CESPU
INSTITUTO UNIVERSITÁRIO
DE CIÊNCIAS DA SAÚDE

Enantioselective Ecotoxicity of Psychotropic Pharmaceuticals and Illicit Drugs

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Dissertation thesis for the degree of Mater of Forensic Sciences and Laboratory Techniques

Gandra, 21 January 2020

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Enantioselective Ecotoxicity of Psychotropic Pharmaceuticals and Illicit Drugs

Dissertation thesis presented to the University Institute of Health Sciences (IUCS, CESPU) to fulfill the requirements necessary to obtain a Master's Degree in Forensic Laboratory Science and Techniques under the guidance of Professor Doctor Cláudia Ribeiro and Professor Doctor Maria Elizabeth Tiritan.

Master in Forensic Laboratory Science and Techniques of the University Institute of Health Sciences (IUCS, CESPU)

Gandra

January 2020

DECLARAÇÃO DE INTEGRIDADE

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ACKNOWLEDGMENT

This dissertation result of a lot of effort and it could not be done without the support and help of those that I am going to announce and that I would like to express my deepest thanks:

I am extremely grateful to my advisers Professor Cláudia Ribeiro and Professor Maria Elizabeth Tiritan for all the knowledge, patience and dedication transmitted, for been always available to help me with everything I could need. In special to Professor Cláudia who oriented me more closely, thank you for everything.

I would like to thank Professor Ricardo Dinis Oliveira, coordinator of master and regent of this curricular unit, for guide me to my advisers of Professor Cláudia Ribeiro and Professor Maria Elizabeth Tiritan.

To all the people that work in the IUCS laboratories, in special to Virginia Gonçalves, a laboratory technician, that helped me with everything I needed. To my laboratory colleague Ariana Pereira, for helping me in the first's steps of these work, thanks for the kindness and willingness to help.

I would also like to extend my deepest gratitude to Professor João Carrola, a professor of Trás-os Montes and Alto Douro University (UTAD) who guide me through the zebrafish assays, for all the support, because always made me feel like home, for all the knowledge that I will take for life. Thank you for everything.

To all the people in UTAD that help me with zebrafish tests, to Luís Félix for the help with his experience, to Germano for helping with the eggs production, to Patrícia Silvia and Ondina Ribeiro for the essential help, that was crucial to ending the assay in time.

I would like to extend my thanks to all my friends that have accompanied me throughout these years.

Last but not the least, I am deeply grateful to my family, that made sure I achieve all my goals. For all the sacrifice made, for always understand me, support me and offering help, for always being there, no matter what. To my beloved mother, my dear father

and my sweet big sister, I would like to thank with all my heart, without them nothing of these could be done.

The results presented in this dissertation are part of the following scientific communications:

Poster communications

F. Teles, M. E. Tiritan, C. Ribeiro, Ecotoxicidade Enantiosseletiva de Fármacos e Drogas Psicotrópicas, XII Jornadas Científicas IUCS and III Congresso APCF, Porto, Portugal, 24-25 May 2018 (Abstract and poster communication in annex 1).

F. Teles, A. Pereira, M. E. Tiritan, C. Ribeiro, Ecotoxicidade Enantiosseletiva de Substâncias Psicotrópicas, XIII Jornadas Científicas IUCS and IV Congresso APCF, Porto, Portugal, 11-12 April 2019 (Abstract and poster communication in annex 2).

F. Teles, JS Carrola, M. E. Tiritan, O. Ribeiro, L. Felix, C. Ribeiro, Ecotoxicidade Enantiosseletiva da Cetamina e Norcetamina no Peixe Zebra (*Danio rerio*) - Dados Preliminares, 3ª Reunião Internacional Rede Académica das Ciências da Saúde da Lusofonia, Braga, Portugal, 26-28 March 2020, (Abstract in annex 3).

Article in international peer-reviewed journal

A. Pereira, C. Ribeiro, F. Teles , R. Gonçalves , V. Gonçalves , J. A. Pereira, J. S. Carrola, C. A. Pires, M. E. Tiritan , Ketamine and norketamine: multimiligram enantioseparation and enantioselective aquatic ecotoxicity studies. (Submitted to Chemosphere, 2020).

ABSTRACT

In the last years, the overall pharmaceuticals and illicit drugs consumptions have been increasing. Subsequent to this rise, the discharges into the environment also increase due to the excretion after consumption or to the bad disposal of surpluses and expired products. These substances are considered pseudo-persistent due to the continuous entrance in the environment. Therefore, it is necessary to understand the impact of these pseudo-persistent substances in aquatic fauna. About 56% of these substances are chiral, however, the ecotoxicological assays are usually performed only with racemates. In this work ecotoxicological tests were carried out, according to national and international standards (ISO, OECD), in order to evaluate the effect psychotropic substances namely ketamine (K), its metabolite norketamine (NK) and the respective enantiomers and racemate amphetamine (AMP), methamphetamine (MAMP) and methylenedioxymethamphetamine (MDMA) and (*S*)-MAMP in ecologically relevant aquatic organisms at different concentrations. The effect of K and NK racemates and enantiomers were assessed in the fish *Danio rerio* , the crustacean *Daphnia magna* and the protozoan *Tetrahymena thermophila* and the following parameters were evaluated: mortality, malformations, larvae length, behavior response and the analysis of oxidative stress for *D. rerio* , percentage of mortality/ immobilization for *D. magna* and the percentage of growth inhibition for *T. thermophila*. The effects of racemate AMP, MAMP and MDMA and of the (*S*)-MAMP were assessed for *D. magna* and *T. thermophila* in order to determine the half maximal effective concentration (EC_{50}) for each compound.

Regarding *D. rerio* assays, the enantiomer (*R*)-K showed higher percentage of mortality and malformations than (*S*)-K, showing possible enantioselective effects. Larvae exposed to (*R,S*)-K exhibit a more agitate behavioral, presenting an increase in the percentage of time active. Although the K enantiomers induce more effects on mortality and malformation, oxidative stress showed higher reactive oxygen species production (ROS) for (*R*)-NK followed by (*R,S*)-NK and lower activity of antioxidant enzymes as catalase and glutathione while both K enantiomers and (*R,S*)-K showed higher activity. In contrast, both K enantiomers showed a higher percentage of mortality than NK racemate and enantiomers in *D. magna* being the (*S*)-K enantiomer the more toxic. Considering *T. thermophila* (*S*)-NK was more toxic than (*R*)-NK at the lower

concentrations and (S)-K had a higher growth inhibition. These results show that different organism may show different sensitivity to the test compounds, and that the effects can be enantioselective demonstrating the importance of these studies for an accurate environmental risk assessment.

Considering amphetamine and amphetamine like substances, the (*R,S*)-MAMP showed higher percentage of mortality for *D. magna* (80%) in the selected range of concentrations (10 to 35 mg/L) and EC_{50} values were 20.8, 30.2 and 34.5 mg/L for racemate MAMP, MDMA and AMP, respectively and 28 mg/L for (*S*)-MAMP. EC_{50} for *T. thermophila* were 23, 26 and 27.5 mg/L for MDMA, AMP and MAMP racemates.

These results show the different susceptibility of organisms and highlighting the importance of enantioselective ecotoxicological assays.

RESUMO

Nos últimos anos, o consumo de fármacos e de substâncias ilícitas têm vindo a aumentar. Consequentemente a este aumento, as descargas para o meio ambiente também aumentaram, devido à excreção após consumo, ou pelo mau descarte de excedentes e produtos vencidos. Estas substâncias são consideradas pseudo-persistentes, devido à entrada contínua no meio ambiente. Portanto, é necessário entender o impacto que estas substâncias pseudo-persistentes têm na fauna aquática. Cerca de 56% dessas substâncias são quirais, no entanto, os ensaios de ecotoxicidade são normalmente realizados somente com os racematos. Neste trabalho foram realizados ensaios ecotoxicológicos, de acordo com as normas nacionais e internacionais (ISO, OCDE), com vista a avaliar o efeito da cetamina (K), do seu metabolito norcetamina (NK), e respetivos enantiómeros, dos racematos anfetamina (AMP), da metanfetamina (MAMP) e da metilenodioximetamfetamina (MDMA) e do enantiómero (S)-MAMP em organismos aquáticos, ecologicamente relevantes a diferentes concentrações. Os efeitos da K e NK, racematos e enantiómeros, foram avaliados no peixe *Danio rerio* , no crustáceo *Daphnia magna* e no protozoário *Tetrahymena thermophila*, e os seguintes parâmetros foram avaliados: mortalidade, malformações, comprimento das larvas, avaliação do comportamento e analise do stress oxidativo para o *D. rerio*, percentagem de mortalidade/ imobilização para a *D. magna* e a percentagem da inibição do crescimento para o *T. thermophila*. Os efeitos dos racematos AMP, MAMP e MDMA e do enantiómero (S)-MAMP foram avaliados para a *D. magna* e *T. thermophila* de forma a determinar o valor de concentração que provoca um efeito de 50 % (CE_{50}) para cada composto.

Considerando os ensaios realizados com o *D. rerio*, o enantiómero (*R*)-K apresentou maior percentagem de mortalidade e malformações do que o (*S*)-K, mostrando possíveis efeitos enantiosseletivos. Alevins expostos ao (*R,S*)-K exibiram um comportamento mais agitando, apresentando um aumento na percentagem de tempo ativo. Apesar de os enantiomeros da K induzirem mais mortalidade e malformações, análise do stress oxidativo demonstrou maior produção de espécies reativas de oxigénio (ERO) após exposição à (*R*)-NK e (*R,S*)-NK e menor atividade para enzimas antioxidantes como a

catálase e glutationa enquanto os enantiomeros da K e racemato apresentaram maior atividade.

Pelo contrário, ambos enantiómeros da K mostraram maior percentagem de mortalidade do que os enantiómeros e racemato da NK na *D. magna* sendo que o enantiómero (*S*)-K que teve maior toxicidade. Considerando o *T. thermophila,* o enantiómero (*S*)-NK apresentou maior toxicidade do que o enantiómero (*R*)-NK nas concentrações mais baixas e o (*S*)-K teve a maior inibição de crescimento. Estes resultados demonstram que organismos diferentes podem ter suscetibilidades difererentes aos compostos testados e que os efeitos podem ser enantiosseletivos demonstrando a importância destes estudos para uma correta avaliação do risco ambiental.

Considerando as anfetaminas e substancias aparentadas, o (*R,S*)-MAMP apresentou maior mortalidade para a *D. magna* (80%) e os valores de CE_{50} foram de 20.8, 30.2 e 34.5 mg/L para os racematos de MAMP, MDMA e AMP, respetivamente e 28 mg/L para o (*S*)-MAMP. O CE_{50} para o *T. thermophila* foi de 23, 26 e 27.5 mg/L para os racematos de MDMA, AMP e MAMP. Estes resultados demonstram diferente suscetibilidade dos organismos testados e destacando a importância da enantiosselectividade nos ensaios ecotoxicológicos.

Estes resultados mostram a diferente suscetibilidade dos organismos testados para a mesma substância. A enantiosseletividade também foi observada nos enantiómeros da K, realçando a importância dos ensaios ecotoxicológicos nos enantiómeros puros.

Palavras-Chave

Ecotoxicidade; Enantiosseletividade; *Danio rerio*; *Daphnia magna*; *Tetrahymena thermophila*.

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LIST OF ABREVIATIONS, SYMBOLS AND ACRONYMS

AcHE Acetylcholinesterase

AMP Amphetamine

BSA Bovine serum albumin prtein

CAT Catalase

CNS Central nervous system

DCFH-DA dichlorofluorescin-diacetate

EC$_{50}$ Half maximal effective concentration

EDTA ethylenediaminetetraacetic acid

GSH Glutathione

GSSG Oxidized glutathione

GST Glutathione S-transferase

HEPES Hydroxyethyl piperazine ethanesulfonic acid

hpf Hours post-fertilization

ISO International organization for standardization

K Ketamine

LC$_{50}$ Half lethal concentration

LDH Lactate dehydrogenase

LPO Lipid peroxidation

MAMP Methamphetamine

MDA Malondialdehyde

MDMA Methylenodioxymethamphetamine

MeOH Methanol

NADH 1,4-dihydro-nicotinamide adenine dinucleotide

NK Norketamine

NMDA *N*-methyl-*D*-aspartate

OD Optical density

OECD Organization for economic co-operation and development

ROS Reactive oxygen species

SOD Superoxide dismutase

TBARS Thiobarbituric acid reactive substance

1. INTRODUCTION

1.1 Contaminants in the aquatic environments: pharmaceuticals and illicit drugs

The presence of pollutants, namely pharmaceuticals and other biologically active substances, such as illicit substances, in various environmental compartments, especially in the aquatic systems has been an important issue in these recent years owing to their high consumption and continuous discharge (Coelho et al., 2019; Goncalves et al., 2019; Patel et al., 2019). Besides, due to the continued growth of human population it is expected that the consume of pharmaceuticals and illicit substances continues to increase in the next years (Galindo-Miranda et al., 2019; Kümmerer, 2010).

Different classes of pharmaceuticals and illicit drugs have been found in the environment. In fact, these substances, their metabolites as well as transformation products may reach water bodies as a result of their excretion after human consumption due the inefficiency of the wastewater treatment plants (WWTPs) system or as a result of other activities as industrial discharges (Cosenza et al., 2018; Fent et al., 2006). On the other hand, psychotropic pharmaceuticals and illicit drugs have been receiving special attention in the last years since detection and quantification of these substances can give information about epidemiological studies such as the consumption habits of a specific population including the use of illicit drugs (Goncalves et al., 2019; Mackuľak et al., 2019). Nonetheless, information about their impact on non-target species is less understood. Pharmaceuticals and illicit drugs as cocaine, morphine, methamphetamine (MAMP), amphetamine (AMP), 3,4-methylenedioxyamphetamine (MDMA), ketamine (K), as well as their metabolites among many others have been reported in various water systems in low ng up to µg/L (Coelho et al., 2019; Goncalves et al., 2019; Kümmerer, 2010; Li et al., 2016; Yadav et al., 2019). Due to their constant release even at low concentrations in the aquatic environment, besides the acute effects, they are most likely to induce chronic toxic effects on aquatic non-target organisms from different trophic levels, and negatively affecting the ecosystems (Ferrari et al., 2003; Gworek et al., 2019). Knowledge about the impact of these pollutants on living organisms is of

highly importance for risk assessment and further establishment of measures for environmental protection. **Figure 1** shows the different sources of pharmaceuticals and illicit drugs in water resources and consequences of their occurrence.
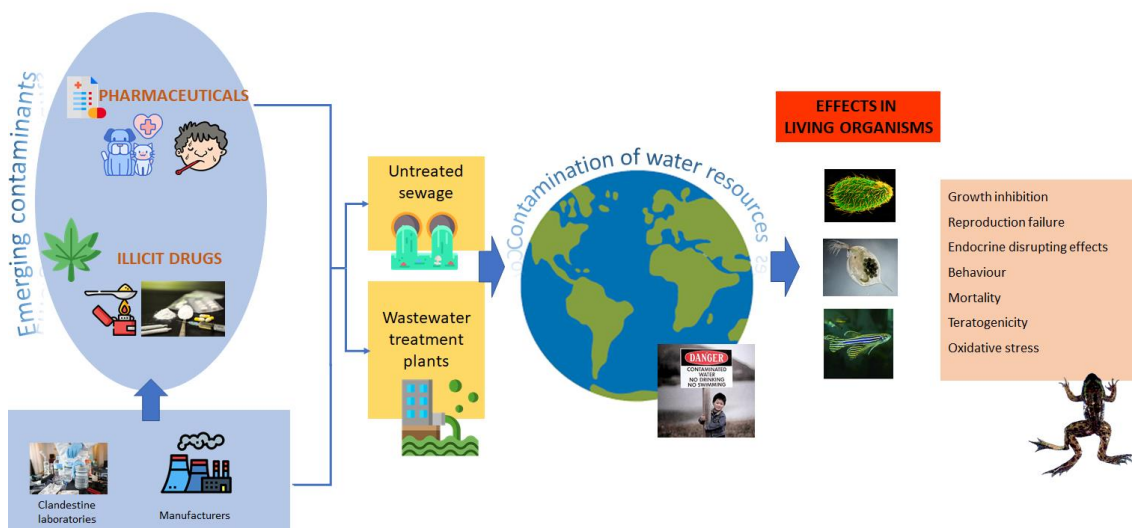
Figure 1 – Sources of pharmaceuticals and illicit drugs in water resources and consequences of their occurrence.

1.2 Chiral Pharmaceuticals and illicit drugs: enantioselective ecotoxicity

Most pharmaceuticals and illicit drugs are chiral. In fact, 56% of drugs and more than 1500 pollutants are chiral (Basheer, 2018). Chiral compounds are molecules with two non-superimposable mirror images, given by their three-dimensional asymmetry. Chiral pharmaceuticals are available either as racemate or enantiomerically pure (Ribeiro et al., 2018).

