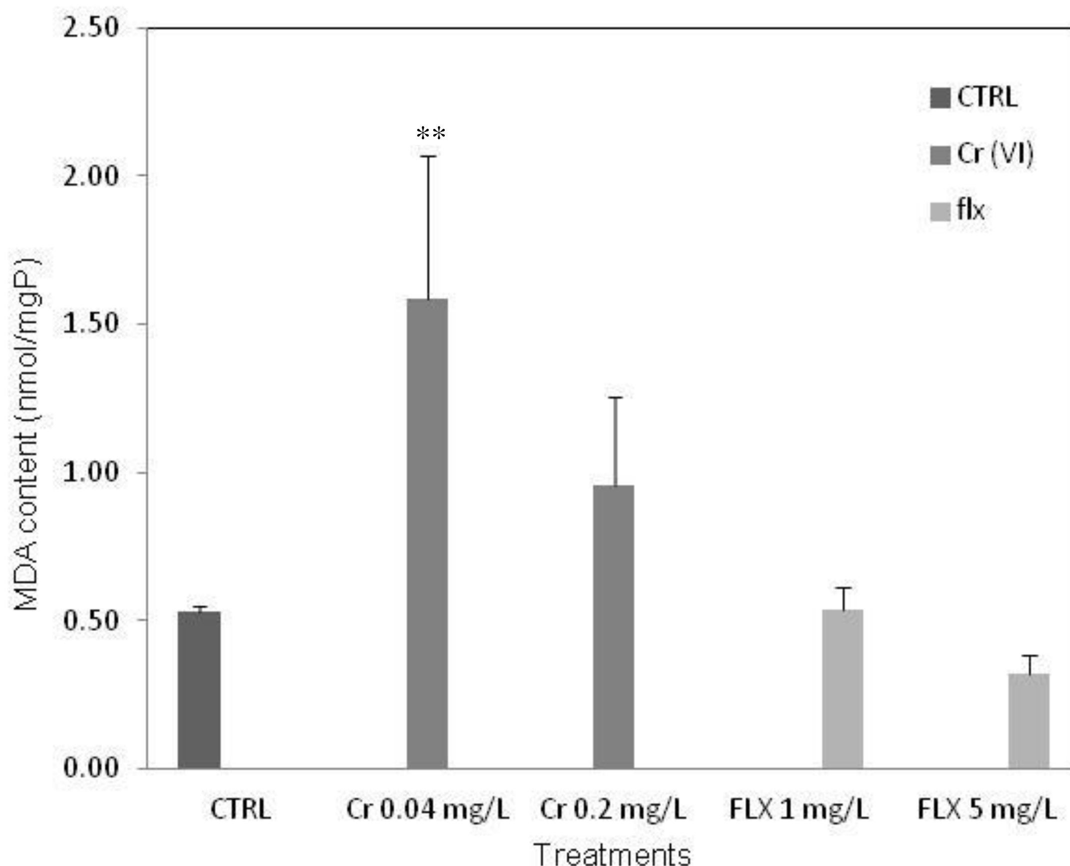


Fig. 4.13. MDA content in *Daphnia* exposed to fluoxetine and chromium(VI). Asterisk indicates a value significantly different from control: ** p < 0.01.

4.3.3 Glutathione S-transferase activity

GST activity in the control treatment was about 134 nmol/min/mg protein. Slightly higher GST activities, which would represent an activation of detoxification reactions occurring after pharmaceutical exposure, were observed in *Daphnia* exposed to 1.0 and 5.0 mg/L fluoxetine (Fig. 4.14), although the differences from the control were not significant.

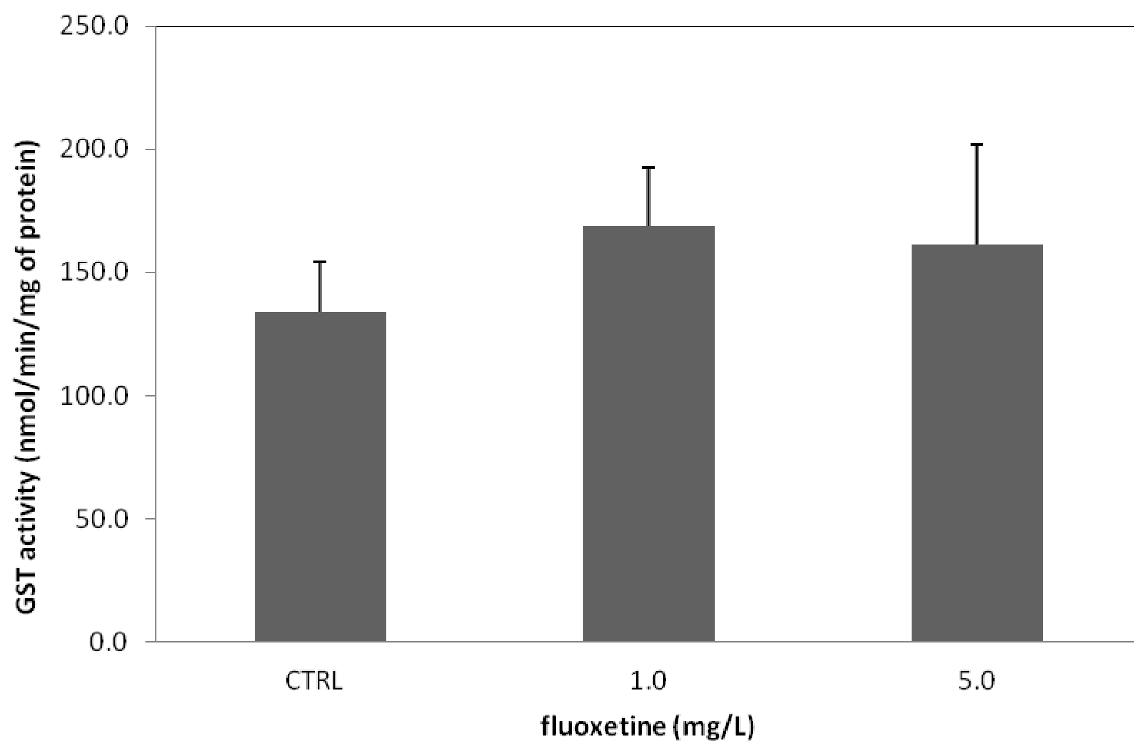


Fig. 4.14. GST activity in Daphnia exposed to fluoxetine.

5. Discussion

5.1 Single Toxicants Exposure

5.1.1 24-h acute immobilization toxicity test with reference toxicant (potassium dichromate)

Repeatedly, throughout this study, the sensitivity of *Daphnia magna* was assessed by 24 h immobilization tests with the reference substance potassium dichromate.
To satisfy the validity criteria dictated by ISO (2012), EC50 values should fall in the range 0.6 mg/L to 2.1 mg/L. EC50 values estimated throughout the study ranged from 1.05 to 1.68 mg/L indicating that the sensitivity of the organisms used, even with a certain degree of variability, remained within the range considered acceptable.
In our experience, the chemical composition of the dilution water has a strong influence on potassium dichromate toxicity. In fact, in experiments previously carried out in our laboratory, according to the same procedures and using the same exposure conditions except that Guizza water (see section 3.1.1) was adopted as the test medium, all the estimates EC50 (0.54 mg/L, 0.61 mg/L, 0.50 mg/L) were below or just slightly above the lower limit set by the protocol.

5.1.2 48-h acute immobilization toxicity test on pharmaceuticals

Acute tests with single pharmaceuticals were carried out in order to identify the EC50 values. This information for propranolol and fluoxetine was used to calculate concentrations in terms of toxic units and as the basis to properly design the acute mixtures exposure experiment.
The three experiments with propranolol alone provided EC50s in the range 6.5 to 7.3 mg/L. Taking into consideration also the single pharmaceutical EC50 values estimated in the mixture tests when propranolol was added as single pharmaceutical, the values ranged from 6.5 to 9.5 mg/L.
Stanley et al. (2006) found an EC50 of 1.67 mg/L for *D. magna* exposed to propranolol for 48hours, which is roughly equivalent to the values 1.6 and 2.75 mg/L reported by Huggett et al. (2002) and Ferrari et al. (2004), respectively. These values

are lower than those found in the present study and this may be due to different procedures and different media used during the tests.