It is well known that enantiomers may undergo different pharmacological (pharmacokinetic, pharmacodynamics) and toxicological activities (Basheer, 2018; Ribeiro et al., 2018). These substances can suffer stereoselective metabolism in humans due to the chiral nature of organism systems. The biodegradation in WWTP can also be stereoselective due to microbiological processes during the treatment of effluents, which leads to changes in their enantiomeric fraction (EF). Consequently, these

substances can be found in environmental at different enantiomeric compositions. Various studies have been reporting the enantioselectivity of various classes of pollutants namely pesticides in different species. For example, the (*S*)-metolachlor effect on oxidative stress, in *Scenedesmus obliquus* stimulated more reactive oxygen species (ROS) than the racemate (Liu et al., 2017). The embryonic exposure to *cis*-bifenthrin (BF) to *D. rerio* suggested that 1*S*-*cis*-BF has higher risk to induce oxidative stress, apoptosis and immunotoxicity than 1*R*-*cis*-BF (Jin et al., 2013). The estrogenic and thyroid enantioselective endocrine disrupting effects of nine pesticides were investigated using three *in vitro* methods (Luciferase reporter gene assay, E-screen and T-screen assay) (Song et al., 2017). More than 70% of the selected pesticides showed high differences among enantiomers endocrine disrupting effects (enantioselectivity). Regarding chiral pharmaceuticals, Stanley et al 2007 studied the enantiotoxicity of fluoxetine (FLX) and of the β-blocker a propranolol (PHO) in the *Pimephales promelas* (Stanley et al., 2007). In that studied it was showed that (*S*)-FLX is more toxic to *P. promelas* than (*R*)-FLX, after seven-days of exposure concerning survival of the fish (LC$_{50}$ (*rac*)=198 µg/L; LC$_{50}$ (*R*-FLX)=216 µg/L; LC$_{50}$ (*R*-FLX)=212 µg/L) and (*S*)-PHO affected the growth of *P. promela* and is more toxic than *R* enantiomer. De Andres et al. 2009 reported the enantioselective toxicity of dopa, FLX and atenolol in three organisms, the microalgae *Pseudokirchneriella subcapitata*, the crustacean *D. magna* and the *T. thermophila*. The (S)-enantiomer was the most toxic, in all substances tested, to the three test organisms. *T. thermophila* was the most sensitive species to the enantiomers of FLX and atenolol (De Andres et al., 2009).

A recent report showed the enantioselective toxicity, distribution and bioaccumulation of venlafaxine and *O*-desmethylvenlafaxine in loach *Misgurnus anguillicaudatus* co-exposed to microplastics (Qu et al., 2019). Concerning psychoactive chiral drugs there are few studies regarding the enantioselective effects in different trophic levels aquatic organisms.

1.2.1 Psychoactive Chiral Drugs

Ketamine

Ketamine (K) is a phenylcyclohexylamine derivative widely use as anesthetic, available as a racemate. The enantiomers (*S*)-K or esketamine and (*R*)-K or asketamine are represented in Figure 2. It was first developed by Calvin Stevens in 1962 and became commercially available in 1964 to replace the anesthetic phencyclidine (Morgan et al., 2012). It is a non-competitive antagonist of the glutamate receptor *N*-methyl-*D*-aspartate (NMDA). Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. Due to K side effects it has been use for specific situations as veterinary and pediatric anesthetic. Side effects include delusions, hallucinations, delirium and confusion, the main reasons why K is consumed in recreational environment (Dinis-Oliveira, 2017; Morgan et al., 2012).

K is metabolized to three metabolites, being 80% metabolized to the active metabolite norketamine (NK, Figure 2), 15% into hydroxy-norketamine and 5% are metabolized in hydroxy-ketamine. NK has about 20 to 30% potency when compared with K (Dinis-Oliveira, 2017; Lin et al., 2014; Malinovsky et al., 1996; Mion et al., 2013). NK also binds to the phencyclidine site on the NMDA receptor complex, but with lower affinity compared with K but evidencing anesthetic potential (Holtman et al., 2008). Some reports have been shown that K and NK enantiomers have different potencies. For example, (*S*)-K is two times stronger than the racemic form, and four times stronger than (*R*)-K (Mion et al., 2013; Schmid et al., 1999).
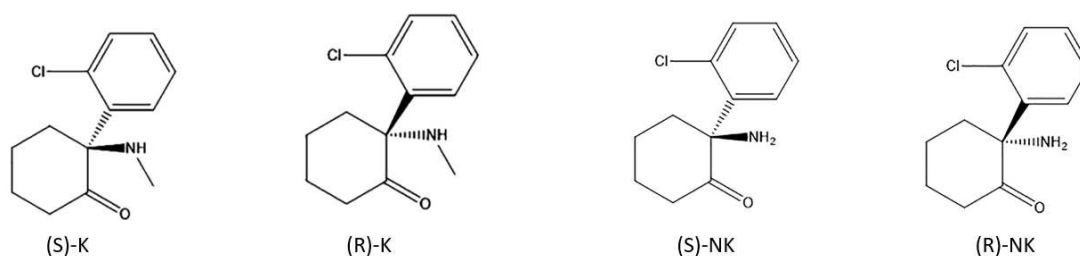
Figure 2 - Chemical structure of ketamine and norketamine enantiomers.

Zhang et al. 2019 reported that (*R*)-K may have less detrimental sides effects and can induce antidepressant effects that last longer, in animal models, when compared with

the (*R,S*)-K and (*S*)-K forms (K. Zhang et al., 2019). It was also demonstrated that (*S*)-NK exhibits rapid and sustained antidepressant effects, without showing behavioral and biochemical abnormalities that are present in (*S*)-K. Once (*S*)-NK maintained a similar potency to (*S*)-K but without its side effect (*S*)-NK could be safer than (*S*)-K (Zhang et al., 2019). Some studies reported the occurrence of either K and NK in environmental matrices as WWTPs and surface waters (Lin et al., 2014; Mackuľak et al., 2019). Félix et al. 2014 found that *D. rerio* embryos exposed for 20 minutes to K (racemate) showed changes in development, increased morphological abnormalities and higher mortality (Félix et al., 2014). To date, there are no studies evaluating the enantioselective effects of K and its metabolite, NK in different aquatic organisms. With the recent FDA approval for an intranasal spray based on esketamine, (*S*)-K, (Spravato) more patients will have access to the treatment of resistant depression being more urgent understand the effects of these compounds in the environment taking into account that the enantiomers show different potency and side effects (Luu et al., 2019; Swainson et al., 2019).

Amphetamine and amphetamine like substances

Amphetamine and amphetamine like substances such as MAMP and the MDMA are phenylethylamine derivates structurally related with great potential for abuse, addiction and toxicity (Dinis-Oliveira et al., 2015; Ribeiro et al., 2018). Chemicals structures are represented in Figure 3. These substances are designed to interfere with the central nervous system (CNS), being responsible for suppress feelings, stimulate mood and increase self-confidence (Liao et al., 2015; Musshoff et al., 2012).
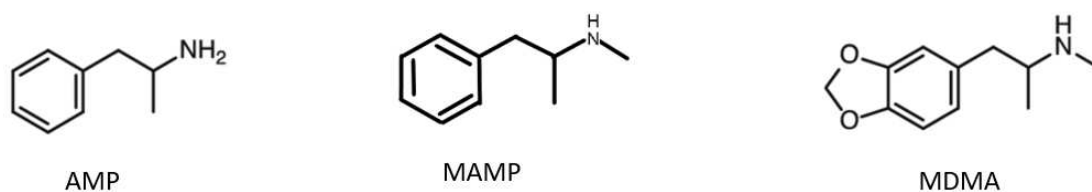
AMP   MAMP   MDMA

Figure 3 - Chemical structure of AMP, MAMP and MDMA.

MAMP affect heartbeat and body temperature regulation, attention, mood and responses associated with alertness or alarm conditions. The methyl group present in MAMP is responsible for the potentiation of the effects when compared to AMP (Freye, 2010). MAMP activate dopamine D1 receptors, inducing glutamate release increasing this way the dopamine and glutamate extracellular concentration and produce oxidative stress (Mark et al., 2004). The enantiomer (*S*)-MAMP has greater potency than (*R*)-MAMP in approximately 2-fold more potent in inhibiting vesicular uptake and approximately 3-fold more potent in evoking vesicular release. Illicit distribution can occur as racemate and as the pure (*S*)-MAMP. (Bardo et al., 2019; Partilla et al., 2006).

Nowadays the medical user of AMP and amphetamine-like substance are restricted, being only used to treat attention deficit disorders, hyperactivity and narcolepsy (Dinis-Oliveira et al., 2015). Recently, these compounds also have been considered environmental contaminants due to their continuous disposal into the environment. In these recent years, the occurrence of these substances in the aquatic system has been an important issue as it can give information about epidemiological studies concerning the estimative of drug consumption by a specific community. In addition, the enantiomeric profile of these drugs has been used to investigate the consumption of pharmaceuticals and illicit drugs, differentiate between consumption or direct disposal of drugs and the synthetic pathways and even possible discharges of clandestine laboratories. After consumption, these drugs undergo stereoselective metabolism and thus parent compounds and their metabolites are excreted into the sewage system in different EF (Gao et al., 2018; Goncalves et al., 2019; van Nuijs et al., 2011).

1.3 Toxicity Assays

Water quality evaluation of aquatic systems should include information about chemical and ecological status. According to water quality guidelines, ecological assessment should be evaluated covering exposure test of organisms from different trophic levels, at least three levels. Due to their occurrence, persistence and adverse effects, various substances were included in a list of priority substances as well as their environmental quality standards that should be evaluated in other to assess their environmental impact

in the ecosystems (Directive, 2008/105/EC, 2013/39/EU). Nevertheless, a Watch List encompassing 10 substances/group of substances was published and various other substances are under investigation for new data about occurrence, fate, biodegradation and adverse effects in order to support future prioritization policies (Decision, 2015/495/EU). In this sense, data about the adverse effect of pollutants including pharmaceuticals and illicit drugs is of highly importance for risk management. Ecotoxicological assays should comprise acute and chronic tests in order to include the most sensitive *endpoints* of different species. Acute studies are among the most used for study the toxicity of substances. These assays have a shorter period comparatively with their life cycle and different parameters can be evaluated such as mortality, immobility, among other *endpoints*. Most common organisms include microalgae's, bacteria like *Vibrio* sp, protozoan, the crustacean, *Daphnia magna* and fish (Ferrari et al., 2003; Lele et al., 1996; Tatarazako et al., 2007).

The most common toxicity endpoints used are the mortality evaluation, reproduction capacity and behavioral assays. To carry out the ecotoxicity assays, is essential select the test organism. Standardize protocols using certain organisms known as "sentinel organisms" or bioindicators are now available to evaluate the toxic effects of chemicals. These organisms are selected due to their relevance in the ecosystems (OECD, 2004, 2011, 2013, 2017). These assays are used to predict safety levels in the environment.

1.3.1 Aquatic Organisms

1.3.1.1 *Danio rerio*

The *D. rerio*, usually known as zebrafish, belongs to the family of freshwater fishes *Cyprinidae* and is a native tropical fresh water species of south Asia (Spence et al., 2008). It is a small fish (3-5 cm) showing an easy bred. Females can spawn every two weeks, embryos are fully developed in 96 hours post-fertilization (hpf) and the life cycle is short (about 3 mouths) (Parng et al., 2002). The outside fertilization and development allow easy access to embryos and a close following of their development, which is possible due to the transparency of the embryo, making clearly visible all developmental stages.

Figure 4 shows *D. rerio* life cycle. Briefly after fertilization the embryo starts developing. Between 4 to 8 hpf several processes occur, among them the epiboly which consist in the migration of cells over the yolk sac. The gastrulation occurs between 8 to 11 hpf, and the tree primary embryonic germ layers are formed through cell movements. Then, the somitogenesis starts anterior to posterior, i.e., from the head toward the tail of the embryo. Is around the 24 hpf that a heartbeat can be observe, and with 2 days post-fertilization the larvae hatch. With 5 days the larvae begin search for food and with around 3 months reach to adult phase (Willemsen et al., 2011).

The D. rerio become very popular as a research model in different scientific areas namely for in vivo assessment of chemical toxicity. There are many reasons that justify the use of the *D. rerio* as a research model, the main ones are the fact of they have a low cost to maintain, is possible to have a large number of animals in a small space and the females can generate hundreds of eggs, making possible to have large samples sizes. As mention above, they have a fast development and because the eggs are transparent it is possible to observe the embryogenic development and organ morphology. Another fact that help to increase the *D. rerio* popularity is that, until 5 days after fertilization, it is considered as a nonprotected animal, overcoming the ethical issues (Lele et al., 1996; Santos et al., 2018; Willemsen et al., 2011; Zou et al., 2017) .

One more feature that make *D. rerio* a model organism is the fact that its genome sequence is well known (Howe et al., 2013) and they are closer related to humans then invertebrates. In fact, *D. rerio* have many genes homologous to the humans (He et al., 2014; Zou et al., 2017).
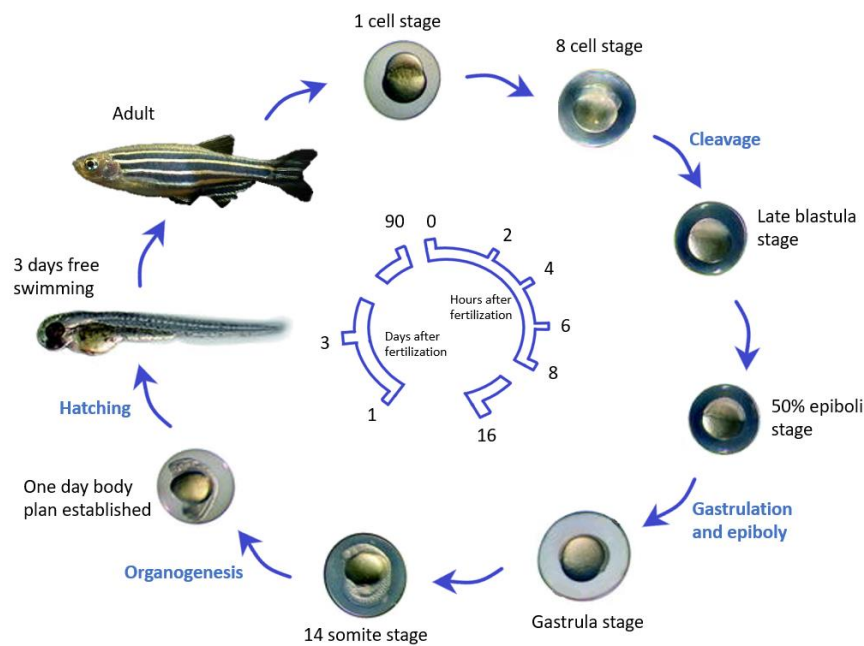
Figure 4 – *D. rerio* life cycle (adapted from Willemsen et al., 2011).

Different endpoints and effects are evaluated in *D. rerio*. *D. rerio* embryo toxicity assays are common as the early stages of life are particularly susceptible to the adverse effects of drugs and chemicals. The most common studied endpoints are the mortality (coagulation of fertilized eggs, lack of somite formation and lack of heartbeat are indicators of mortality mentioned by OECD 236), malformations and length. Some studies have been reported considering other *endpoints* and life stages studying behavioral alterations, reproductive changes and the effect on the oxidative stress parameters (Félix et al., 2016; Félix et al., 2014; Felix et al., 2017; He et al., 2014).

1.3.1.2 *Daphnia magna*

D. magna is a small freshwater crustacean, also known as water flea, which belongs to *Cladocera* order. It can reach about 5 mm, feeds of seaweed like *Spirulina sp.* and can reproduce asexually and sexually depending of the environmental conditions. *Daphnia* life cycle is represented in Figure 5. Briefly, the life cycle is affected by the environmental conditions, like temperature and food resource (Ribeiro et al., 2020). The predominant mode of reproduction is asexually, by parthenogenesis where genetically identical females are produced, and a clonal lineage are formed (Tatarazako et al., 2007; Vanoverbeke et al., 2007). When resources are abundant and the environmental

conditions are prosperous the entire *Daphnia* population consist in females, and the parthenogenesis allows a rapid expansion (Tatarazako et al., 2007). Whenever the conditions deteriorate, sexual reproduction starts, and males are produced and dormant and resisting eggs are made. Fertilized eggs are involved by a protective envelope called *ephippium*. These resistant eggs can survive under unfavorable environmental conditions for decades and hatch when conditions turn favorable again (De Meester, 1996; Jansen et al., 2017; Tatarazako et al., 2007; Vanoverbeke et al., 2007).

In ecotoxicology, *D. magna* occupies a prominent position as a recommended species for toxicologically assessing of substances (e.g., ASTM 1980; ISO 1996; OECD 1998; ISO 2000; OECD 2004), water (e.g., ASTM 1980; USEPA 2002), sediment (e.g., USEPA and USACE 1998) samples. *D. magna* became a test model because this organism can be easily maintaining in laboratory, without being necessary any expensive supplies and equipment, they have a good ecological relevance (are the main part of the diet of fish and invertebrate predators), are known to be sensitive to many chemicals and the short life cycle allow a faster observation of the responses to the testing compounds.
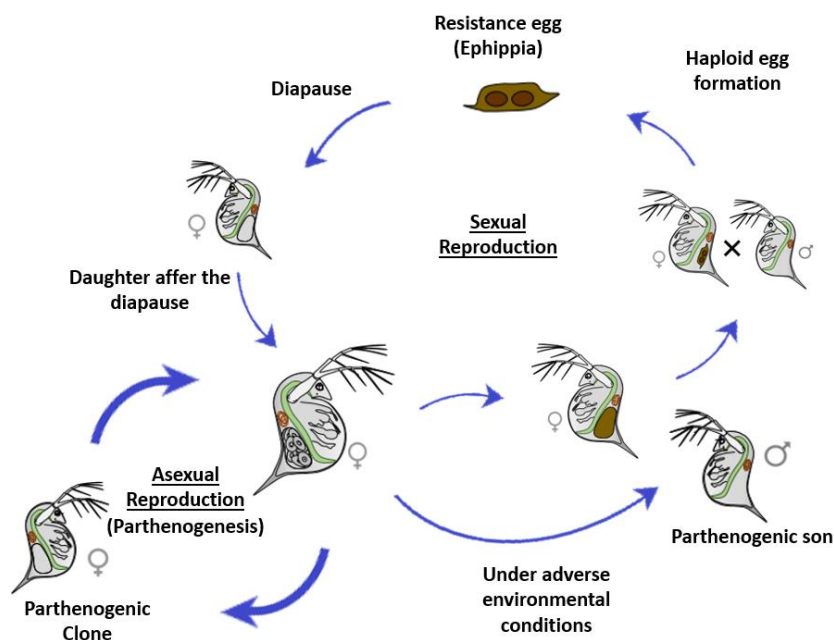
Figure 5 - *D. magna* life cycle (adapted from Ribeiro et al., 2020)

Two type of assays can be done with *D. magna*, acute and chronic. The acute assay evaluates the percentage of immobility/ mortality and is two time points: 24 and 48 hours of exposure. The chronic assay is carried out for 21 days and evaluate if the reproduction ability is modified.

1.3.1.3 *Tetrahymena thermophila*

T. thermophila is a unicellular ciliated protozoan typically found in freshwater environments, like rivers and streams (Cheng et al., 2019). *T. thermophila* can be grown until 50 µm long and maintained in a varied range of conditions. This organism has the body protected by a semi-rigid and structurally complex cortex, containing multiple layers (Wloga et al., 2012). Like *D. magna*, *T. thermophila* can alternate between sexual and asexual reproduction. *T. thermophila* life cycle is shown in Figure 6. Briefly, the vegetative development corresponds to asexual reproduction and occurs in two semi-independent nuclear division pathways: the replication of the somatic macronucleus by an amitotic fission and the replication of the germline micronucleus by a mitosis. The conjugation, or the sexual reproduction, is a complex process that consist on mating pairs undergo a 12 hours developmental program involving six nuclear divisions (Cole et al., 2012).
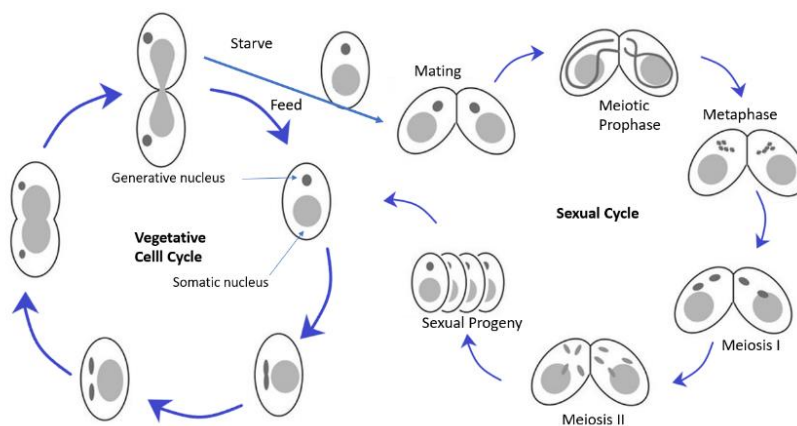
Figure 6 - *T. thermophila* life cycle (adapted from Howard-Till et al., 2017) .

Under optimal conditions has a rapid growth rate, though they can be maintained for several months in a slow growing vegetative culture with limited loss of function (Cassidy-Hanley, 2012). *T. thermophila* is responsible for the recycling of organic matter, being an extreme important organism in the community of aquatic microfauna.

Until now, *Tetrahymena* is the ciliate genera most widely employed in toxicity assay and the species *T. thermophila* is used in the commercial protozoan toxicity test kits. Though these tests have been widely used at a research level, they are still not fully standardized at OECD and ISO guidelines. The tests measure the phagocytic activity of ciliates grazing on bacteria by comparing the turbidity decrease in the samples incubated with the test chemical and control samples without toxicants.

1.3.2 Biomarkers as tools for assess toxicological effects of exposure to pharmaceuticals and illicit drugs

1.3.2.1 Oxidative stress biomarkers

Exposure to environmental contaminants including biological active substances such as pharmaceuticals can cause various adverse effects in aquatic organisms including biochemical disturbances or adaptive responses. Oxidative stress is a normal phenomenon in the body, and various enzymes are involved in maintaining the *in vivo* redox homeostasis. Nevertheless, exogenous substances including pharmaceuticals can alter biochemical homeostasis leading to the rise of reactive oxygen species (ROS) levels beyond the normal or physiological threshold levels, i.e., higher than the detoxifying capability of the local tissues. Therefore, oxidative stress is often defined as an imbalance of pro-oxidants and antioxidants. This is a harmful condition in which increases in free radical production, and/or decreases in antioxidant levels can lead to potential damage.

Unbalance between ROS and antioxidants (Gagné, 2014; Zitka et al., 2012) include changes in antioxidant enzyme activity, damaged DNA bases, protein oxidation products, and lipid peroxidation products.

Elimination of exogenous substances from the cell is catalyzed by several enzymes, i.e., phase I and II enzymes. Enzymes of phase I metabolism catalyze, via the introduction of a polar moiety, the formation of more hydrophilic forms. Phase II enzymes are involved in conjugating metabolized exogenous substances by adding endogenous hydrophilic molecules, thereby easing excretion. The activity of phase I enzymes can lead to an increase in ROS production or the generation of reactive, redox cycling intermediates. ROS by-products include the superoxide anion (O_2^-), the hydrogen peroxide (H_2O_2) and the hydroxyl radicals (OH) (Schieber et al., 2014). Antioxidant enzymes eliminate ROS and reactive chemical intermediates. Superoxide dismutase(Ford et al.) is one of the enzymes that eliminate toxic superoxide radicals. SOD catalyzes oxygen radicals to produce H_2O_2, which will be transformed into water and oxygen by catalase or glutathione peroxidase (Durak et al., 1993; Gagné, 2014).

Reduced glutathione (GSH) is a tripeptide thiol, synthetized from glutamate, glycine and cysteine, mainly in the liver and acts as a major factor in metabolic protective functions. It can react directly with ROS species, thereby detoxifying them. In addition, GSH is used as a conjugating molecule by glutathione S-transferase (GST) to ease excretion of xenobiotics. GSH is oxidized to glutathione disulfide (GSSG) in a nonenzymatic pathway. The GSH:GSSG ratio is used as a cellular toxicity marker, once is know that in a healthy cell this ratio is greater than 100:1 and in response to oxidative stress the GSH cellular concentration significantly reduces (Gagné, 2014; Slaninova et al., 2009; Wu et al., 2004; Zitka et al., 2012). GST is an important phase II conjugation enzyme during metabolism. It conjugates with exogenous compounds allowing an easy excretion from the organism. GST is an important regulator of glutathione homeostasis (Roth et al., 2011; Zhang et al., 2018).

Lactate dehydrogenase (LDH) is a tetrameric enzyme that catalyzes the transformation of pyruvic into lactic acid, under anaerobic conditions, and vice versa (Jovanovic et al., 2010; Le et al., 2010).

Acetylcholinesterase is one of two cholinesterase's presents in vertebrates and have a role in the nervous system, being found mainly at neuromuscular junctions and cholinergic synapses. AcHE hydrolyze acetylcholine into choline and acetate (Lionetto et al., 2013; Parlak, 2018).

Cell membranes are sensitive to ROS damage. An indirect thiobarbituric acid-reactive substances (TBARS) assay can be used to measure lipid peroxidation. In this assay, the end-product of lipid peroxidation, malondialdehyde (MDA), reacts with thiobarbituric acid (TBA) to form a pink MDA-TBA complex that is measured spectrophotometrically. TBARS are formed as a by-product of lipid oxidative damage (i.e., as degradation products of fats) and can be detected by the MDA measure. MDA is one of the most abundant aldehydes generated during secondary lipid peroxidation nonetheless, other low-molecular weight end products can be formed from the decomposition of some primary and secondary lipid peroxidation products. Also, not all peroxidation reactions generate MDA. Deleterious effects of MDA include induced intracellular oxidative stress, leading to membrane lesions in erythrocytes. MDA is also genotoxic as it can react with DNA to form highly mutagenic adducts and thus showing mutagenic and carcinogenic properties. As biological effects tools, biomarkers are thus widely employed in environmental monitoring and risk assessment of aquatic pollutants.

2. AIMS

The aim of this work was to investigate the possible enantioselective ecotoxicological effects of selected psychotropic pharmaceuticals and illicit drugs towards ecological relevant organisms belonging to three different trophic levels namely the protozoan, *T. thermophila*, the crustacean *D. magna* and the fish *D. rerio*.

Acute and chronic tests were conducted at different concentration levels including reported environmental relevant levels. Acute assays were conducted using the crustacean D. magna and the fish Danio rerio and chronic assays were conducted using the protozoan, *T. thermophila*.

Selected compounds were K and its metabolite NK and their enantiomers. The racemate of K and NK and the pure enantiomers were evaluated concerning their enantioselective effects to three organisms: T. *thermophila*, *D. magna* and *D. rerio*.

Ecotoxicological effects of psychotropic pharmaceuticals and illicit drugs, namely the racemate of AMP, MAMP and MDMA and the enantiomer (*S*)-MAMP, were investigated using T. *thermophila* and *D. magna.*

The growth inhibitions, percentage of mortality, developmental toxicity, behavioral alterations and oxidative stress were the *endpoints* chosen for these evaluations.

3. MATERIALS AND METHODS

3.1 Reagents and Materials

Standard of K was obtained from the pharmaceutical NIMATEK, kindly given from Dechra Veterinary Products (Bladel, Netherlands), and NK was acquired from LGC Standards (Luckenwalde, Germany). Individual standards stock solutions were prepared at 1 mg/mL in methanol (MeOH) purchased from Fisher Scientific UK (Leicestershire, United Kingdom) and stored in amber bottles at -20º C. Enantiomers of both K and NK were obtained by a semipreparative enantioseparative liquid chromatography – diode array method from the racemates and the absolute configuration of the isolated enantiomers of NK was determined by electronic circular dichroism, in a previously work (Pereira et al. 2020). Racemates AMP and MAMP were acquired from Lipomed (Arlesheim, Switzerland) and (*S*)-MAMP was acquired from Sigma-Aldrich (Steinheim, Germany). MDMA was synthetized in the Laboratory of Toxicology from the Faculty of Pharmacy of the University of Porto (Porto, Portugal). For the ecotoxicological assays two test kits were used: DAPHTOXKIT F^{TM} MAGNA and PROTOXKIT F^{TM} acquired from MicroBioTests Inc., (Gent, Belgium). Test organisms were incubated in an Infors HT Ecotron incubator (Fisher Scientific, Portugal), according the protocols of the kits. Absorbance was measure using an UV/Vis spectrometer (ATI Unicam, Leeds, England). Test reference were done using potassium dichromate ($K_2Cr_2O_7$) obtained from José Manuel Gomes dos Santos, LDA (Odivelas, Portugal).

For the *D. rerio* assays the following reagents were used: sodium chloride (NaCl), oxidized glutathione (GSSG), NADH acquired from PanReac Applichem, D(+)-saccharose ($C_{12}H_{22}O_{11}$) from Fisher Scientific UK (Leicestershire, United Kingdom), NEM from Alfa Aesar (Haverhill, MA, USA), tris base from Nzytech, sodium dodecyl sulphate, SDS ($C_{12}H_{25}O_4SNa$) from Himedia, hydrogen peroxide 30% (H_2O_2) from VWR (Radnon, PA, USA), pyruvic acid sodium salt ($C_3H_3NaO_3$) from Biosynth. Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$), potassium dihydrogen phosphate (KH_2PO_4), di-sodium hydrogen phosphate anhydrous (Na_2HPO_4), magnesium chloride ($MgCl_2$) and dimethylsulfoxide (DMSO) were acquired from Merck (Darmstadt, F.R, Germany). Xanthine, sodium hydroxide (NaOH), *N*-(2-hydroxyethyl)piperazine-N'-(2-

ethanesulfonic acid), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) ($C_8H_{18}N_2O_4S$), ethylenediaminetetraacetic acid, EDTA ($C_{10}H_{14}N_2O_8Na_2.2H2O$), potassium phosphate dibasic (K_2HPO_4), potassium chloride (KCl), the standards of superoxide dismutase (Ford et al.), catalase (Lionetto et al.), glutathione (GSH), malondialdehyde (MDA) and dichlorofluorescin (DCF) were acquired from Sigma Aldrich (St Louis, MO, USA). The dichlorofluorescin-diacetate (DCFH-DA) were acquired by Cayman Chemicals (Ann Arbor, MI, USA).

All other reagents used in the *D. rerio* assay were purchased from the brand with highest purity found in the market.

For protein quantification and oxidative stress test a microplate spectrophotometer Power Wave XS2 by BioTek with the Gen5 software was used. For quantification of glutathione (GSH), oxidized glutathione (GSSG) and reactive oxygen species (ROS) a fluorescence spectrophotometer Cary Eclipse by Varian, with Cary Eclipse Advanced Reads Application was used.

The stereomicroscope Nikon SMZ800 was used for the determination of the alevins length of the *D. rerio* and the Cannon G7X for the behavioral test.

3.2 Ecotoxicological Assays

3.2.1 *Danio rerio*

The *D. rerio* tests were performed according to OECD test Guideline 236 (OECD, 2013) and Portuguese legislations on animal welfare.

Maintenance

The *D. rerio* maintenance was conducted as described by Félix et al 2014,2017. Briefly, wild-type (AB strain) adults' *D. rerio* were kept in 20 L glass aquaria (Figure 1S, annex), in a maximum density of 40 animals. The aquariums were kept in a semi-closed water system with both mechanical and biological filtration, at 28 ± 0.5°C, in a 14:10 h light: dark cycle. The fish were fed twice a day with a commercial diet (Zebrafeed by Sparos)

supplemented with *Artemia sp. nauplii*. *D. rerio* embryos were obtained from spawning adults grouped in tanks overnight. Spawning was induced in the morning, with the beginning of the light period. Before being arbitrarily distributed to 6 wells plate, the newly fertilized eggs were collected and rinsed with chloramine T (Félix et al., 2014; Felix et al., 2017).

Exposure test

Figure 7 shows the experimental design. Briefly, live embryos with 2-3 hpf and normal developed were selected. About 60 embryos per group were randomly
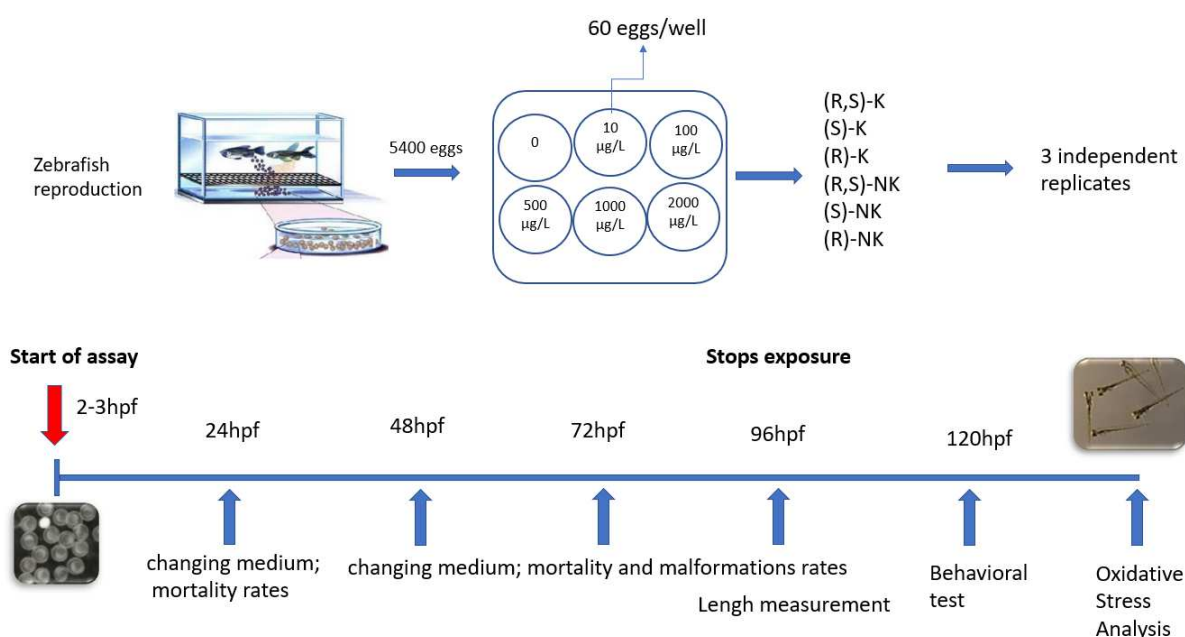
Figure 7 – *D. rerio* experimental design.

distributed for a six well plate with 5 mL of exposure solutions, i.e., controls (medium), K and NK racemates and isolated enantiomers in the concentrations of 10, 100, 500, 1000 and 2000 µg/L. The range of K and NK racemates and enantiomers concentrations was selected based on Félix et al 2014 work and a preliminary assay (Félix et al., 2014). Three independent replicates for each assay were done. The work solutions were daily renovated, and the mortality and malformations rate were recorded (dead animals were removed). After the 96 hpf the work solutions were removed, and the larvae were washed three times with medium. Then, ten larvae of control and each concentration were arbitrarily selected and with the stereomicroscope Nikon SMZ800 (1.7x) and using

the Progress Capture Pro-2.8.8 software, photographs were taken to measure larvae length.