Regarding fluoxetine, the EC50 values provided by the experiments, considering also the single pharmaceutical EC50 found in the mixture tests, ranged from 7.6 to 9.1 mg/L.

Brooks et al. (2003) found a LC50 value (50% Lethal Concentration) of 0.820 mg/L for *D. magna* exposed to fluoxetine for 48 hours. Also in this case, the difference in comparison with this study might be explained by the use of a different reconstituted hard water (US EPA, 1991). Our hypothesis is that the substances can react in different way with the dissolved salts as already explained in section 5.1.1.

Acute toxicities of propranolol and fluoxetine appear to be similar, when we compare the results of the present study. Even though there is not complete agreement with previous reports, it is confirmed that both pharmaceuticals exert acute effects only at levels that are several orders of magnitude higher than environmental concentrations, which range from ng/L to low µg/L, according to the literature. The only possible exceptions, at a local scale, could be accidental releases or improper disposals.

Regarding carbamazepine, the experiments provided two EC50 values, of 105.5 and 103.3 mg/L. Jos et al. (2003) exposing *D. magna* to carbamazepine for 48 hours found an EC50 of 97.8 mg/L; Kim et al. (2007) reported an EC50 value > 100 mg/L. Both results are in line with ours.

The acute EC50 values found in the present and previous studies are considerably higher than the environmental concentrations of carbamazepine, up to 1075 ng/L in surface water (Heberer, 2002) and 6.3 µg/L in municipal STP effluents (Ternes, 1998). However, it is important to remember that the highest removal efficiency for carbamazepine in a WWTP (Waste Water Treatment Plants) was 53%, observed by Paxéus (2004), while it is normally poorly removed (below 9%, Fent et al., 2006). Such low removal efficiency of carbamazepine can be explained by its properties. First, it is resistant to biodegradation at low concentrations. Stamatelatou et al. (2003) conducted a biodegradability test of carbamazepine in CH_3COONa cultured activated sludge in both sea and fresh water. They observed no biodegradation of carbamazepine at an initial concentration of 0.5 mg/L in either sea or fresh water. In the classification scheme for pharmaceutical biodegradation, the removal status of carbamazepine is classified as ‘‘no removal” (Joss et al., 2006). Furthermore, it is hardly attached onto sludge. Its distribution coefficient between water and secondary

sludge (Kd) is 1.2 L kg_{ss}^{-1} (Ternes et al., 2004), far from the value 500 L kg_{ss}^{-1} required for significant sorption onto sludge. Therefore the bulk of carbamazepine remains associated with the aqueous phase. Furthermore, Vernouillet et al. (2010) observed the ability of CBZ to bioaccumulate in aquatic organisms through food contamination, although the high concentration of CBZ (150 mg/L) used experimentally is unlikely to be found in the environment (Fent et al., 2006).

We conclude that although water concentrations are lower than the EC50 experimentally established, carbamazepine is pseudo-.persistent in the environment and able to bioaccumulate. Therefore, animals are chronically exposed to the pharmaceutical and studies on long term effects are advisable.

5.1.3 21-d chronic reproduction toxicity test on pharmaceuticals

Chronic tests with single pharmaceuticals were carried out in order to identify the EC50 values for reproduction. This information was used to calculate concentrations in terms of toxic units and as the basis to properly design the chronic mixtures exposure experiment.

Single chemical experiments with propranolol provided two EC50 of 0.592 and 0.883 mg/L. If we take into consideration also the single pharmaceutical EC50 found in the mixture tests the values becomes ranged from 0.592 to 0.909 mg/L.

Experimental data showed that increasing the concentration of the substance, a decrease in reproduction occurred. Compared to the average number of newborns in the control treatment, a decrease in the number of offspring was observed even at the lowest tested concentrations: at 0.25 mg/L the reduction was 6 % and 19 % for the first and second tests, respectively. This is in contrast with Stanley et al. (2006), that observed a significant increases in reproduction at 0.05, 0.20 and 0.40 mg/L in *D. magna* exposed to the racemic propranolol for 21 days. The mechanistic cause of this increase was unknown at that time (Stanley et al., 2006). On the contrary they detected a significant decrease in reproductive output at 0.8 mg/L and values similar to the control at concentration of 0.10 mg/L.

Results in line with the present study are presented by Dzialowsky et al. (2006) who observed a significant decrease in reproduction in D. magna exposed to 0.11 and 0.44 mg/L in subchronic 9-day tests. They also identified heart rate as the most

sensitive among the tested endpoints, since it decreased even at the lowest tested concentration (0.055 mg/L).

Regarding fluoxetine, the chronic EC50 for reproduction ranged from 0.216 to 0.245 mg/L, considering the single chemical experiments and the single chemical EC50 from the mixtures experiment. These values are much lower than those found for propranolol. Comparing acute to chronic tests, the EC50 values decreased for both substances by one order of magnitude. However the greatest variation was observed for fluoxetine, for which the chronic EC50 was 30 fold smaller than the acute EC50.

In addition, it is important to note that, while propranolol determined a monothonic decrease in reproduction with increasing concentration, the trend was not as regular for fluoxetine. In fact, at 0.025 mg/L, the lowest tested concentration, a slight increase in the number of newborns per *D. magna* was observed. In our study this result is not statistically significant, however the trend is in agreement with previous evidence that low concentrations of fluoxetine lead to hormesis; in particular an increase of reproductive output was observed. Fong (1998) reported that 0.036 mg/L fluoxetine increased zebra mussel fecundity, and Brooks et al. (2003) found that *Ceriodaphnia dubia* produced significantly more offspring when exposed to 0.056 mg/L fluoxetine. Flaherty and Dodson (2005) reported that 0.036 mg/L 30-d chronic fluoxetine exposure elicited a significant increase (3 times higher than control) in the total number of *D. magna* offspring produced. An increase in *D. magna* fecundity following chronic fluoxetine exposure may be an example of the phenomenon known as overcompensation hormesis, an adaptive response to low levels of stress that results in improved fitness for a finite period (Calabrese and Baldwin, 2002).

Stanley et al. (2007) after exposing *D. magna* for 21 d to 0.01, 0.05, 0.1, 0.25, 0.5, 1.0 mg/L R-, rac- and S-fluoxetine (nominal concentrations), identified 0.430, 0.429, 0.444 mg/L respectively as the LOEC (Lowest Observed Effect Concentration, measured concetrations). For the pure enantiomers, but not for the racemic mixture, they also observed a minimal, not statistically significant, increase in the mean number of offspring produced per organisms at low concentrations: at 0.010 mg/L for R-fluoxetine and at 0.051 mg/L for S-fluoxetine (measured concentrations).

This results are in agreement with our study and point out that hormetic effects of fluoxetine on reproduction deserve further clarifications.

5.2 Mixture exposure

The experimental results of the acute and chronic mixture tests were analyzed by MixTox, a statistical approach devised to predict the combined effects of chemical and to identify the type of interaction among them. Mixtox can be applied only if all the single substances in the mixture show monotonic dose-response relationship. This holds in our case: increasing the concentrations of both pharmaceuticals, the toxic effect monotonically increased as well. In addition, although the lower concentrations of FLX in the mixture (0.030 and 0.042 mg/L) are indicated in the literature as the “hormesis zone” (section 5.1.3), according to our data the hormesis effect is not statistically significant, therefore excluded or at least negligible.

The application of MixTox is based on the choice between two reference models: concentration addition (CA) and independent action (IA). In the present study the CA model was adopted, considering propranolol and fluoxetine to have similar mode of action (see section 1.3.4).
The MixTox analysis detected an antagonism deviation from the conceptual model of Concentration Addition for both acute and chronic test.
This has implications as to environmental risk assessment, were one of the major concerns is the possible amplification of effects when multiple contaminants, pharmaceuticals in particular, are present in the environment. In our particular case, the contrary was actually observed. We are aware that the experiments were performed in environmentally unrealistic conditions: mixtures in the aquatic environment will never be constituted only by two pharmaceuticals. In addition, the detected environmental concentrations are always much lower than those tested. These consideration, however, do not reduce the importance of the tests because they provide new information, also as a starting point for further exploration.
One major issue raised in the present study was how well the reference models would predict joint effects of a chemical mixture, and how the MoA of compounds must be known before a clear decision on which conceptual model to use becomes useful.
Franzellitti et al. (2013) with their studies on the mussel *Mytilus galloprovincialis* have considered both pharmaceuticals acting with the same MoA, and detected that the effects on mantle/gonads of mussels exposed to mixtures of fluoxetine + propranolol

were quite different than those exerted by the exposure to single chemicals. This finding was consistent with an antagonistic effect displayed by PROP on the 5-HT pathway in mantle/gonads as a result of a prevalent serotoninergic control in these tissues.

The available knowledge on the mode of action of the two pharmaceuticals in *D. magna* has been increased by recent studies on membrane receptors, illustrated in section 1.3.4. Therefore, a hypothesis can be formulated to explain the mechanism underlying the antagonism between propranolol and fluoxetine. The therapeutic action of fluoxetine as a SSRI is to increase serotoninergic neurotransmission at synapses by blocking 5-HT reuptake transporters, thus increasing extracellular levels of 5-HT (Fong et al., 2003). Propranolol is an β-adrenergic receptor antagonist (Weir, 2009), but it is also an effective antagonist for type 1 5-HT receptors (5-HT1), which are negatively coupled to the cAMP transduction pathway (Alexander and Wood, 1987).

Studies on propranolol carried out in mussels show that, by blocking β-adrenergic receptors, PRP blocks the action of adrenergic agonists which increases cAMP, thus it indirectly decreases intracellular levels of cAMP (Franzellitti et al., 2011). By blocking 5-HT1 receptors, PRP prevents binding of serotonin on this class of receptors. Fluoxetine provokes an increase in the concentration of serotonin which in turn would cause a decrease in the concentration of intracellular cAMP through 5HT1 receptors (Fazzino et al., 2009).

In conclusion, both pharmaceuticals alone induce a decrease of cAMP levels. This phenomenon could induce a decrease in reproduction; in fact cAMP pathway is involved in a variety of physiological functions related to reproduction as shown in mussels, i.e., it modulates the availability of energy for the development of the gonads thus being essential for the reproduction of mussels (Fabbri and Capuzzo, 2010). In other bivalves, it is also well demonstrated that serotonin injection induces gamete releases (Fabbri and Capuzzo, 2010), again by acting on the cAMP pathways.

Regarding the immobilization effect, it is widely demonstrated that cAMP is involved in modulation of motility. Specifically, an increase in cAMP level stimulates motility of flagellum (Negri et al. 1996) and vibratile cilia (Fabbri and Capuzzo, 2010), and also stimulates muscle contraction (Menezes-Rodrigues et al., 2013).

When propranolol and fluoxetine are present simultaneously, both compounds can affect the same molecular target, directly, FLX, by increasing the availability of the agonist molecules or indirectly, PRP, by binding to the receptor and preventing its occupation by the agonist. We may hypothesize that in *Daphnia* exposed to the mixture propranolol binds the 5-HT1 receptor and the effect of serotonin to decrease cAMP level induced by fluoxetine is reduced (fluoxetine would result less effective), thus a lower decrease in cAMP is observed (Fig. 5.1).

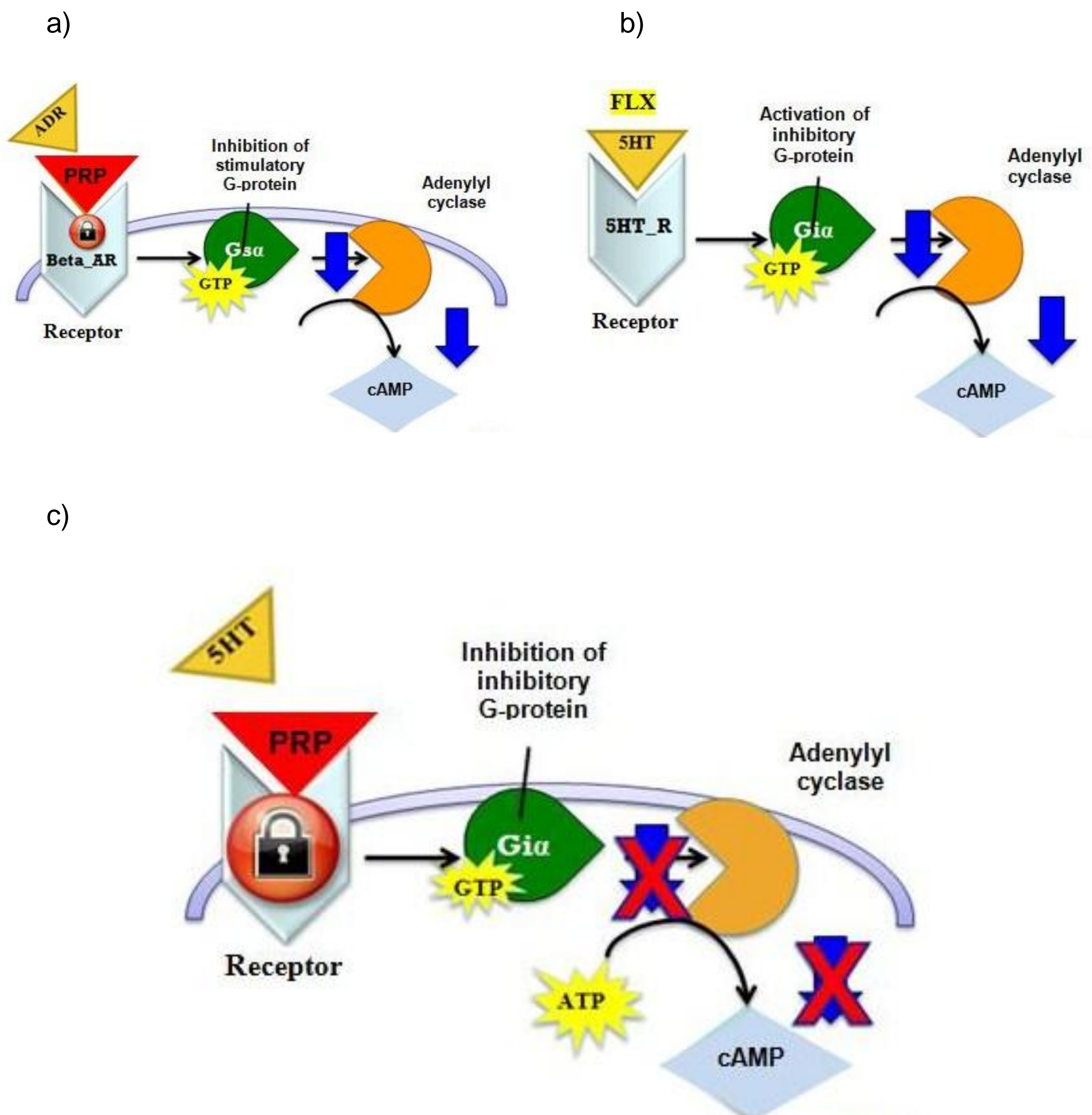


Fig. 5.1. Representation of how propranolol (a), fluoxetine (b) and the mixture propranolol + fluoxetine (c) may affect cAMP pathway.