Behavioral testing

After 120 hpf, behavioral testing was performed. This test consists of recording larvae in 6-well plates, each well was filled with 5 mL of melted 0.5% agarose. Once solidified, a circular portion was stamped out using a sharp stainless-steel ring (27 mm diameter, 5 mm deep and 1.5 mm thick) creating this way a circular swimming area that improve the optics at the edge of each well preventing shadows and blind spots. All groups were equally present in each well-plate, minimizing difference in experimental timing. Three independent replicates of 1 larvae were used (Felix et al., 2017). The plates were position over a laptop screen and the camera (Cannon G7X) were perpendicular position are shown in Figure 8.
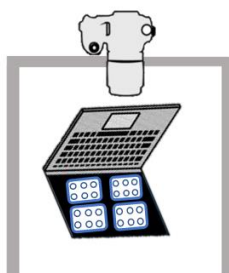
Figure 8 - Scheme showing the camera position used to film the larvae in the behavioral test.

Oxidative Stress

The remaining larvae were transfer into an eppendorf tube with 400 µL of oxidative stress buffer (0.32 mM of sucrose, 20 mM HEPES, 1 mM $MgCl_2$ and 0.5 mM of phenylmethyl sulfonylfluoride) and stored at -20°C until oxidative stress determination. For oxidative stress seven enzymes and lipid oxidative parameters were tested: SOD, CAT, GST, GSH, GSSG, thiobarbituric acid reactive substances (TBARS), lactate dehydrogenase (LDH), acetylcholinesterase AcHE and reactive oxygen species (ROS).

The content of the eppendorf tube was homogenized using a TissueLyser II from Qiagen with the frequency of 30 for 1 minute, and to assure the rupture of the tissues disposable steel spheres (4.5mm) was used. Then, samples were centrifuge for 10 min, at 12000 g at 4°C and the supernatant collected. For quantification of the protein concentration a Tack 3 from the BioTek and 2μL of sample were used. This method quantifies the bovine serum albumin (BSA) protein.

For determination SOD, CAT, GST and LDH activities, 10 μL of sample were added to each well, followed by the buffer and then a first 2 min read using a microplate spectrophotometer. Then, a catalyzer was added and another 3min read was done. The reads were done in a microplate spectrophotometer Power Wave XS2 by BioTek with the Gen5 software at 30°C. The extensive protocol used can be found in Table 1S of annex.

Briefly, SOD activity was determined spectrophotometrically according to Durak et al 1993, with a wavelength of 560 nm (Durak et al., 1993). The calibration curve was done with SOD standards in a concentration range of 0-30 U/mL, and the activity were express in U/mg using the following formula:

$$\text{Formula 1:} \quad v = a \times \frac{V_{cuvette}}{V_{sample} \times \rho}$$

v- enzyme activity, express in U/mg

a-specific activity, calculate with the calibration curve

Vcuvette-assay volume (μL)

Vsample-sample volume (μL)

ρ-sample concentration of protein (mg/mL)

CAT activity was determined spectrophotometrically according to Claiborne, 1985, with a wavelength of 240 nm (Claiborne, 1985). The calibration curve was done with CAT standards in a concentration range of 0-6 U/mL, and the activity was expressed in U/mg using the above formula.

AcHE and LDH were determined spectrophotometrically, at 30°C, according to Rodriguez Fuentes et al. 2015 and Domingues et al. 2010, respectively (Domingues et al., 2010; Rodriguez-Fuentes et al., 2015). For AcHE, the activity was determined using the TNB

extinction coefficient (ε) of 13.6 mM^{-1}cm^{-1} at 405 nm, and for LDH the NADH extinction coefficient used was 6.22 mM^{-1}cm^{-1} at 340 nm. The activities were express in μmol TNB/min.mg protein for AcHE and in μmol NADH/min.mg protein for LDH using the following formula:

Formula 2:
$$v = \frac{r_A}{l \; x \; \varepsilon \; x \; V_B} \; x \; \frac{Vcuvett}{Vsample \; x \; \rho}$$

v- enzyme activity
r_A- obtained slope (abs.min^{-1})
l- optical path length (0.8cm)
ε- extinction molar coefficient to the respective wavelength
V_B- stoichiometric coefficient of the reaction (=1)

Vcuvette-assay volume (μL)

Vsample-sample volume (μL)

ρ-sample concentration of protein (mg/mL)

The GST activity was determined spectrophotometrically according to Habig 1981, with a wavelength of 340 nm and using the CDNB extinction coefficient of 9.60 mM^{-1}cm^{-1} (Habig et al., 1981). The activity was express in μmol CDNB/min.mg protein using the formula 2.

The GSH levels were determined fluorometrically, using a Varian Cary Eclipse (Varian, USA) spectrofluorometer equipped with microplate reader. According to Misra et al 2009 both GSH and GSSG were measured at 320 nm (excitation wavelength) and 420 nm (emission wavelengths) (Misra et al., 2009). A calibration curve was done with GSH and GSSG standards in a concentration range of 0-500 μM, and the activity expressed in μmol GSH/mg protein for GSH and in μmol GSSG/mg protein, using the formula 1.

TBARS activity was determined spectrophotometrically according to Wallin et al 1993, at 530 nm and 600 nm (Wallin et al., 1993). A calibration curve was done with MDA standards in a concentration range of 0-500 μM, and the activity was expressed in μmol MDA/mg protein using the formula 1.

According to Deng et al the ROS levels was determined fluorometrically at 485 nm (excitation wavelength) and 530nm (emission wavelength) (Deng et al., 2009). ROS

accumulation was estimated based in a calibration curve done with DCF, in a concentration range of 0-500 µM, and using the formula 1.

3.2.2 *Daphnia magna*

For the *Daphnia* acute tests, we used the MicroBioTests DaphtoxKit F magna that are in accordance with the European Standards, OECD test Guideline 202 (OECD, 2004). This kit uses the dormant eggs (ephippia) of the crustacean *D. magna.* These eggs are protected by a chitinous capsule called ephippium, and can be stored for long periods of time, in darkness at 5°C (± 2°C), without losing their viability. Figure 9 shows the experimental design.

Standard Freshwater was prepared dissolving, in distilled water, solutions of concentrated salts (sodium bicarbonate ($NaHCO_3$) 67.75 mg/L; calcium chloride dihydrate ($CaCl_2.2H_2O$) 294 mg/L; magnesium sulfate heptahydrate ($MgSO_4.7\ H_2O$) 123.25 mg/L and potassium chloride (KCl) 5.75 mg/L). This medium was aerated for about 20 minutes with the aid of an aeration pump and a magnetic stirrer, before use.
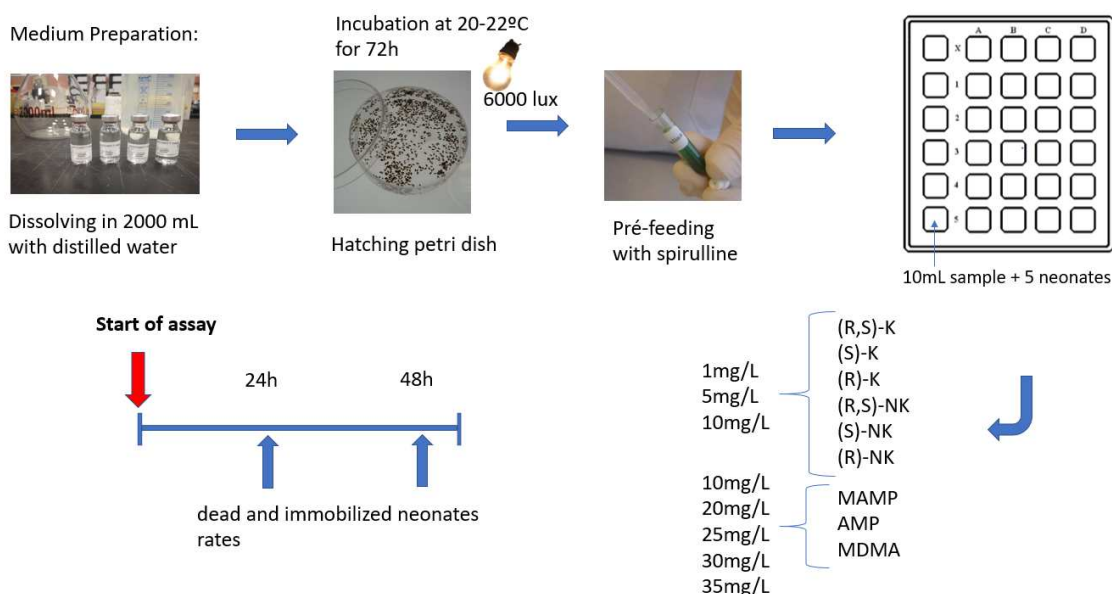

Figure 9 - *D. magna* experimental design.

To hatch the ephippia, 3 days prior to the start of the test, the contents of one tube with ephippia was placed into the microsieve and clean meticulously with distilled water to make sure all storage medium was removed. Then, the ephippia were transfer to the

hatching petri dish, previously filled with 15 mL pre-aerated Standard Freshwater, all ephippia were guaranteed to be submerged and incubated for 72 h, at 20-22°C under continuous illumination of min. 6000 lux. To perform a complete test, 120 neonates younger than 24 h were feed with a suspension of spirulina microalgae 2 h prior to perform the toxicity test.

10 mL of each test concentration 1, 5 and 10 mg/L for K and NK enantiomers and 10, 20, 25, 30 and 35 mg/L for racemates AMP, MAMP and MDMA and (*S*)-MAMP or Standard Freshwater for the control were added into each well. Each test concentration was performed in 4 replicates. DaphtoxKit test plates were provided on the left side with a column of "rinsing wells", that prevented dilution of the toxicant during the transfer of the test organisms. Five neonates were transferred with a micropipette into each well in a total of 20 neonates for each concentration and control. The plates were put the incubator at 20°C, in the darkness. After 24 h and 48 h incubation the number of dead or immobilized neonates were recorded and calculated the mean and the percentage effect at each substance concentration.

In order to check the correct execution of the test procedure and the sensitivity of the test animals the protocol previously described were performed using the reference toxicant potassium dichromate ($K_2Cr_2O_7$) in the following concentration: 0.32, 0.56, 1, 1.8 and 3.2 mg/L. For the assay be validated the mortality in the controls should not exceed 10%.

3.2.3 *Tetrahymena thermophila*

For *Tetrahymena* test a MicroBioTests ProtoxKit F was used based on European Standards OECD test Guideline 244 (OECD, 2017). This kit uses ciliates in a stock culture vials which are maintained alive, at room temperature, for several months. The test is based on the turnover of substrate into ciliate biomass, a normal proliferating cell cultures clear the substrate suspension in 24 h. Optical density (OD) measurements of the turbidity quantify the degree of growth inhibition. Figure 10 shows the experimental design.

To prepare the ciliate inoculum 500 µL from the ciliate stock culture were transfer using a sterile syringe and add to 1 mL of distilled water. Then the OD was measured at 440 nm to assure the concentration of ciliates. For that the dilution factor (F) and the dilution volume (V) using the following formulas:

$$F = \frac{ODvalue}{0.040}$$

$$V = 0.5 \times (F-1)$$

Transfer the V mL, calculate before, of distilled water to 500 µL of the diluted ciliate stock, having this way the ciliate inoculum.

For preparation of the substances solutions at the different concentration and control a Standard Freshwater, was prepared dissolving in distilled water sodium bicarbonate (NaHCO$_3$) 96 mg/L, calcium sulfate dihydrate (CaCl$_2$.2H$_2$O) 60 mg/L (two flasks), magnesium sulfate heptahydrate (MgSO$_4$.7H$_2$O) 123 mg/L and potassium chloride (KCl) 4 mg/L.
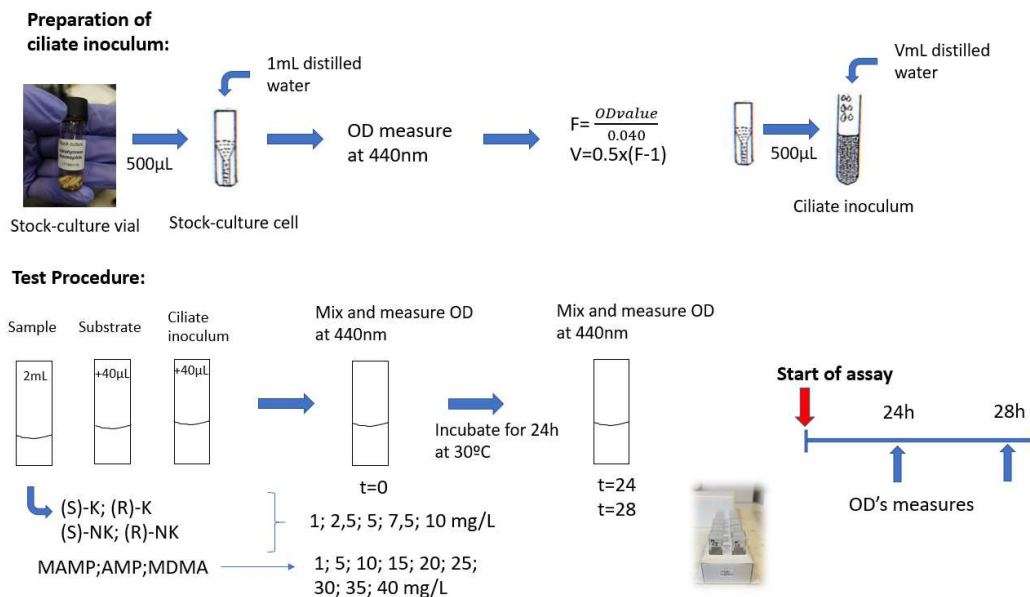
Figure 10 - *T. thermophila* experimental design.

For this test two replicates for each test concentrations were performed. Each test cells contain 2 mL of sample, with the follow concentrations 1, 2.5, 5, 7.5 and 10 mg/L for K and NK enantiomers and 1, 5, 10, 15, 20, 25, 30 and 35 mg/L for racemates MAMP,AMP and MDMA and (*S*)-MAMP (Standard Freshwater for the controls), 40 µL of substrate

previously prepared by adding reconstitution medium into substrate vial and mixing thoroughly, and 40 µL of ciliate inoculum. Before reading the OD (at 440 nm) the test cells were mixed, to make sure all ciliates were in suspension and not deposited in the bottom. First measurement corresponded to the T0h. The test cells were put in an incubator, in darkness at 30°C for 24h -28h. After 24 and 28 h, the OD was measured.

To calculate the percentage of growth inhibition following equation was used:

$$\% \text{ growth inhibition (c1-c5)} = (1 - \frac{\Delta OD(c1-c5)}{\Delta ODc0}) \times 100$$

where $\Delta OD(C1-C5)$ is the difference between the absorbance after the incubation (T24h or T 28h) and prior to the test (T0h) and $\Delta OD(C0)$ is the difference in absorbance in the control tests.

In order to check the performance and validate the assay a to test protocol was carried out using the potassium dichromate ($K_2Cr_2O_7$), in the following concentrations: 5.6, 10, 18, 32 and 56 mg/L and the OD decrease in the controls must be at least of 60%.

3.3 Statistical Analysis

Statistical analysis was performed using the IBM SPSS program, version 24 for Windows. Due to the non-normality of the data (tested with the Shapiro-Wilk test) and the small sample size, the Kruskal-Wallis test non-parametric test was used to analyze the significance of the differences between compounds and between concentrations, followed by multiple comparison tests (Dunn's test) to identify the compound/ concentration pairs with statistically significant differences. A significance level of 5% was considered, that is, the differences were considered statistically significant when the significance value of the tests was less than 0.05 ($p < 0.05$).

4.RESULTS AND DISCUSSION

4.1 Enantioselective ecotoxicity assays of ketamine and norketamine

The possible enantioselective effects of K and NK were investigated using three ecological relevant organisms belonging to different trophic levels, the fish *D. rerio*, the crustacean *D. magna* and the protozoan *T. thermophila*. Acute fish toxicity tests with *D. rerio* embryo, acute assays with *D. magna* and chronic tests with *T. thermophila* were performed.

4.1.1 *Danio rerio*

The *D. rerio* embryo toxicity assay was performed with newly fertilized eggs exposed to the selected range of concentration of K and NK racemate and isolated enantiomers, i.e., from 10 to 2000 μg/L and different endpoints were assessed: larvae length, malformation (Figure 2S in the annex) and mortality of the embryos, oxidative stress process and behavior responses. Statistical analysis data considering the differences between compounds and between concentrations can be found in annex 4.