Although the adrenergic or serotoninergic receptors present in *Daphnia* are not known in details, we may hypothesize that the antagonism between propranolol and fluoxetine revealed in the mixture takes place in line with the effects described above, suggesting that a lower reduction of cAMP (due to the mixture exposure as above hypotesized) lead to a reduction of immobilization or a reduction of the reproduction decrease compared to the effects of single pharmaceuticals.
Even though MixTox indicated that Fluoxetine and Propranolol act as antagonists in Daphnia and we proposed a possible mechanism, this result was not obvious in advance and different outcomes are possible for other species. For instance Drake and Gordon (1994) published a case study concerning a 53 years old man demonstrating that the addition of fluoxetine to his normal daily care for anxiety with propranolol caused a complete heart block. The hypothesis suggested by the authors was that since there is good evidence that 5-HT receptors are present in the human atrium, fluoxetine involves the enhancement of the action of serotonin compromising the atrio-ventricular conduction.
We must admit that the two pharmaceuticals can have multiple mechanisms of action, and influence reproduction and heart functionality in different ways in different organisms, therefore we are not able to extrapolate which are the physiological mechanisms that determine the actual antagonism between the two pharmaceuticals in *D. magna*; however the above hypothesis appear plausible for what regards the receptors involved.

5.3 Biomarkers

Before testing the effects of fluoxetine on AChE, MDA and GST on *D. magna* it was necessary to optimize the procedures and particularly the exposure conditions. It was very important to find the number of animals per sample that allowed an accurate measurement of the enzymatic activities. This number had to be a good compromise between the sensitivity of enzymatic determinations and costs (in money, time and human effort) needed to culture the daphnids required to run the experiment. Consequently, all further tests were performed on samples consisting of 40 individuals aged 7-8 d at the end of the 72 h exposure.

5.3.1 Acetylcholinesterase activity

Literature data on the effects of fluoxetine on AChE on *D. magna* are not available. In our experimental conditions, no effect of fluoxetine on AChE was observed. AChE basal activity in unexposed animals was in agreement with Jemec et al. (2007). AChE activity is selectively inhibited by organophosphate and carbamates, but also metals can influence this enzyme (Ishaaya, 2001): in fact Diamantino et al. (2000) observed a significant inhibition of AChE activity in *Daphnia magna* exposed to 150 µg/L Cr(VI) for 48 hours. However Jemec et al. (2007) observed no effect on AChE activity in organism exposed up to 280 µg/L Cr(VI) for 48 hours, while the AChE activity in animals exposed to low concentrations of Cd^{2+} increased slightly as a result of a possible hormesis. Jemec et al. (2007) supposed that there were no direct linkage between biomarkers and immobility of the organisms: several authors (Day and Scott, 1990; Jemec et al., 2007) have concluded that the immobility after acute exposure in this organisms is not always directly linked with the degree of the enzyme inhibition. Immobility were proportional (dose-response) to the concentration of the tested substances, while AChE activities were independent of the tested concentrations. Thus, in this case response of biochemical biomarkers were less sensitive than higher level endpoints (immobilization). Therefore, the applicability of this biomarker in routine acute toxicity tests is limited. On the other hand Jemec et al. (2008) reported that biochemical biomarker in chronic study were in some case equally or more sensitive than reproduction. This effect was more pronounced for

Cr(VI), which had more influenced the AChE after chronic exposure, while none was changed after acute exposure. For instance, AChE activity significantly decreased and increased in *D. magna* chronically exposed to concentrations above 35 µg/L of Cr^{6+} and to 0.082 µg/L of Cd^{2+}, respectively.

It is know that carbamates and organophosphates, widely used as active ingredients of insecticide formulations, are inhibitors of AChE. AChE inhibitors disrupt the nerve transmission by competitively binding to the esteric site of AChE (Fukuto, 1990), this inhibiting the breakdown of the neurotransmitter acetylcholine to acetate and choline. Accumulation of acetylcholine leads to paralysis (immobilization) and eventually to death (Boelsterli, 2007). *D. magna* is very sensitive to organophosphorous pesticides (Guilhermino et al., 1996), and it is widely used in aquatic risk assessment because often water bodies around agricultural fields receiving organophosphorous and carbamates pesticide treatments. Many studies demostrated that AChE activity in *D. magna* responds to a organophosphorous or carbamates exposure. For instance, Barata et al. (2004) in *D. magna* after 48 h exposure observed a significant decrease in AChE activity from a basal value of 3.5 nmol/min/mg protein to values < 1 nmol/min/mg protein for the organophosphorus chlorpyrifos and malathion, and a value slight below 2 nmol/min/mg protein for the carbamate carbofuran pesticides.

Studies reported above indicate that organophosphorus, carbamate and heavy metals can inhibit the AChE activity, however, also other environmental pollutants, such pharmaceuticals can inhibit AChE activity, for instance ibuprofen tested in bivalves by Milan et al. (2013). Regarding the response of AChE activity to fluoxetine, Munari et al. (2014) exposed clams for 7 days to 0, 1, 5, 25, 125 and 625 mg/L fluoxetine. AChE activity was shown to decrease significantly in the gills of clams exposed to 1 and 5 mg/L compared with controls. It is well known that AChE plays an important role in the functioning of the neuromuscular system by preventing continuous muscular contraction. Munari et al. tested the hypothesis that fluoxetine is neurotoxic to *V. philippinarum*. The results of their study partially support this hypothesis. Indeed, gill AChE activity decreased significantly in clams exposed to the lowest concentrations of fluoxetine, whereas enzyme activity returned to control values in clams exposed to the highest fluoxetine concentrations. Their controversial result was also recorded in a recent study on mussels (*M. galloprovincialis*) that were exposed to fluoxetine (75 ng/L) for 15 days (Gonzalez-Rey and Bebianno, 2013). In that study, SSRI FLX potential to cause neurotoxic effects response was tested by

assessing the activity of the essential neurotransmission modulator, enzyme acetylcholinesterase in mussel gills. The enzyme activity increased significantly after 3 days of exposure, followed by progressive inhibition, reaching a significantly lower activity than controls after 15 days of exposure. In that experiment AChE activity was clearly altered. Conversely, AChE activity was shown to increase significantly in the muscle of crabs (*C. maenas*) after 7 days of exposure to fluoxetine (120 and 750 mg/L) (Mesquita et al., 2011).

Based on both reported studies and our results we can conclude that AChE activity may vary markedly in response to fluoxetine exposure, depending on the exposure concentration and duration and on the species and tissues analyzed. Since in our study no effect was observed and there is no further literature support, we presently exclude effects by fluoxetine on AChE activity. We also suggest that this biomarker is suitable for evaluating pesticide exposure, while its suitability for metals or pharmaceuticals need to be verified.

We could assume that the applicability of this biomarker in *D. magna* is limited and other studies to test lower concentration are essential.

5.3.2 Malondialdehyde content

The MDA content in control treatment was 0.530 ± 0.002 nmol/mg of protein. As well as AChE activity, also the MDA content did not change in *Daphnia* exposed to fluoxetine. To check the consistency of these results, a test with chromium(VI), which is a metal known to cause oxidative stress, was carried out. In fact *D. magna* exposed to 0.04 mg/L of chromium(VI) showed a significant increase in MDA content in comparison to the control. MDA content is considered a good general indicator of lipid peroxidation and its accumulation a marker for oxidative stress. When the antioxidant system response is compromised by an ROS excess, lipid peroxidation (LPO) occurs, resulting in the damage of phospholipids membrane (Valavanidis et al., 2006). Franzellitti et al. (2013) measured MDA content in mussels exposed for 7 days to nominal 0.03, 0.3, 3, 30 and 300 ng/L fluoxetine and they observed a significant decrease in its content in digestive gland after treatment with 0.3 and 30 ng/L fluoxetine. They explained that the lack of clear antioxidant responses in digestive gland can be a consequence of a preferential lysosomal sequestration of

fluoxetine, which in turn would prevent further cellular effects of the pharmaceutical, including lipid peroxidation.

Galecki et al. (2009) measured MDA content in erythrocytes of depressed patient before and after a 3 month fluoxetine treatment. They observed that the MDA level was significantly higher in depressed patients (low levels of serotonin) before treatment (0.739 nmol/gHb) compared to the control group (0.549 nmol/gHb), while MDA values in the depressed group after treatment (increased levels of serotonin) were statistically different (0.607 nmol/gHb) still higher than control, but lower than those found in depressed patients. Although we are aware of the evolutionary gap between humans and *Daphnia*, this results can be read in line with ours since we did not find any difference in MDA content in *D. magna* treated with fluoxetine, but we observed a slight not significant decrease at the highest concentration.

Regarding the oxidative effect of Cr(VI), Fabbri and Capuzzo (2006) detected a significant decrease in labilization time in lysosomes of the digestive glands of *Mytilus galloprovincialis* exposed to 10 µg/L chromium(VI) for 7 d. Decrease in labilization time, is considered an index of cellular damage and consequently, of stress. In the same conditions they found a significant increase in cAMP content in the gills. Jemec et al. (2008) observed a decrease in protein content in *D. magna* exposed for 21 days to 1.1, 17.5, 52.5 µg/L of chromium(VI), a reflection of the general detrimental effect physiological state of the organism.

Moreover, we observed that at 0.2 mg/L Cr(VI), MDA content did not increase significantly in comparison with the control. Our hypothesis is that such high concentration inhibits many process, including peroxidation. This results is in line with the study by Jemec et al. (2008), who observed a significant increase in GST activity in *D. magna* exposed for 21 d at low concentration of Cr(VI), 0.52, 4.3, 17.5 µg/L, but they found values similar to the control for the highest concentration 52.5 µg/L Cr(VI). A further hypothesis is that at high concentration MDA, which is an intermediate product, decreases progressively increasing the lipofuscin production, in fact lipofuscins represent an end point in the lipid peroxidation process and their accumulation is easily detectable in cells of stressed organisms. When ROS levels production exceeds antioxidant defences, the peroxidation end-products are accumulated in lysosomes as insoluble granules containing autofluorescent pigments usually referred to as lipofuscins. The bulk of lipofuscin granules is constituted by oxidatively modified proteins and lipid degradation products, along with

carbohydrates and metals (Buratti, 2011). All lipofuscin pigments are not degradable. During the exposure of organisms to pollutants, this biomarker typically shows a continuously increasing trend, which reaches a maximum level that is determined by the rate of secretion of lipofuscin-rich residual bodies into the external fluids.

To conclude, although MDA content was not different from the control in *D. magna* exposed to fluoxetine, it was significantly increased in *D. magna* exposed to chromium(VI). Since test on heavy metal has established that this biomarker is suitable for organisms as *D. magna*, we simply conclude that fluoxetine does not affect MDA content at least at the tested concentrations.

5.3.3 Gluthatione S-transferase

GST activity in the control treatment was about 134 nmol/min/mg protein, while slight higher activities were observed in *D. magna* exposed to 1.0 and 5.0 mg/L fluoxetine, although the differences from the control were not statistically significant. These results were in line with the Jemec et al. (2007) study in which they observed control values similar to ours and no significant change in GST activity in *D. magna* exposed for 48 hours to up to 280 µg/L of Cr(VI) and up to 7 µg/L of diazinon (one of the most used insecticides). Although literature data on the effect of Cr(VI) on GST are inconsistent (Jemec et al., 2007), GST activity was expected to increase due to possible oxidative stress caused by Cr(VI) (Stohos and Bagchi, 1995). Also Choi (2000) did not observe any effect in *Chironomus riparius* exposed to 1.75 mg/L for 24 h. In another study, Barata et al. (2005) observed that GST activity increases in *D. magna* exposed for 48 h to five substances known to lead ROS production: cadmium (2, 5 µg/L), copper (5, 20 µg/L), the pesticides endosulfan (200, 400, 600 µg/L) and paraquat (2, 5 µg/L), and the quinine menadione (50, 200 µg/L). They observed also that organic substances induced greater GST activity stimulation than metals.
Moreover, Jemec et al. (2008), during chronic 21 days test, observed a significant increase in GST activity in *D. magna* exposed at low concentration of Cr(VI) (0.52, 4.3, 17.5 µg/L).
Regarding the effect of fluoxetine on GST activity, as for the other biomarkers analyzed in this study, we do not have information about effect of fluoxetine in *D. magna*, thus we can only refer to fluoxetine effects in other organisms. In a recent

study Gonzales-Rey and Bebianno (2013) exposing mussels *M. galloprovincialis* to 75 ng/L of fluoxetine up to 15 days, detected a similar GST activity in both tissues, gills and digestive glands, although with irregular patterns. In controls, GST activities in both tissues decreased; after the first week in gills and immediately after the 3rd day in digestive gland. In exposed mussels, GST activity remained unaltered in gills, whereas in digestive gland it fluctuates decreasing to control levels after the first week of exposure and varying to significantly higher activities than controls after the 3rd and 15th day of exposure and increasing thereafter. Phase II detoxification enzyme GST promotes reduced glutathione (GSH) conjugation with parental electrophilic compounds enabling its transformation to more extractable hydrophilic metabolites (Halling-Sørensen et al., 1998).

The above results on GST activities in *D. magna* after an acute exposure to organic substances or metal are discordant therefore further studies should be conducted.

In the present investigation, GST activity was not increased by fluoxetine at the tested concentrations, although fluoxetine effects on GST activity was observed in *M. galloprovincialis.* We may conclude that *D. magna* does not counteract possible effects by fluoxetine through the detoxification system represented by GST, or at these concentrations fluoxetine does not affect the crustacean. As a whole, although other studies have reported antioxidant enzyme response in *D. magna,* accurate information on antioxidant defense system against contaminants are absent.

6. Conclusion

The main aim of the present work was to evaluate alteration of life cycle endpoints on D. magna exposed to mixtures of propranolol and fluoxetine, two pharmaceuticals widely detected in freshwater environment. To gain further knowledge, also three widely used biomarkers were applied, i.e. acetylcholinesterase inhibition, MDA levels, and GST activity.

The issue of pharmaceutical in the aquatic environment is of concern worldwide, and in the latest years received great attention within the scientific community. However, pharmaceutical residues are never alone in water, and their possible interaction when animals are exposed to mixtures is difficult to study and indeed rarely investigated. Mixture effects are indeed the most urgent topic requiring investigation in this field.

The MixTox tool allowed the conclusion that an antagonistic effect was caused in Daphnia exposed to the mixtures, i.e. the effect of the mixture on immobilization and reproduction was lower than the effects of the two pharmaceuticals applied alone. Two different tests, acute and chronic were performed on D. magna, and both reached the above conclusion. This result is based on the choice of concentration addition as the reference model that defines the absence of interaction between the two chemicals. This, in turn, is founded on the assumption that the two pharmaceuticals share a common mode of action in Daphnia.

Although, D. magna physiology is not known in details, this approach let us hypothesize that exposure to pharmaceutical mixtures also affected cell signaling, including cAMP, serotoninergic and adrenergic receptors, which suggest an evolutionary conservation of the basal biological targets between humans and crustaceans. According to this hypothesis, the involvement of cAMP is both the common element in the mode of action of fluoxetine and propranolol and the explanation for antagonism.

Studying the early stress response with molecular biomarkers, no effect was detected in D. magna exposed to fluoxetine. Nevertheless, further analysis should be carried to search for early warning biomarkers in D. magna.

Although pharmaceutical concentrations used during the study are much higher than the actual concentrations found in the environment, the research is not less important, and data obtained must be regarded as indications to be used in ecological risk assessment. In this regard, one of the major concerns is the possible

amplification of effects when multiple contaminants in particular, are present in the environment. In this particular case, the contrary was actually observed.

More studies should be conducted to better investigate the physiology of D. magna and the effects of pharmaceutical mixtures, crucial for risk assessment approaches.

7. References

Adams, S.M., 2002. Biological indicators of aquatic ecosystem stress. American Fisheries Society, Bethasda, Meryland.

Alexander, B.S., Wood, M.D., 1987. Stereoselective blockade of central [3H]5-hydroxytrypamine binding to multiple site [5HT1A, 5HT1B, and 5HT1C by mianserin and propanolol. J Pharm Pharmacol 39, 664-666.

Allan, I.J., Vrana, B., Greenwood, R., Mills, G.A., Roig, B., Gonzalez, C., 2006. A "toolbox" for biological and chemical monitoring requirements for the European Union's Water Framework Directive. Talanta 69(2), 302-22.