Larvae length

The larvae length was recorded after 96 hpf. Determination of this parameters is important to evaluate the general growth rate. There were no significant alterations on the larvae length in all tested compounds, i.e., K and NK racemates and corresponding enantiomers in the selected range of concentration (Figure 11). Nevertheless, considering mean larvae length assessed for each concentration and compound (Figure 12), a significant difference was observed among NK racemate and its enantiomers. In fact, exposure to both NK enantiomers caused a significant decreased in larvae length compared to (*R,S*)-NK. Nevertheless, there was no significant differences among enantiomers and thus no enantioselective effect was observed (Table 1S, annex 4). These results are in accordance with Félix et al 2014. In fact, there was not significant alterations in the body length in the 144 hpf larvae, exposed to 0.2, 0.4, and 0.8 mg/mL of (*R,S*)-K (Félix et al., 2014).
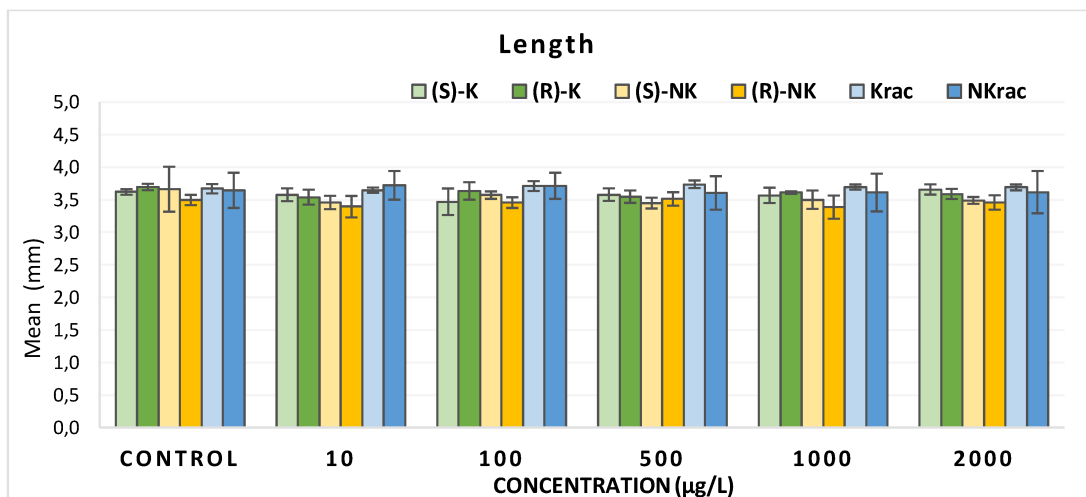
Figure 11– Larvae length measures (mm) for D. rerio after 96 hpf for each compound and concentration.
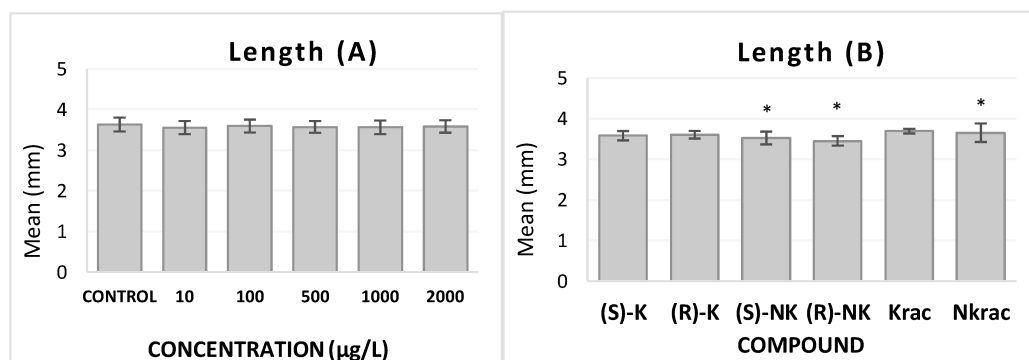
Figure 12 - Mean larvae length (in mm) for D. rerio after 96 hpf for each concentration (A) and compound (B). The * represents statistically significant difference between the (R,S)-NK and its enantiomers (*p < 0.001)

Mortality

Figure 13 shows the percentage of mortality for each compound at each concentration. There were not significant differences observed for each compound in the selected range of concentrations. This means that mortality was not dependent of the concentration for each compound. Nevertheless, when considering mean total mortality and comparation among the compounds a significant difference was observed (Figure 14). In fact, (*R*)-K showed higher % of mortality rather than all other compounds. Besides, a significant difference was observed comparing (*R*)-K with both (*R,S*)-K and (*R,S*)-NK. Nevertheless, no significant difference was observed between enantiomers, i.e. (*R*)-K and (*S*)-K.
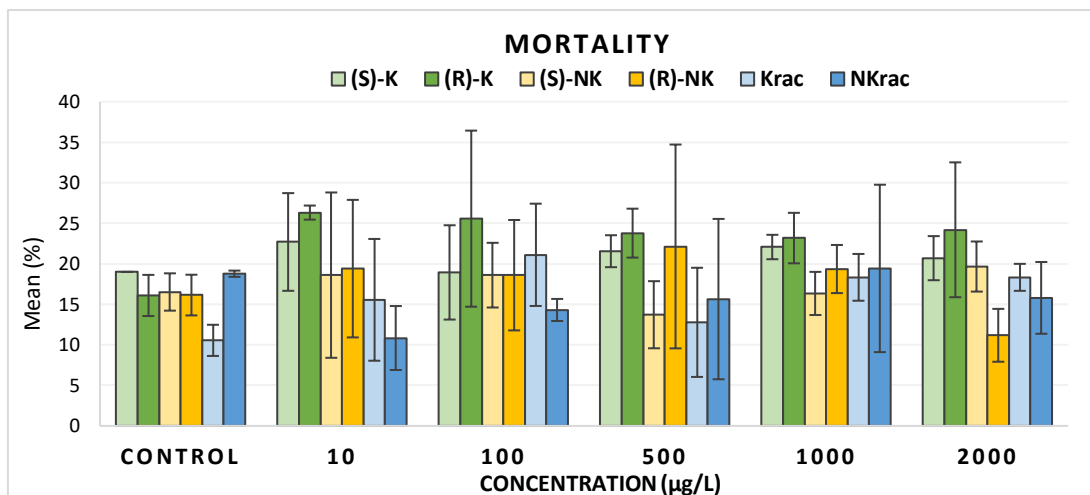
Figure 13 – Mortality (%) for D. rerio larvae after 96 hpf for each compound and concentration.
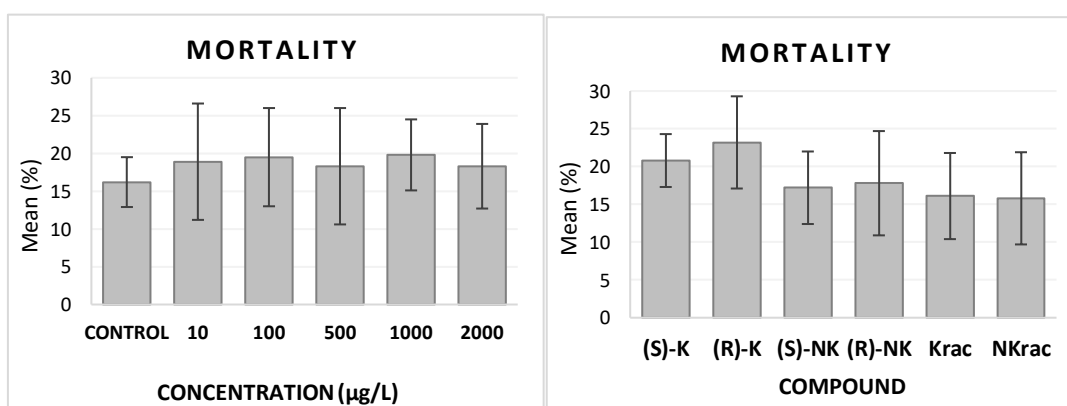
Figure 14 – Mean total mortality (%) for D. rerio larvae after 96 hpf for each compound and concentration.

Malformations

Figure 15 shows the percentage of malformation for each compound in the range of the selected concentration. There were significant differences for all compounds among control and tested concentrations, nevertheless, malformations were not dependent of the concentrations. Considering mean values (Figure 16) it was possible to observe that both K enantiomers presented significant differences from the others compounds and (*R*)-K induce more malformations on *D. rerio* embryos showing the highest toxicity. However, no significant differences were observed among both enantiomers.
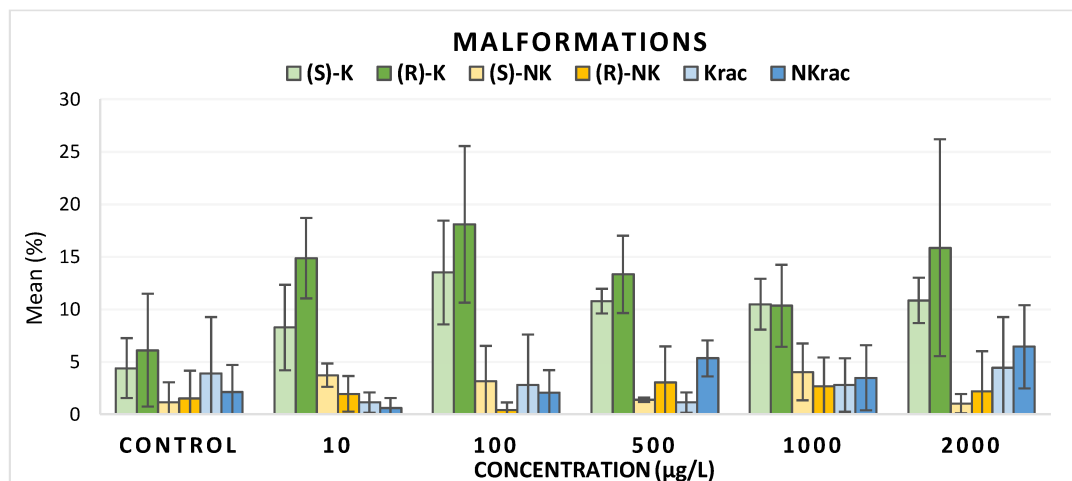
Figure 15 - Percentage of malformations for D. rerio larvae after 96h hpf for each compound and concentration.
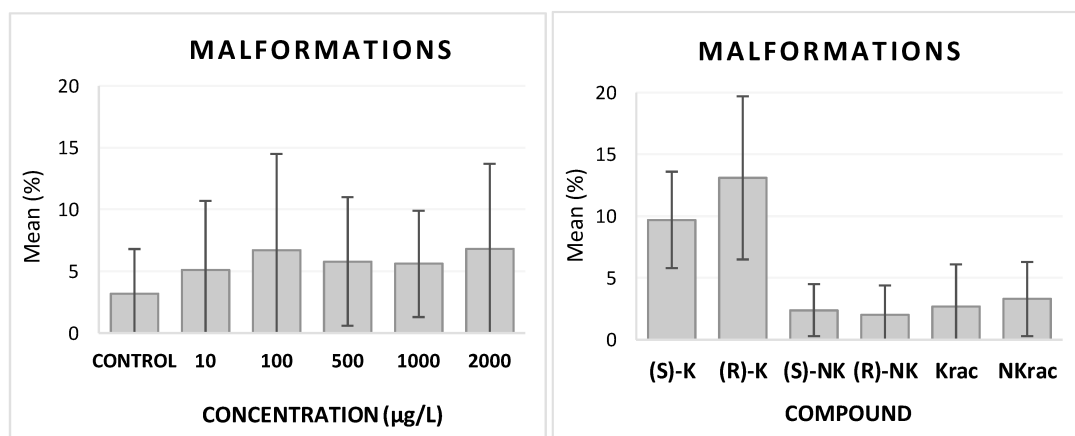
Figure 16 – Mean percentage of malformations for D. rerio larvae after 96h hpf for each concentration and compound.

Behavioral testing

Neurotoxicants substances can lead to changes in the organism's behavior (e.g. predator escape, feeding and mating behavior) decreasing fitness which may cause populations decline and consequently severe impact on ecosystems. Early life stages are more prone to xenobiotics and some detrimental effects are only visible later in their lives. In other to investigate the behavior response of *D. rerio* exposed to both K and NK racemates and enantiomers, five parameters were assessed: speed, total distance moved, percentage of time active, distance to center and the absolute turn angle.

K racemate induced a significant decrease of speed, total distance moved and of the absolute turn angle (Figure 17a/b, 18a/b and 19a/b), however, a significant increase in the percentage of time active was noted (Figure 20b). This means that the larvae exposed to (*R,S*)-K are more agitated, although they stay in the same spot. For all five parameters analyzed the effect was independent of the concentrations, as show in Figures 17a, 18a, 19a, 20a and 21a. It is interesting to notice that both K enantiomers showed the higher speed but lower activity in contrast to the racemate. No enantioselective effect was observed.
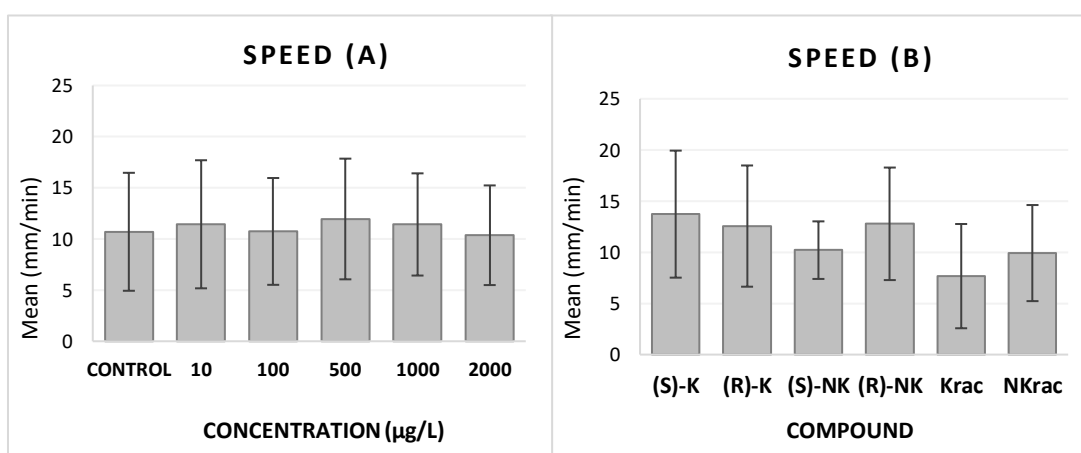
Figure 17a – Mean total speed of larvae after exposure to K and NK racemate and enantiomers by concentration and compound.
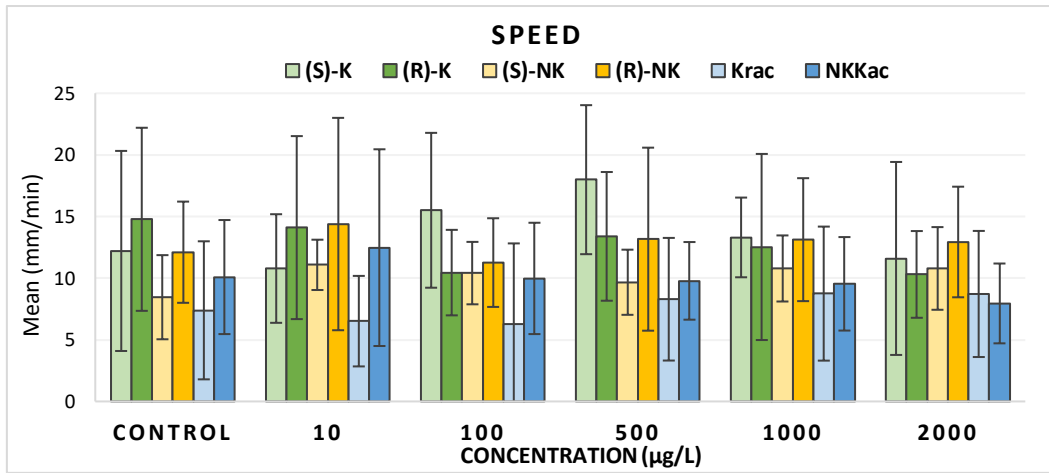
Figure 17b – Speed evaluation (in millimeters per minute) of D. rerio larvae after 120 hpf.
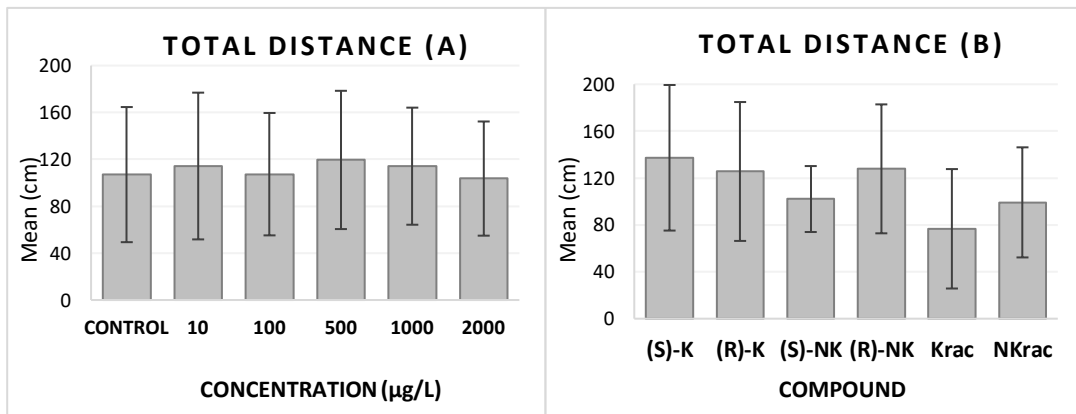
Figure 18a - Mean total distance of larvae after exposure to K and NK racemate and enantiomers by concentration and compound.
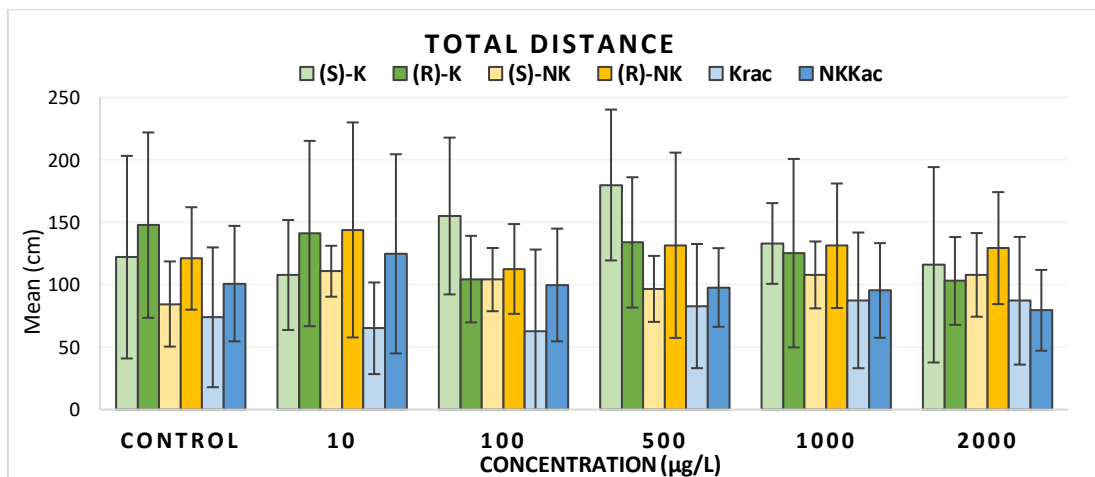
Figure 18b – Total distance moved evaluation (in cm) of D. rerio larvae with 120 hpf.

Absolute turn angle has been use as a measure of motor coordination (Bridi et al., 2017). (*R,S*)-K had less absolute turn angle, with significant differences between (*R,S*)-NK and both enantiomers, although no difference was showed between enantiomers.
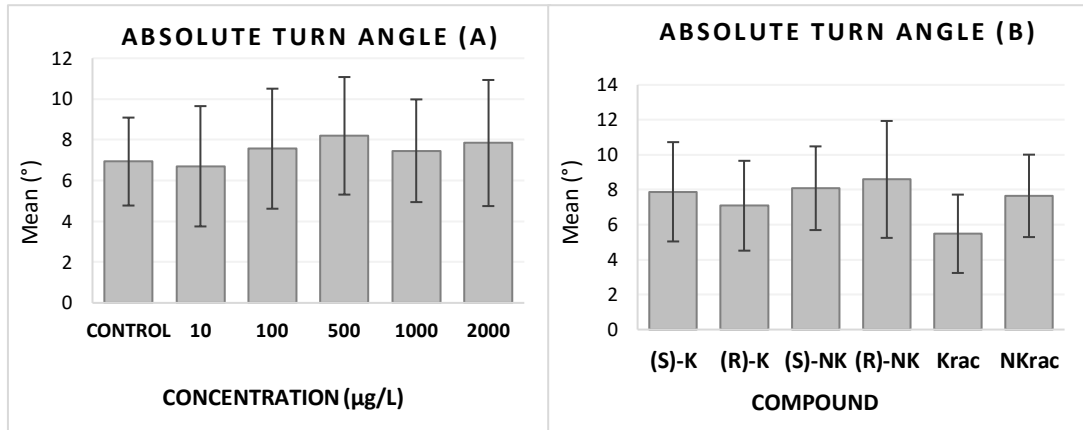
Figure 19a– Mean total absolute turn angle after exposure to K and NK racemate and enantiomers by concentration and compound.
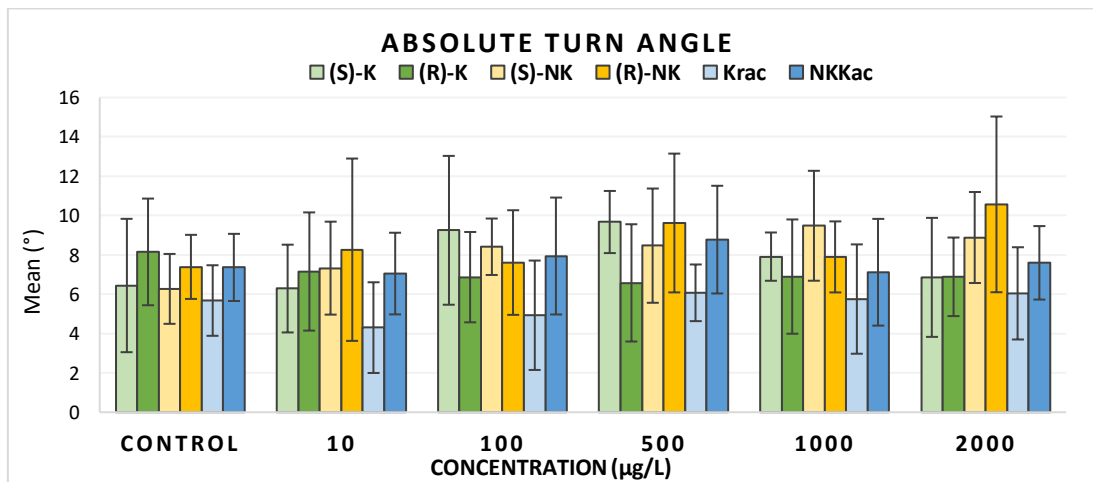
Figure 19b – Mean absolute turn angle evaluation of D. rerio larvae after 120hpf.
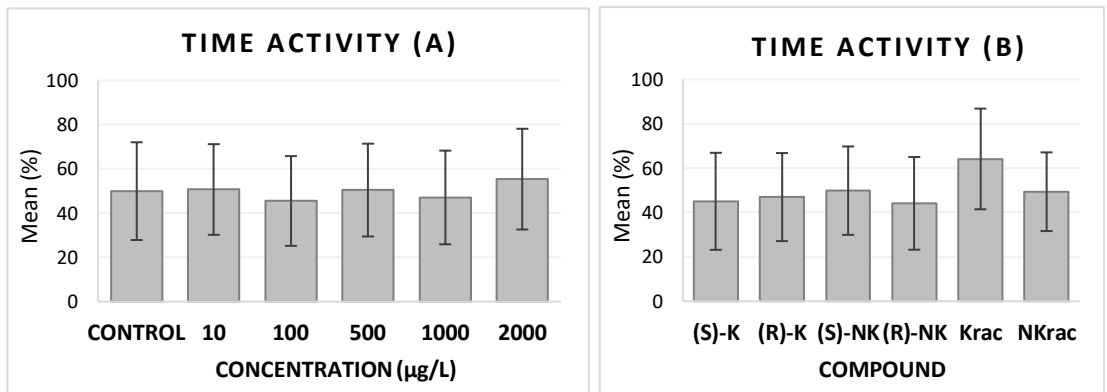
Figure 20a - Mean total time activity of larvae after exposure to K and NK racemate and enantiomers by concentration and compound.
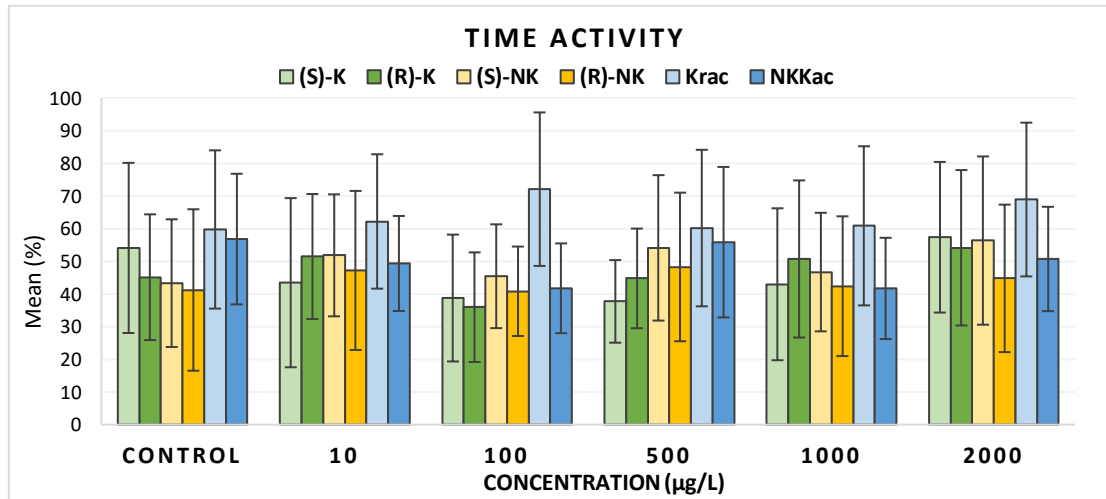
Figure 20b – Percentage of time D. rerio larvae, with 120hpf, were active.

The distance to the center did not suffer alterations unlike Félix et al 2016 work, where only the distance to the center was significantly altered (Félix et al., 2016).
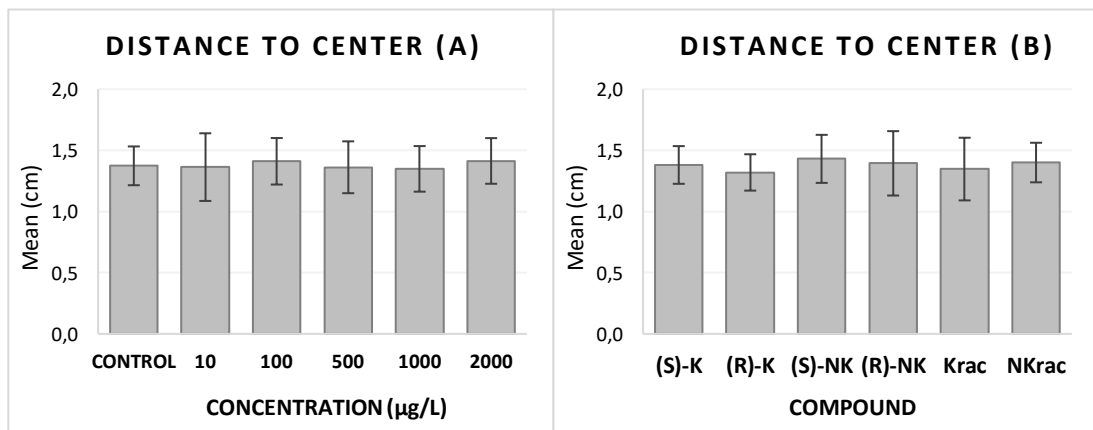
Figure 21a - Mean total distance to center after exposure to K and NK racemate and enantiomers by concentration and compound.
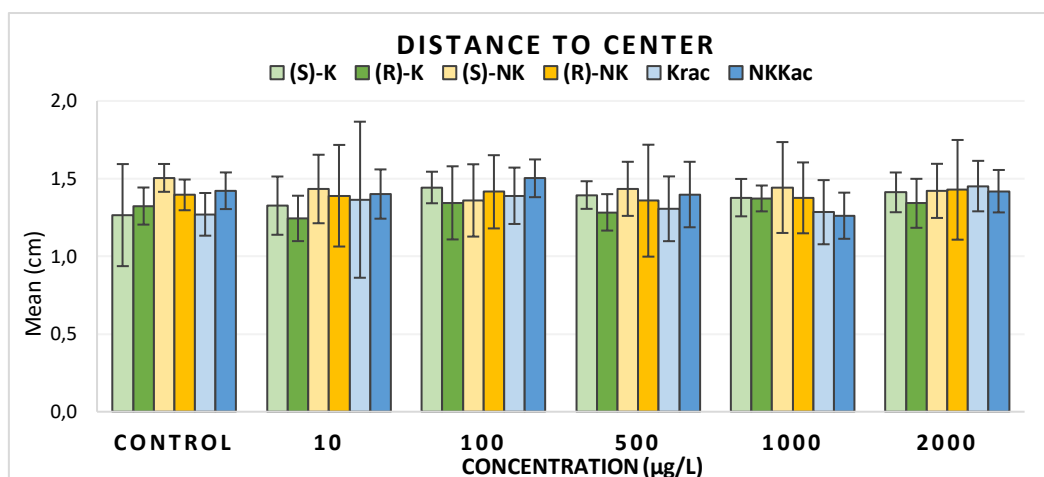
**Figure 21b-** Distance to the center of the well evaluation (in cm) of D. rerio larvae with 120hpf.

Oxidative Stress

The enzymatic activity of several biomarkers including nervous systems enzymes as AcHE, LDH, antioxidant enzymes (SOD, CAT, GST, GSSG) and lipid peroxidation (LPO) (TBARS) were assessed.

ROS content and SOD, CAT activities in the control and after 7 days of exposure are shown in Figure 22**.** It is known that exposure to xenobiotics may induce ROS production in organisms, causing oxidative stress. The production of ROS increased in a dose-dependent way, and (*R*)-NK presented the higher ROS production. A significant difference was observed among ROS content in embryos exposed to (*R*)-NK comparatively to (*R,S*)-NK and both K enantiomers. Nonetheless, no significant differences were found between NK enantiomers. Also, no significant differences were observe between (*R,S*)-K and K enantiomers. Both SOD and CAT are key antioxidant enzymes protecting cells from oxidative damage. SOD is the primary enzyme of cellular antioxidant defense, which catalyzes the dismutation of superoxide (O_2^-) into molecular oxygen and hydrogen peroxide (H_2O_2). Although H_2O_2 is not a ROS, it is a powerful and toxic oxidizing agent that plays an important role in oxygen toxicity. Regarding SOD activity, all compounds showed a general dose dependent activity of SOD, i.e., the highest concentrations showing the highest SOD activity levels except for (*R*)-NK, where no significant differences were found among concentrations. Both NK enantiomers and racemate showed the higher activities being (*R,S*)-NK the compound with the highest

activity of SOD nevertheless, (*R*)-NK showed the highest levels of ROS content. This result may indicate that other detoxifying mechanism can be activated, or that (*R*)-NK can cause damage to the cells. No enantioselective effects were found for NK enantiomers. No significant differences between the higher concentrations and the control for (*R,S*)-NK was shown. This could mean that a threshold limit may have been reach, and another detoxification pathway may be using. Also, significant difference were observed among (*R,S*)-NK and both (*R,S*)-K and its enantiomers.

Figure 22 shows the CAT activity in the control and after 7 days of exposure. It can be observed that (*S*)-NK showed the highest CAT activity comparatively with (*R*)-NK and (*R,S*)-NK. It is interesting to note that, (*R*)-NK and (*R,S*)-NK showed the highest levels of ROS content and SOD respectively. In fact, (*R*)-NK and (*R,S*)-NK showed the lower CAT activity and a significant difference was observed among both enantiomers showing a enantioselective effect. It seems that, (*R*)-NK cannot activate this protect system and thus corroborating the highest levels of ROS. The highest levels of CAT were found for both K enantiomers and racemate.   These could mean that another detoxification pathway is being used, like GSH. Both CAT and GSH play an important role in the reduction of H_2O_2. In fact, (*R,S*)-NK and (*R*)-NK induce GSH activity in 459.9 and 411.5 µmol GSH/mg protein, respectively. The oxidized form of GSH, the GSSG, have higher activity than GSH, in all tested concentrations. The GSH activity did not reach into 600 µmol GSH/mg, but the GSSG activity is above 600 µmol GSSG/mg. These higher values of GSSG over GSH, exhibit a decline over the normal GSH:GSSG ratio (100:1), indicating an existence of oxidative stress (Slaninova et al., 2009). Lower levels of GSSG were found for bot K racemate and enantiomers. This is also in accordance with the highest levels found for GST activity found for both K racemate and enantiomers.
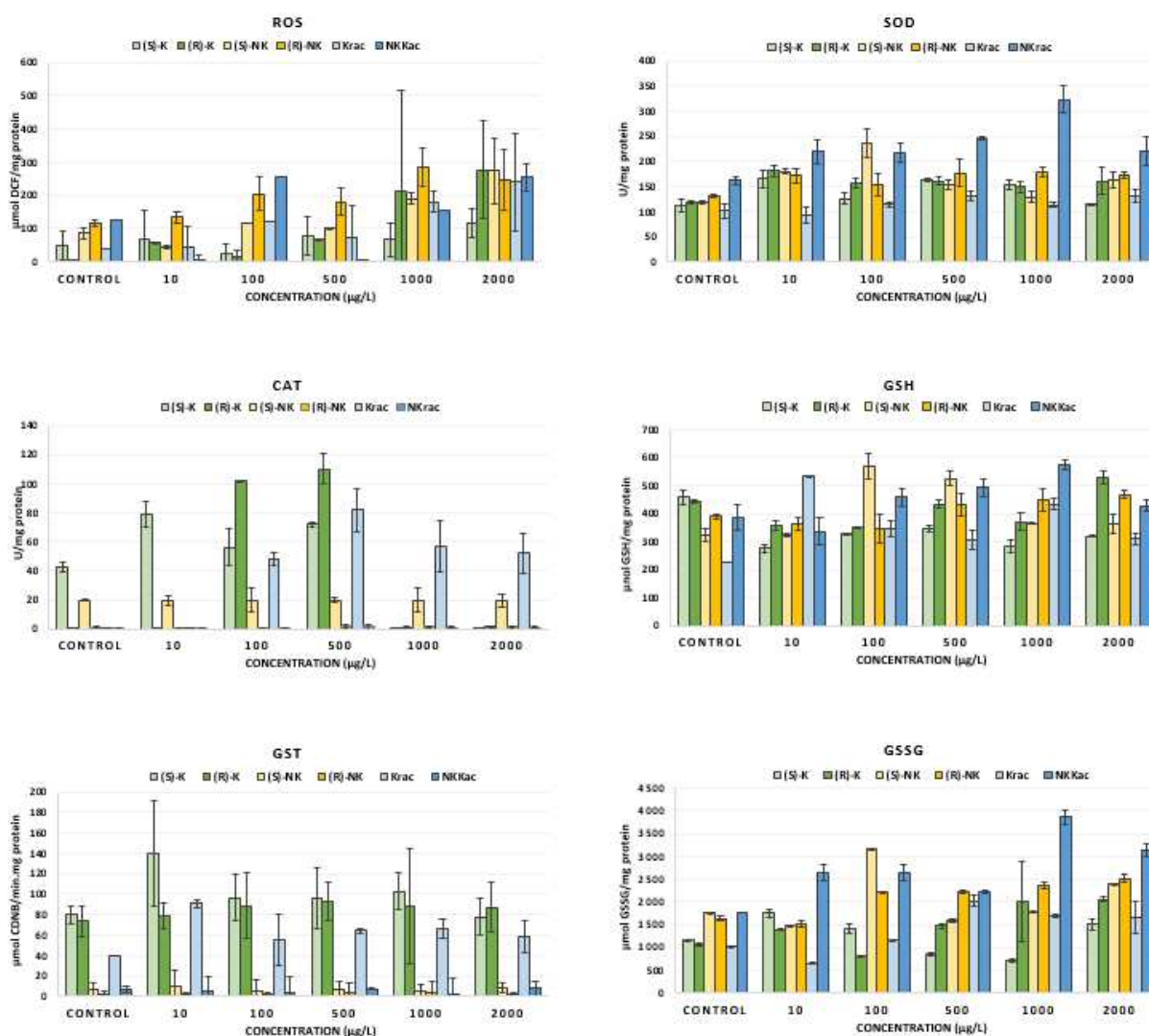
Figure 22 – Effects of both K and NK racemates and enantiomers on ROS, SOD, CAT, GSH, GST and GSSG activity after 7 day of exposure.