Altamura, A.C., Moro, A.R., Percudani, M., 1994. Clinical pharmacokinetics of fluoxetine. Clin Pharmacokinet. 26(3), 201-14.

Andreozzi, R., Marotta, R., Pinto, G., Pollio, A., 2002. Carbamazepine in water: persistence in the environment , ozonation treatment and preliminary assessment on algal toxicity. Water Res. 36, 2869-2877.

Banni, M., Bouraoui, Z., Ghedira, J., Clearandeau, C., Jebali, J., Boussetta, H., 2009. Seasonal variation of oxidative stress biomarkers in clams *Ruditapes decussatus* sampled from Tunisian coastal areas. Environ Monit Assess 155(1-4), 119-28.

Barata, C., Baird. D.J., Nogueira, A.J., Soares, A.M., Riva, M.C., 2006. Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna* Straus. Implications for multi-substance risks assessment. Aquat Toxicol. 78(1), 1-14.

Barata, C., Solayan, A., Porte, C., 2004. Role of B-esterases in assessing toxicity of organophosphorus (chlorpyrifos, malathion) and carbamate (carbofuran) pesticides to *Daphnia magna* Aquatic Toxicology 66 (2004) 125–139

Barata, C., Varo, I., Navarro, J.C., Arun, S., Porte, C., 2005. Antioxidant enzyme activities and lipid peroxidation in the freshwater cladoceran *Daphnia magna* exposed to redox cycling compounds. Comp Biochem Physiol C Toxicol Pharmacol. 140(2), 175-86.

Blazer, V.S., Iwanowicz, L.R., Iwanowicz, D.D., Smith, D.R., Young, J.A., Hedrick, J.D., Foster, S.W., Reeser, S.J., 2007. Intersex (testicular oocytes) in smallmouth bass from the Potomac River and selected nearby drainages. Aquat Anim Health. 19(4), 242-53.

Bliss, C.I., 1939. The toxicity of poisons applied jointly. Annals of Applied Biology 26, 585-615.

Boelsterli, U.A., 2007. Mechanistic Toxicology the Molecular Basis of How Chemicals Disrupt Biological Targets. CRC Press, Boca Raton.

Brooks, B.W., Foran, C.M., Richards, S.M., Weston, J.J., Turner, P.K., Stanley, J.K., Solomon, K.R., Slattery, M., La Point, T.W., 2003. Aquatic ecotoxicology of fluoxetine. Toxicol Lett. 142(3), 169-83.

Brooks, B.W., Turner, P.K, Stanley, J.K., Weston, J.J., Glidewell, E.A., Foran, C.M., Slattery, M., La Point, T.W., Huggett, D.B., 2003. Waterborne and sediment toxicity of fluoxetine to select organisms. Chemosphere 52(1), 135-42.

Brun, G.L., Bernier, M., Losier, R., Doe, K., Jackman, P., Lee, H.B., 2006. Pharmaceutically active compounds in atlantic canadian sewage treatment plant effluents and receiving waters, and potential for environmental effects as measured by acute and chronic aquatic toxicity. Environ Toxicol Chem. 25(8), 2163-76.

Buratti, S., 2011. Pharmaceutical residues in aquatic systems: mode of action and effects on mussel physiology. Tesi di Dottorato in Scienze Ambientali, tutela e gestione delle risorse naturali. Università di Bologna, Campus di Ravenna.
Calabrese, E.J., Baldwin, L.A., 2002. Defining hormesis. Hum Exp Toxicol. 21(2), 9-7.

Calamari. D., Zuccato, E., Castiglioni, S., Bagnati, R., Fanelli, R., 2003. Strategic survey of therapeutic drugs in the rivers Po and Lambro in northern Italy. Environ. Sci. Technol. 37(7), 1241-1248.

Campos, B., Pina, B., Carlos, B. C., 2012. Mechanisms of action of selective serotonin re-uptake inhibitors in *Daphnia magna.* Environ. Sci. Technol. 46, 2943-2950.

Choi, J., Roche, H., Caquet, T., 2000. Effects of physical (hypoxia, hyperoxia) and chemical (potassium dichromate, fenitrothion) stress on antioxidant enzyme activities in *Chironomus riparius* mg. (diptera, chironomidae) larvae: Potential biomarkers. Environmental Toxicology and Chemistry 19(2), 495-500.

Chou, T.C., 2006. Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies. The American Society for Pharmacology and Experimental Therapeutics, Pharmacol Rev 58, 621–681.

Christen, V., Hickmann, S., Rechenberg, B., Fent, K., 2010. Highly active human pharmaceuticals in aquatic systems: A concept for their identification based on their mode of action. Aquat Toxicol. Aquatic Toxicology 96, 167-181.

Daughton, C., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ. Health Perspect. 107, 907-938.

Day, K.E., Scott, I.M., 1990. Use of acetylcholinesterase activity to detect sublethal toxicity in stream invertebrates exposed to low concentrations of organophosphate insecticides. Aquatic Toxicology (Amsterdam). 18(2), 101-113

Diamantino, T.C., Guilhermino, L., Almeida, E., Soares, A.M., 2000. Toxicity of sodium molybdate and sodium dichromate to *Daphnia magna* Straus evaluated in

acute, chronic, and acetylcholinesterase inhibition tests. Ecotoxicol Environ Saf. 45(3), 253-9.

Directive 2000/60/EC of the European Parliament and the Council of 23 October 2000. Establishing a framework for Community action in the field of water policy. Official Journal of the European Union L 327.

Directive 2013/39/EU of the European Parliament and the Council of 12 August 2013. Amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. Official Journal of the European Union L 226.

Dobell, C., 1932. Antony van Leeuwenhoek and his “little animals”. Constable, Ltd. (Dover reprint 1960).

Drake, W. M., Gordon, G.D., 1994. Heart block in a patient on propanolol and fluoxetine. The Lancet, 343, 8894; ProQuest Central. pg. 425.

Drewes, J.E., Heberer, T., Reddersen, K., 2002. Fate of pharmaceuticals during indirect potable reuse. Water Sci. Technology 46, 73-80.

Dzialowski, E.M., Turner, P.K., Brooks, B.W., 2006. Physiological and reproductive effects of beta adrenergic receptor antagonists in *Daphnia magna*. Arch Environ Contam Toxicol. 50(4), 503-10.

Ebert, D., 2005. Ecology, Epidemiology, and Evolution of Parasitism in *Daphnia*. University of Basel, Switzerland. Bethesda (MD).

Eli Lilly and Co, annual report, 2001.

EMEA, Guideline on the Environmental Risk Assessment of Medicinal Products for Human Use, The European Agency for the Evaluation of Medicinal Products: Committee for Medicinal Products for Human Use, EMEA/CHMP/SWP/4447/00, 2006.

Environment Canada, 2005. Guidance document on statistical methods for environmental toxicity tests. Method development and applications section, Environment Canada, Report EPS 1/RM/46. Ottawa, Ontario, Canada.

Ecology, Epidemiology, and Evolution of Parasitism in *Daphnia* Copyright © 2005, Dieter Eber.

Edmondson, W.T., 1987. *Daphnia* in experimental ecology: notes on historical perspective. In Peters, R.H. and De Bernardi R. (Eds.), *Daphnia.* Mem. Ist. Ital. Idrobiol., 45, 11-30.

Fabbri, E., Capuzzo, A., 2006. Adenylyl cyclase activity and its modulation in the gills of Mytilus galloprovincialis exposed to Cr^{6+} and Cu^{2+}. Aquatic Toxicology 76, 59-68.

Fabbri, E, Capuzzo, A., 2010. Cyclic AMP signaling in bivalve molluscs: an overview. J Exp Zool A Ecol Genet Physiol. 313(4), 179-200. Review.

Fazzino, F., Urbina, M., Cedeno, N., Lima, L., 2009. Fluoxetine treatment to rats modifies serotonin transporter and cAMP in lymphocytes, CD4+ and CD8+ subpopulations and interleukins 2 and 4. International Immunopharmacology 9 (4), 463-467.

Fent, K., 2003. Ecotoxicological problems associated with contaminated sites. Toxicol Lett. 140-141, 353-65.

Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol., 76(2), 122–159.

Ferrari, B., Mons, R., Vollat, B., Fraysse, B., Paxéus, N., Lo Giudice, R., Pollio, A., Garric, J., 2004.Environmental risk assessment of six human pharmaceuticals: are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? Environ Toxicol Chem. 3(5),1344-54.

Ferrari, B., Paxeus, N., Lo Giudice, R., Pollio A., Garric, J., 2003. Ecotoxicology and Environmental Safety 55, 359-370.

Flaherty, C.M., Dodson, S.I., 2005. Effects of pharmaceutical in *Daphnia* survival, growth and reproduction. Chemosphere 61, 200-207.

Fong, P.P., Philbert, C.M., Roberts, B.J., 2003. Putative serotonin reuptake inhibitorinduced spawning and parturition in freshwater bivalves is inhibited by mammalian 5-HT2 receptor antagonists. Journal of Experimental Zoology Part A Comparative Experimental Biology 298, 67–72.

Fong, P.P., 1998. Zebra mussel spawning is induced in low concentrations of putative serotonin reuptake inhibitors. Biol. Bull. 194 (2), 143-149.

Franzellitti, S., Buratti, S., Donnini, F., Fabbri, F., 2010. Exposure of mussels to a polluted environment: insights into the stress syndrome development. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 152, 24–33.

Franzellitti, S., Buratti, S., Valbonesi, P., Capuzzo, A., Fabbri, E., 2011. The β-blocker propranolol affects cAMP-dependent signaling and induces the stress response in Mediterranean mussels, *Mytilus galloprovincialis.* Aquatic Toxicology 101, 299-308.

Franzellitti, S., Buratti, S., Valbonesi, P., Fabbri, E., 2013. The mode of action (MoA) approach reveals interactive effects of environmental pharmaceuticals on *Mytilus galloprovincialis*, Aquatic Toxicology 140– 141 (2013) 249– 256.

Fukuto, T.R., 1990. Mechanism of action of organophosphorus and carbamate insecticides. Environmental Health Perspectives 87,245–254.

Galecki, P., Szemraj, J., Bieñkiewicz, M., Florkowski, A., Galecka, E., 2009. Lipid peroxidation and antioxidant protection in patients during acute depressive episodes and in remission after fluoxetine treatment. Pharmacological reports, 61, 436-447.

García-Morales, I., Sancho Rieger, J., Gil-Nagel, A., Herranz Fernández, J.L., Antiepileptic drugs: from scientific evidence to clinical practice. Neurologist. 6(1), 20-8.

Geller, W., Muller, H., 1981. The filtration apparatus of cladocera –filter mesh-sizes and their implications on food selectivity. Oecologia, 49, 316-321.

Goldin, A. and Mantel, N., 1957. The employment of combinations of drugs in the chemotherapy of neoplasia: a review. Cancer Res 17, 635–654.

Gonzalez-Rey, M., Bebianno, M.J., 2013. Does selective serotonin reuptake inhibitor (SSRI) fluoxetine affects mussel *Mytilus galloprovincialis*? Environ. Pollut. 173, 200-209.

Gorbi, G., 1987. Utilizzazione di *Daphnia magna* in tossicologia ambientale. Atti del corso di formazione: utilizzazione di *Daphnia magna* in tossicologia ambientale. Reggio Emilia.

Greco, W.R., Bravo, G., Parsons, J.C., 1995. The search for synergy: a critical review from a response surface perspective. Pharmacol Rev 47, 331–385.

Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, A.M.V.M., 1996. Inhibition of acethylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. Chemosphere 32, 727–738.

Halling-Sørensen, B., Nielsen, S.N., Lanzky, P.F., Ingerslev, F., Holten Lutzhoft, H.C., Jorgensen, S.E., 1998. Occurrence, fate and effects of pharmaceuticals substance in the environment e a review. Chemosphere 36 (2), 357-393.

Halliwell, B., Gutteridge, J.M.C., 1999. Free radicals in biology and medicine. Oxford University Press , New York, USA.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol Lett. 131(1-2), 5-17.

Hejnol, A., Scholtz, G., 2004. Clonal analysis of distal-less and engrailed expression patterns during early morphogenesis of uniramous and biramous crustacean limbs. Dev Genes Evol. 214, 473-85.

Henschel, K.P., Wenzel, A., Diedrich, M., Fliedner, A., 1997. Environmental hazard assessment of pharmaceuticals. Regul. Toxicol. Pharmacol. 25, 200-225.

Huggett, D.B., Brooks, B.W., Peterson, B., Foran, C.M., Schlenk, D., 2002. Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (B-blockers) on aquatic organisms. Arch Environ Contam Toxicol. 43(2), 229-35.

Ishaaya, I., Kontsedalov, S., Mazirov, D., Horowitz, A.R., 2001. Biorational agents--mechanism and importance in IPM and IRM programs for controlling agricultural pests. Meded Rijksuniv Gent Fak Landbouwkd Toegep Biol Wet. 66(2a), 363-74.

ISO 6341:1996. Water quality-Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea), Acute toxicity test.

ISO 10706:2000. Water quality-Determination of long term toxicity of substances to *Daphnia magna* Straus (Cladocera, Crustacea).

Jemec, A., Drobne, D., Tisler, T., Trebse, P., Ros, M., Sepcić, K., 2007. The applicability of acetylcholinesterase and glutathione S-transferase in *Daphnia magna* toxicity test Comp Biochem Physiol C Toxicol Pharmacol. 144(4), 303-9.

Jemec, A., Tisler, T., Drobne, D., Sepcić, K., Jamnik, P., Ros, M., 2008. Biochemical biomarkers in chronically metal-stressed daphnids. Comp Biochem Physiol C Toxicol Pharmacol. 147(1), 61-8.

Jonker, M.J., Svendsen, C., Bedaux, J.J., Bongers, M., Kammenga, J.E., 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. Environ Toxicol Chem. 24(10), 2701-13.

Jørgensen SE, Halling-Sørensen B., 2000. Drugs in the environment. Chemosphere. 40(7), 691-9.

Jos, A., Repetto, G., Ríos, J.C., del Peso, A., Salguero, M., Hazen, M.J., Molero, M.L., Fernández-Freire, P., Pérez-Martín, J.M., Labrador, V., Cameán, A., 2005. Ecotoxicological evaluation of the additive butylated hydroxyanisole using a battery with six model systems and eighteen endpoints. Aquat Toxicol. 71(2), 183-92.

Jos, A., Repetto, G., Rios, J.C., Hazen, M.J., Molero, M.L., del Peso, A., Salguero, M., Fernandez-Freire, P., Perez-Martin, J.M., Camean, A, 2003. Ecotoxicological evaluation of carbamazepine using six different model systems with eighteen endpoints. Toxicology in Vitro 17, 525-532.

Joss, A., Zabaczynski, S., Göbel, A., Hoffmann, B., Löffler, D., McArdell, C.S., Ternes, T.A., Thomsen, A., Siegrist, H., 2006. Biological degradation of pharmaceuticals in municipal wastewater treatment: proposing a classification scheme. Water Res. 40, 1686–1696.

Kim, Y., Choi, K., Jung, J., Park, S., Kim, P.G., Park, J., 2007. Aquatic toxicity of acetaminophen, carbamazepine, cimetidine, diltiazem and six major sulfonamides, and their potential ecological risks in Korea. Environ Int. 33(3), 370-5.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environ Sci Technol. 36(6), 1202-11.

Lange, R., Dietrich, D., 2002. Environmental risk assessment of pharmaceuticals drug substances-conceptual considerations. Toxicol. Lett. 131, 97-104.

Loewe, S., Muischnek, H., 1926. Combinated effects I announcement –implements to the problem. Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmacol. 114, 313-326.

Loureiro, S., Svendsen, C., Ferreira, A.L.G., Pinheiro, C., Ribeiro, F., Soarest, A.M.V.M., 2010. Toxicity of three binary mixtures to *Daphnia magna*: comparing chemical modes of action and deviations from conceptual models. Environmental Toxicology and Chemistry 29, No 8, 1716-1726.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193, 265-275.