AcHE and LDH activities in the control and after 7 days of exposure to K and NK racemate and isolated enantiomers in the range of 10 to 2000 µg/L is shown in Figure 23. Compared to the control, both NK racemate and isolated enantiomers increased enzymatic activity at 2000 µg/L for both AcHE and LDH. High levels of LDH activity were observed for (*R*)-K at 10 and 500 µg/L compared to the racemate K, (*S*)-K enantiomer and both NK racemate and enantiomers. AcHE plays an important role in the regulation of the cholinergic system as its responsible for the hydrolysis of the acetylcholine in the cholinergic synapses ending the nerves impulse. Other studies reported that some

pharmaceuticals, such as diazepam, could inhibit cholinesterase activity in a crustacean (*Artemia parthenogenetica*) and lead to neurotransmission impairment (Nunes et al., 2006).
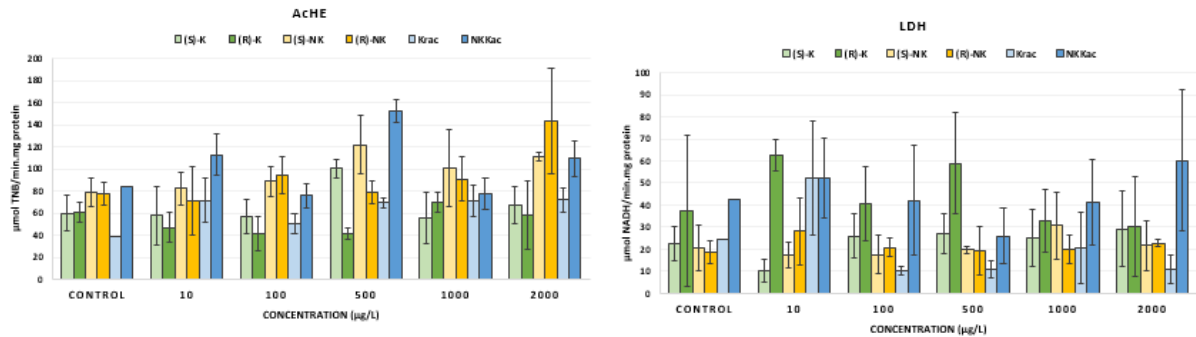
Figure 23 - Effects of both K and NK racemates and enantiomers on AcHE and LDH activity after 7 day of exposure.

TBARS content in the control and after 7 days of exposure is shown in Figure 24. TBARS was evaluated by the measure of MDA content. MDA is a byproduct of the decomposition of unsaturated fatty acid peroxides that are generated from ROS. The formation of MDA is considered to be a sign of occurrence of LPO. In the present study, exposure to both NK racemate and enantiomers showed the highest levels of TBARS while no enantiomer differences and alteration after exposure to K racemate and enantiomers were observed. These results are in accordance with the high GSSG occurrence for both NK racemate and enantiomers. Choi et al 2010 after expose *D. rerio* adults to silver *nanoparticles* verified an increased levels of MDA and a decreased of CAT activity (Choi et al., 2010).
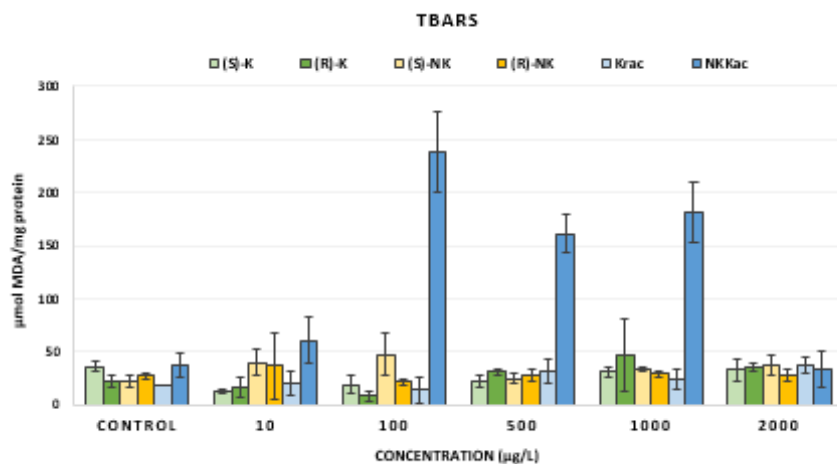
Figure 24 - Effects of both K and NK racemates and enantiomers on MDA content after 7 day of exposure.

There is no information about the enantioselective oxidative stress responses of *D. rerio* to K and NK. Thus, the enzymatic activity of several biomarkers including antioxidant enzymes (SOD, CAT, GST, GSSG), nervous systems enzymes as AcHE, LDH and lipid peroxidation (LPO, TBARS) were assessed. Oxidative stress is a common mechanism caused by an imbalance between the production of ROS and the ability of biological system to readily detoxify the reactive intermediates or easily repair the resulting damage. ROS is an important biomarker for assessing toxicity. The higher ROS content was found for (*R*)-NK while SOD higher activities were found for both (*R*,*S*)-NK and (*R*)-NK, being (*R*,*S*)-NK the compound which induced the highest levels. It was interesting to notice that CAT activity was higher in (*S*)-NK rather than (*R*)-NK and (*R*,*S*)-NK indicating that other detoxifying mechanisms such as GSH may be activated. In fact, high levels of GSH and also GSSG were found for both NK enantiomers and racemate and the ration GSH:GSSG indicated oxidative damage. Also, TBARS showed high levels for both NK and NK showing LPO damage. Li et al also showed high levels of ROS in in rat PC12 cells after exposure to acetofenate and a decrease of SOD and CAT activity and that effects were enantioseletive. In fact, it was demonstrated that (*S*)-(+)-acetofenate possessed the strongest effects in induction of ROS and a decrease in SOD and CAT activities, and increase in MDA levels.

4.1.2 *Daphnia magna*

D. magna is a commonly used test organism for studying aquatic toxicology due to its ecological relevance. Main reasons are its ubiquitous occurrence, it's an active filter of water ingesting the toxics from water or absorbing them by their exoskeleton and form an important link in food chains. Further, it is easily cultured in laboratory conditions. The OECD and ISO describe guidelines and procedures for *D. magna* toxicity. Thus, acute immobilization toxicity assays were performed for 48 h, according to OECD and ISO guidelines. The number of dead or immobilized neonates were recorded at 24 and 48 h of exposure to the selected pharmaceuticals and illicit drugs, namely K, NK, AMP, MAMP and MDMA. For each concentration and control, a minimum of 20 *D. magna* was used*.* The mortality percentage were calculated based on the swimming test organism on each well versus each concentration tested. The number of dead plus immobilized organism did not exceed 10% in the controls, and a reference test with $K_2Cr_2O_7$ was performed to validate the assays.

Regarding enantiomers of K and NK, both were tested at 1, 5 and 10 mg/L to investigate possible enantioselective effects. In these range of concentrations, the NK enantiomers did not show toxicity to *D. magna.* Nevertheless, for both K enantiomers, at the higher concentration tested (10 mg/L), high toxicity were presented 95% for (*S*)-K and 100% for (*R*)-K and at the 5 mg/L a significant difference was observed between the enantiomers toxicity, being the (*S*)-K more toxic than the (*R*)-K (Figure 25).
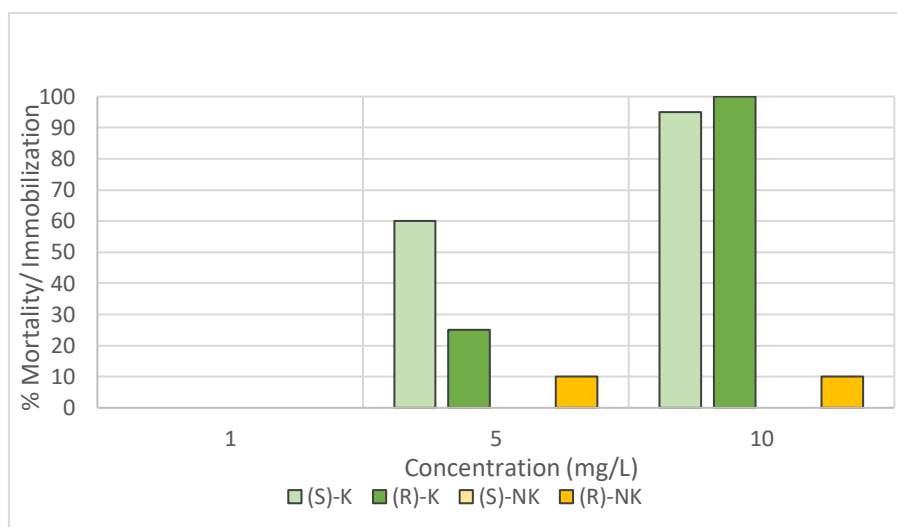
Figure 25 - Percentage of mortality of K and NK enantiomers for *D. magna*

These assays with *D. magna* showed that K enantiomers are more toxic than both NK enantiomers. Further, the (*S*)-K enantiomer showed higher toxicity (mortality) at 5 mg/L than (*R*)-K showing possible enantioselective effect. Further studies should be done at lower and higher concentrations than 5 mg/L to investigate the enantioselective effects. There are not studies concerning the enantioselective effect of K and NK enantiomers in aquatic organisms, nevertheless, studies in mice have showed the higher potency and the occurrence of more side effects of (*S*)-K rather than for the racemate and (*R*)-K which corroborates the obtained results. These results are of high importance as (*S*)-K has been used for the treatment of several psychological disorders, chronic depression and pain (Luu et al., 2019; Swainson et al., 2019b).

4.1.3 *Tetrahymena thermophila*

Possible enantioselectivity toxicity of K and NK to T. thermophila was performed within the following range of concentrations: 1, 2.5, 5, 7.5 and 10 mg/L with the isolated enantiomers. This range was selected based on the range of concentrations selected to *D. magna* assays. Figure 26 show the percentage of growth inhibition of both enantiomers.
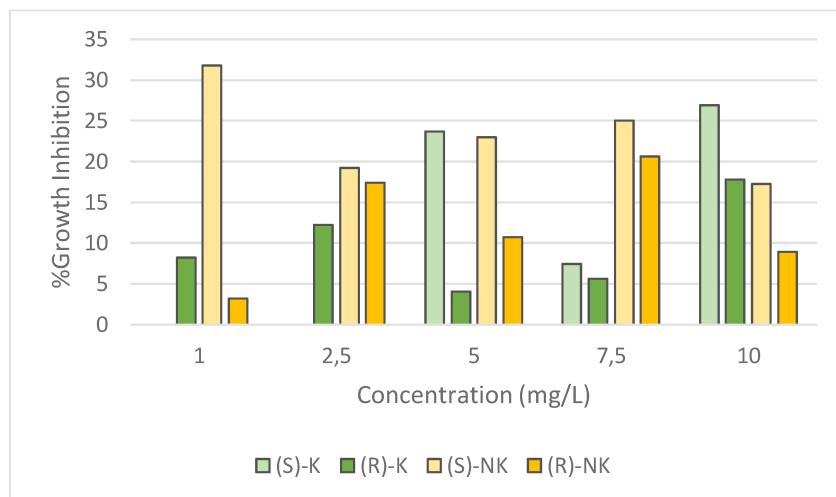
Figure 26 - Growth Inhibition percentage of K and NK enantiomers for *T. thermophila*

In this range of concentrations, no significant differences in growth inhibition were observed for both K and NK enantiomers. Nevertheless, (*S*)-NK showed higher growth inhibition at the lower concentrations, i.e., at 1 and 2.5 mg/L. At higher concentrations growth inhibition were lower though in the same range. Considering (*R*)- NK at 2.5, 5, 7 and 10 mg/L also showed growth inhibition though lower than (*S*)-NK and no significant differences were observed among them. (*S*)-K showed higher growth inhibition at 5 mg/L and 10 mg/L compared to (*R*)-K. The results also showed significant differences between the control and mean growth inhibition values for (*S*)-K and both enantiomers of NK. No significant growth inhibition was observed to the same range of concentration to (*R*)-K. Nevertheless, at 10 mg/L growth inhibition was similar for both NK enantiomers and (*R*)-K and (*S*)-K showed the highest growth inhibition.

4.2 Enantioselective ecotoxicity assay for amphetamine and amphetamine like substances

4.2.1 *Daphnia magna*

Acute toxicity assays were also performed for (*R,S*)-AMP, (*R,S*)-MAMP, (*S*)-MAMP, and (*R,S*)-MDMA for determination of EC_{50} values. In toxicological tests, lethal and / or sublethal effects may be determined, and for that, concentrations that cause an effect or death in 50% of the organisms can be calculated (EC_{50} and LC_{50}, respectively). The EU Directive 93/67/EEC (Commission on European Community, 1996) established limits for the EC_{50} values, forming three classes of compounds: (I) EC_{50} <1 mg/L, very toxic to aquatic organisms; (II) EC_{50} = 1-10 mg/L, toxic to aquatic organisms and (III) EC_{50} = 10-100 mg/L, dangerous for aquatic organisms. In this work, the EC_{50} were investigated for racemates AMP and MDMA while for MAMP toxicity was evaluated for both racemate and the enantiomer (*S*)-MAMP. Range of concentrations investigated were from 10 to 35 mg/L for all compounds based on preliminary assays. EC_{50} were determined for the three compounds using the probit method. All compounds used for these assays were solubilized in methanol. Thus, a control with the highest concentration of methanol was performed, nevertheless, mortality of this control exceeded the acceptable values of 10% with a mortality of 20%. Thus, results obtained for EC_{50} of all compounds are preliminary and will be confirmed after dilution of the compounds in water.

All racemates, (*R,S*)-MAMP, (*R,S*)-MDMA and (*R,S*)-AMP had a dose-dependent increase on the mortality and showing a percentage of mortality at 35 mg/L of 80%, 60 % and 55% respectively (Figure 27) EC_{50} were 20.8, 30.2 and 34.3 mg/L for (*R,S*)-MAMP, (*R,S*)-MDMA and (*R,S*)-AMP, respectively. From the three compounds tested the (*R,S*)-MAMP was the most toxic to *D. magna.* (Table 2S in the annex shows the percentage of mortality record at 24 and 48 h).
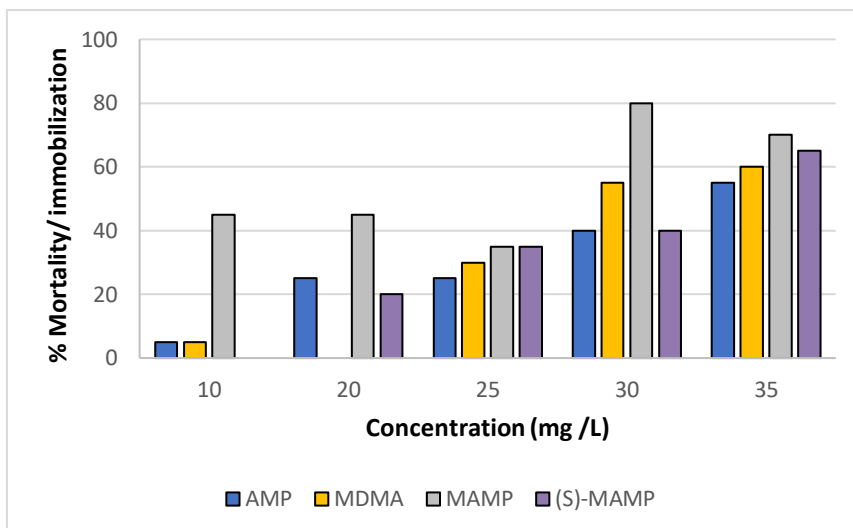
Figure 27 - Percentage of mortality of AMP, MAMP, MDMA and (*S*)- MAMP for *D. magna.*

Considering (*S*)-MAMP enantiomer, EC$_{50}$ was 28 mg/L and thus, showing lower toxicity than its racemate. Regarding these results all tested compounds fill the class III of toxicity, being dangerous to aquatic organisms.