Martin, H., Svendsen, C., Lister, L.J., Gomez-Eyles, J.L., Spurgeon, D.J., 2009. Measurement and modeling of the toxicity of binary mixtures in the nematode *Caenorhabditis elegans* – a test of independent action. Environmental Toxicology and Chemistry 28, 97-104.

McCoole, M.D., Atkinson, N.J., Graham, D.I., Grasser, E.B., Joselow, A.L., McCall, N.M., Welker, A.M., Wilsterman, E.J. Jr, Baer, K.N., Tilden, A.R., Christie, A.E., 2012. Genomic analyses of aminergic signaling systems (dopamine, octopamine and serotonin) in *Daphnia pulex*. Comp Biochem Physiol Part D Genomics Proteomics 7(1), 35-58.

Meadows, S.L., Gennings, C., Carter, W.H. Jr, Bae, D.S., 2002. Experimental designs for mixtures of chemicals along fixed ratio rays. Environ Health Perspect. 6, 979-83. Review.

Menezes-Rodrigues, F.S., Pires-Oliveira, M., Duarte, T., Paredes-Gamero, E.J., Chiavegatti, T., Godinho, R.O., 2013. Calcium influx through L-type channels attenuates skeletal muscle contraction via inhibition of adenylyl ciclases. Eur. J. Pharmacol. 720(1-3), 326-34.

Mesquita, S.R., Guilhermino, L., Guimarães, L., 2011. Biochemical and locomotor responses of Carcinus maenas exposed to the serotonin reuptake inhibitor fluoxetine. Chemosphere 85, 967-976.

Milan, M., Pauletto, M., Patarnello, T., Bargelloni, L., Marin, M.G., Matozzo, V., 2013. Gene transcription and biomarker responses in the clam Ruditapes philippinarum after exposure to ibuprofen. Aquat. Toxicol. 126, 17-29.

Mimeault, C., Trudeau, V.L., Moon, T.W., 2006. Waterborne gemfibrozil challenges the hepatic antioxidant defense system and down-regulates peroxisome proliferator-activated receptor beta (PPARbeta) mRNA levels in male goldfish (Carassius auratus). Toxicology.228(2-3), 140-50.

Mitchell, S. E., 2001. Intersex and male development in *Daphnia magna*. Hydrobiologia, 442, 145-156.

Munari, M., Marin, M.G., Matozzo, V, 2014. Effects of the antidepressant fluoxetine on the immune parameters and acetylcholinesterase activity of the clam Venerupis philippinarum Marine Environmental Research 94 (2014) 32-37.

Nation, J.L. 2002. Insect physiology and biochemistry. CRC Press, Boca Raton, FL.

Negri, P., Grechi, E., Tomasi, A., Fabbri, E., Capuzzo, A., 1996. Effectiveness of pentoxifylline in semen preparation for intrauterine insemination. Hum Reprod. 11(6), 1236-9.

OECD Guidelines for the Testing of Chemicals: Test No. 202, *Daphnia* sp. Acute Immobilisation Test , 2004.

OECD Guidelines for the Testing of Chemicals: Test No. 211, *Daphnia magna* Reproduction Test, 2008.

Pavlaki, M.D., Pereira, R., Loureiro, S., Soares, A.M.., 2011. Effects of binary mixtures on the life traits of *Daphnia magna*. Ecotoxicol Environ Saf. 74(1):99-110.

Paxéus, N., 2004. Removal of selected non-steroidal anti-inflammatory drugs (NSAIDs), gemfibrozil, carbamazepine, b-blockers, trimethoprim and triclosan in conventional wastewater treatment plants in five EU countries and their discharge to the aquatic environment. Water Sci. Technol. 50, 253–260.

Reddy, C.C., Burgess, J.R., Gong, Z.-Z., Massaro, E.J., Tu, C.-P.D., 1983. Purification and characterization of the individual glutathione S-transferases from sheep liver. Arch. Biochem. Biophys. 224, 87-101.

Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Official Journal of the European Union L 136.

Ruppert, E. E., Fox, R. S. & Barnes, R. D., 2004. Invertebrate Zoology, Thomson Brooks And Cole.

Sarkar, A., Ray, D., Shrivastava, A.N., Sarker, S., 2006. Molecular biomarkers: their significanze and application in marine pollution monitoring. Ecotoxicology 15, 333-340.

Secure and Responsible Drug Disposal Act of 2010. One Hundred Eleventh Congress of the United States of America

Seiler, J.P., 2002. Pharmacodynamic activity of drugs and ecotoxicology –can the two be connected? Toxicology Letter 131, 105-115.

Spoladore, R., Fragasso, G., Montanaro, C., Maranta, F., Torlasco, C., Gerosa, S., Margonato A., 2010. Beta-bloccanti e sistema cardiovascolare: attuali acquisizioni e controversie. Recenti Prog Med 10, 429-441.

Stamatelatou, K., Frouda, C., Fountoulakis, M.S., Drillia, P., Kornaros, M., Lyberatos, G., 2003. Pharmaceuticals and health care products in wastewater effluents: the example of carbamazepine. Water Sci. Technol: Water Supply 3, 131–137.

Stanley, J.K., Ramirez, A.J., Chambliss, C.K., Brooks, B.W., 2007. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. Chemosphere 69(1), 9-16.

Stanley, J.K., Ramirez, A.J., Mottaleb, M., Chambliss, C.K., Brooks, B.W., 2006. Enantiospecific toxicity of the beta-blocker propranolol to *Daphnia magna* and *Pimephales promelas*. Environ Toxicol Chem. 25(7), 1780-6

Stohs, S.J., Bagchi, D., Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med. 18(2), 321-36.

Swammerdam, J., 1669. Historia insectorum generalis, of te algemeene verhandeling van de bloedeloose dierkens. t'Utrecht.

Quinn, B., Gagné, F., Blaise, C., 2008. The effects of pharmaceuticals on the regeneration of the cnidarian, Hydra attenuata. Sci Total Environ. 402(1), 62-9.

Ternes, T.A., Herrmann, N., Bonerz, M., Knacker, T., Siegrist, H., Joss, A., 2004. A rapid method to measure the solid–water distribution coefficient (Kd) for pharmaceuticals and musk fragrances in sewage sludge. Water Res. 38, 4075–4084.

Ternes, T. A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water research 32, 3245-3260.

US EPA, 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th edition. Washington, DC, U.S. Environmental Protection Agency.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicology and Environmental Safety 64, 178-189.

Vernouillet, G., Eullaffroy, P., Lajeunesse, A., Blaise, C., Gagné, F., Juneau, P., 2010. Toxic effects and bioaccumulation of carbamazepine evaluated by biomarkers measured in organisms of different trophic levels. Chemosphere 80(9), 1062-8.

Weir, M.R., 2009. Beta-blockers in the treatment of hypertension: are there clinically relevant differences? Postgraduate Medicine 121, 90–98.

Weston, J.J., Huggett, D.B., Algood, J.C., Foran, C.M., Stattery, M., 2001. Determination of fluoxetine (Prozac) and norfluoxetine in the aquatic environment. In: Annual Meeting of the Society of Environmental Toxicology and Chemistry, Baltimore, MD.

Winkler, M., Lawrence, J.R., Neu, T.R., 2001. Selective degradation of Ibuprofen and Clofibric acid in two model river biofilm systems. Water Res. 35, 3197-3205.

Zaffagnini, F., 1987. Reproduction in *Daphnia.* In Peters, R.H. and De Bernardi R. (Eds.), *Daphnia.* Mem. Ist. Ital. Idrobiol., 45, 245-284.

Zhang, Y., Geissen, S.U., Gal, C., 2008. Carbamazepine and diclofenac: removal in wastewater treatment plants and occurrence in water bodies. Chemosphere. 73(8), 1151-61.

Web sources:

http://www.circleofblue.org
http://www.ec.europa.eu/environment/water/water-framework/
http://www.echa.europa.eu/regulations
http://www.ema.europa.eu/ema/
http://www.eureau.org/

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