4.2.2 *Tetrahymena thermophila*

Chronic toxicity assays were also performed for (*R,S*)-AMP, (*R,S*)-MAMP, and (*R,S*)-MDMA for determination of EC$_{50}$ values using the *T. thermophila*. Selected range of concentrations were from 1 to 35 mg/L for all compounds. Figure 28 shows the growth inhibition for the compounds tested. EC$_{50}$ for all compounds were determined. EC$_{50}$ were 23, 26 and 27.5 mg/L for (*R,S*)-MDMA, (*R,S*)-AMP and (*R,S*)-MAMP, respectively. According with the results, these substances are classified in class III, i.e., dangerous for aquatic organisms. In contrast to *D. magna*, (*R,S*)-MDMA presented the highest toxicity, though toxicity range are similar. It is important to investigate the enantioselective toxicity of each compound since these compounds have been found in environmental samples at different EF. These results show that different organisms can have different sensitivity to the tested compounds.
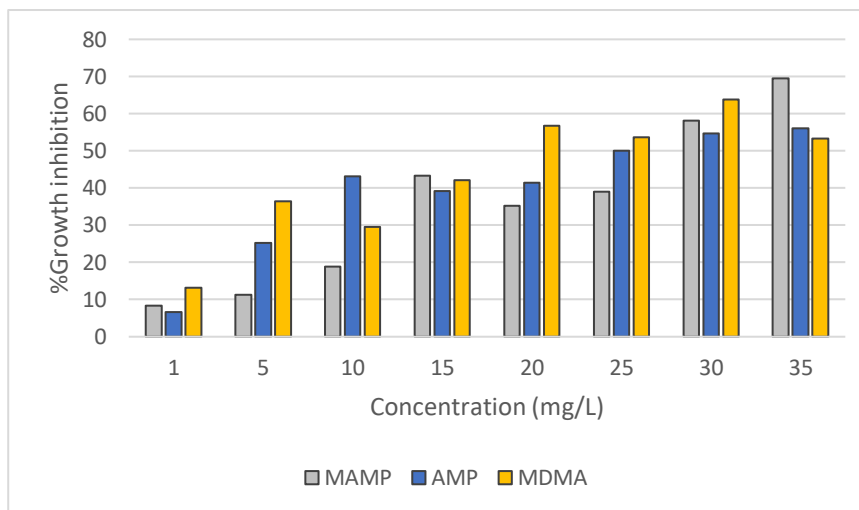
Figure 28 - Growth Inhibition percentage of MAP, AMP and MDMA for *T. thermophila*

There only few studies considering the toxicity of amphetamine and amphetamine like substances to aquatic organisms and there are not studies considering the enantioselective effects. Kyzar et al 2013 studied the effects in brain monoamines of behavior regulation in *D. rerio* after exposure to (*S*)-AMP. (*S*)-amphetamine increases brain monoamines levels and evokes hyperactivity and anxiety and it was demonstrated that (*S*)-AMP at 5 and 10 mg/L lead to acute anxiogenic effects (Kyzar et al., 2013). The locomotor activity of *D. rerio* larvae was characterized by assessing the acute effects affect exposure to (*S*)-AMP (Irons et al., 2010). Low concentrations increased activity, while higher concentrations decreased activity. It is well known that different organism may show different susceptibility to exposure compounds. Also, studies concerning the enantioselective effects of these psychotropic substances were not reported. Both AMP and MAMP can be commercialized in both racemate and enantiomeric pure and different illicit production of these drugs may lead to drugs with different EF. Thus, this kind of studies are urgent and important to investigate their toxicity in the aquatic systems.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

Evaluation and monitoring of the water quality require chemical and ecotoxicological studies. Various bioindicators, may be applied and different endpoints can be evaluated. The enantiotoxicity of various psychotrophic substances to various ecological relevant organism belonging to different trophic levels as *D. rerio* fish, *D. magna* crustacean and the protozoan *T. thermophila* were assessed. These organisms can be used as alert bioindicators in water contaminated with toxic substances. Currently, most ecotoxicological assays regard only racemates and do not consider enantiomers. It is well known that enantiomers may have different biological activities including toxicity. Further, after consumption, pharmaceuticals and illicit drugs suffer enantioselective metabolism in humans and during wastewater treatment causing changes in their EF. Thus, accurate risk assessment of their occurrence in the environment requires enantioselective toxicity assays. Regarding K and its metabolite NK, (*R*)-K induced more mortality and malformations, in *D. rerio*, although NK its enantiomers showed higher ROS and lipid peroxidation and thus, failure of stress oxidative defenses. Behavioral response showed that (*R,S*)-K presented the lower speed but the highest activity demonstrating that this compound causes neurotoxicity. It is interesting to note that both K enantiomers caused higher speed and lower activity and no enantioselective effects were observed. For *D. magna* and *T. thermophila* (*S*)-K showed higher toxicity. No toxicity was observed for *D. magna* exposed to both NK enantiomers while for *T. thermophila* their toxicity were in the same range than (*S*)-K. K and NK assays with both racemates and enantiomers showed that different organisms can show different susceptibility and that different *endpoints* can be affected. *D. rerio* showed higher sensitivity to (*R*)-K and (*R,S*)-K in what concerns morphological and neurological status while NK showed higher toxicity concerning stress oxidative. Both *D. magna* and *T. termophila* showed toxicity for (*S*)-K but differences were observed (*R*)-K and NK racemate and enantiomers towards *D. magna* and *T. thermophila*. Also, ecotoxicological impact of other class of psychotropic substances, the AMP and AMP like substances were investigated for *D. magna* and *T. thermophila*. AMP, MAMP and MDMA exhibit an EC$_{50}$ of 34.3, 20.8

and 30.2 mg/L, respectively, for *D. magna* and 26, 27.5 and 23 mg/L for AMP, MAMP and MDMA, respectively, for *T. thermophila*. (*S*)-MAMP showed lower toxicity than the racemate (28 mg/L) to *D. magna*. Once these substances fall in class III, a similar assay should be done using the enantiomers and different organisms. These results showed that organisms may have different susceptibility to toxicants and that toxicity may be enantioselective. Studies concerning the enantiotoxicity of chiral substance is crucial for an accurate risk assessment. Without this information is not possible to predict risk and establish safety thresholds. Further, ecotoxicological chronic assays should be done not only for *D. rerio* and *D. magna* but also other organisms to evaluate long-term effects, and to considering various *endpoints* including reproductive output. Additionally, it is important to stress, that chemicals do not occur alone in the environment, but as complex mixtures. Thus, even though individual chemicals concentrations can be below the lowest observed effect concentrations or detection limits they may still cause adverse effects due to synergist/ addictive effects.

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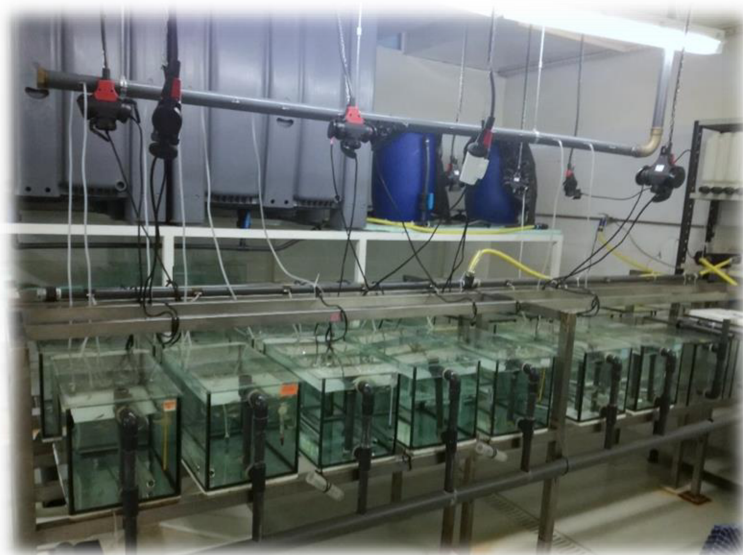
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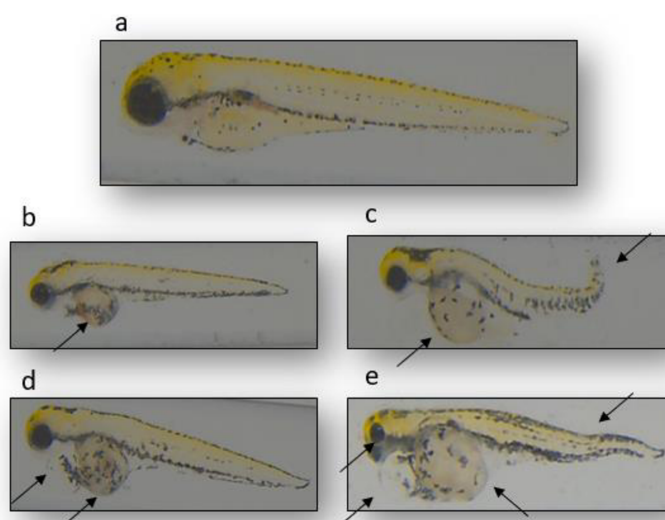

Figure 1S *Danio rerio* aquariums.

Figure 2S Normal 96hpf larvae of *Danio rerio* (**a**) and the most common malformations exhibited in this work: abdominal edema (**b**), abdominal edema with an accentuate spinal curvature (**c**), pericardial and abdominal edema (**d**) and underdeveloped eyes, with both edemas and a slight spinal curvature.

Table1S. Extensive protocol of oxidative stress.

Enzyme	Wavelength	Protocol	References
SOD	560nm	10 µL sample; 170 µL Potassium phosphate buffer 50 mM (KH_2PO_4 and K_2HPO_4) with 0.6 mM hypoxanthine, 1 mM EDTA, and 0.2 mM NBT; read 2 min; 20 µL Xanthine Oxidase 40x diluted in Potassium phosphate buffer 50 mM and 1 mM EDTA; read 3 min; Quantify with SOD standards (0-30 U/mL); Express as U/mg protein.	(Durak et al., 1993)
CAT	240nm	10 µL sample; read 2 min; 90 µL sodium buffer 100 mM (NaH_2PO_4 and Na_2HPO_4) pH 7.4 containing 20 mM H_2O_2; read 3 min; Quantify with CAT standards (0-6 U/mL); Express as U/mg protein.	(Claiborne, 1985)
GST	340nm	10 µL sample; 180 µL potassium phosphate buffer 100 mM (KH_2PO_4 and K_2HPO_4), pH 7.4 containing 1 mM CDNB; read 2 min; 50 µL GSH 25 mM; read 3 min; Express as µmol CDNB/min.mg protein.	(Habig et al., 1981)
GSH	320nm; 420nm	10 µL sample; 180 µL sodium phosphate buffer 100 mM (NaH_2PO_4 and Na_2HPO_4) with 5mM EDTA pH10 µL OPT 1 mg/ml; Incubate 15min room temperature; Reed and quantify using calibration curve 0-1000 µM GSH; Express as µmol GSH/mg protein.	(Misra et al., 2009)
GSSG	320nm; 420nm	10 µL sample; 90 µL NaOH buffer 0.1 N with 0.04 M NEM; Incubate 30min room temperature; 10 µL OPT 1 mg/ml; Incubate 15min room temperature; Reed and quantify using calibration curve 0-1000 µM GSSG (don't add TCA); Express as umol GSSG/mg protein.	(Misra et al., 2009)
TBARS	530nm; 600nm	10 µL sample; 70 µL H_2O; 50 µL Phosphate buffer 50 mM pH 7.4 (NaH_2PO_4 and Na_2HPO_4); 10 µL BHT 1 mM; 75 µL TBA 1.3% in 0,3% NaOH; 50 µL TCA 50%; Incubate 40 min at 60 ºC; cool down in ice for 15 min; 10 µL SDS 20% (2g in 10 mL H_2O warm up at 68 ºC); Reed and quantify using calibration curve 0-50/1000 µM MDA; Express µmol MDA/mg protein.	(Wallin et al., 1993)
LDH	340nm	10 µL sample; 200 µL NADH 0.24 mM; read 2min; 40 µL pyruvic acid sodium salt 10 mM; read 3 min; Express as µmol NADH/min.mg protein.	(Domingues et al., 2010)
AcHE	405nm	10 µL sample; 180 µL DTNB 0.5 mM (2 mg in 10 mL Tris buffer 0.05 M pH 7.4 – 302,85 mg Tris base, 174 µL HCl 37% or 2.1 mL HCl 1M in 50 mL H_2O); read 2min; 10 µL acetylthiocholine iodide 20 mM; read 3min; Express as µmol TNB/min.mg protein.	(Rodriguez-Fuentes et al., 2015)
ROS	485nm; 530nm	10 µl sample; 100 µl PBS pH 7.4 (0.800g NaCl, 0.020g KCl, 0.144g Na_2HPO_4 and 0.024g KH_2PO_4 in 100 mL); 10 µL DCFH-DA 10 mg/mL (in DMSO); Incubate 30 min at 37 ºC; read excitation 485nm and emission 530 nm; Reed and quantify using calibration curve 0-100 µM DCF; Express as µmol DCF/mg protein.	(Deng et al., 2009)

Table 2S. Percentage of mortality at 24 and 48 hours for AMP, MDMA and MAMP on *D. magna*

Concentration (mg/L)	AMP 24h	AMP 48h	MDMA 24h	MDMA 48h	MAMP 24h	MAMP 48h	(S)-MAMP 24h	(S)-MAMP 48h
10	0	5	0	5	0	45	0	0
20	10	25	0	0	0	45	5	20
25	5	25	5	30	5	35	5	35
30	15	40	15	55	20	80	5	40
35	20	55	40	60	5	70	15	65

Annex 1

Annex 1. F. Teles, M. E. Tiritan, C. Ribeiro, Ecotoxicidade Enantiosseletiva de Fármacos e Drogas Psicotrópicas, XII Jornadas Científicas IUCS and III Congresso APCF, Porto, Portugal, 24-25 May 2018 (Abstract and poster communication)

ECOTOXICIDADE ENANTIOSSELETIVA DE FÁRMACOS E DROGAS PSICOTRÓPICAS

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Introdução: De acordo com dados do relatório do Observatório Europeu da Droga e da Toxicodependência de 2017, 1.8 milhões de pessoas consumiram anfetaminas e segundo o Infarmed, o consumo de antidepressivos aumentou 32% nos últimos cinco anos [1, 2]. Devido ao seu elevado consumo e contínuo descarte para o ambiente estas substâncias, biologicamente ativas, assim como os seus metabolitos têm sido frequentemente detetadas em águas residuais, águas de superfície, águas de consumo e inclusivamente em organismos aquáticos [3]. Desta forma, estes contaminantes ambientais podem atingir espécies não alvo com potenciais efeitos nefastos. A maioria destes fármacos e drogas psicotrópicas é quiral e os seus enantiómeros podem apresentar atividades biológicas e tóxicas diferentes. Estas substâncias têm sido encontradas em amostras ambientais quer na forma de racemato quer em diferentes proporções enantioméricas [3]. Estes aspetos levantam problemas ambientais adicionais devido às diferentes atividades dos enantiómeros em relação às suas propriedades toxicológicas e ecotoxicológicas. Alguns estudos têm descrito os efeitos nefastos de alguns antidepressivos em organismos aquáticos como o crustáceo *Daphnia magna* e *Hyalella azteca* e no peixe *Pimephales promela* [4,5]. Stanley et al 2007 demonstrou que a mistura racémica de fluoxetina é mais tóxica para o *P. promela* do que os seus enantiómeros separados e o enantiómero *S* é mais tóxico do que o *R* [5]. No entanto, poucos estudos consideram os enantiómeros de fármacos e drogas psicoativas.

Objetivos: Este estudo tem como objetivo avaliar a toxicidade enantiosseletiva de vários fármacos e drogas antidepressivas e estimulantes em organismos aquáticos.

Material e métodos: Serão utilizados diferentes organismos aquáticos que compreendem vários níveis tróficos. Serão realizados ensaios agudos e crónicos por exposição destes organismos a concentrações ambientalmente relevantes na forma de racemato e dos seus enantiómeros.

Resultados: Os dados obtidos neste estudo permitirão avaliar a toxicidade seletiva em diferentes organismos e avaliar o impacto destes poluentes no ambiente.

Conclusões: A avaliação do impacto de fármacos e drogas psicoativas quirais no ambiente é essencial para uma correta avaliação do risco e para a implementação de medidas de controlo destas substâncias no ambiente.

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ECOTOXICIDADE ENANTIOSSELETIVA DE FÁRMACOS E DROGAS PSICOTRÓPICAS

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INTRODUÇÃO:

Devido ao elevado consumo e contínuo descarte para o ambiente de fármacos e drogas psicotrópicas e os seus metabolitos, estas substâncias têm sido frequentemente detetadas em águas residuais, águas de superfície, águas de consumo e inclusivamente em organismos aquáticos [1].

Desta forma, estes contaminantes ambientais podem atingir espécies não alvo com potenciais efeitos nefastos.

A maioria destes fármacos e drogas psicotrópicas é quiral e os seus enantiómeros podem apresentar atividades biológicas e tóxicas diferentes. Estas substâncias têm sido encontradas em amostras ambientais quer na forma de racemato ou em diferentes proporções enantioméricas [1]. Os enantiómeros da fluoxetina e da anfetamina (Figura 1) são exemplos frequentemente encontrado no ambiente com diferentes proporções enantioméricas.
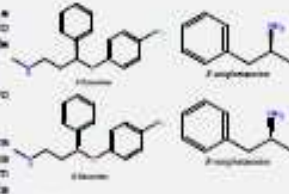
Fig. 1 Enantiómeros da fluoxetina e da anfetamina

OBJETIVOS:

Este estudo tem como objetivo avaliar a toxicidade enantiosseletiva de vários fármacos e drogas psicoativas nomeadamente de antidepressivos e estimulantes em organismos aquáticos.

MATERIAL E MÉTODOS:

Neste estudo serão utilizados organismos aquáticos de diferentes níveis tróficos (Figuras 2, 3, 4 e 5), os quais irão ser submetidos a ensaios agudos e crónicos por exposição a concentrações ambientalmente relevantes, quer na forma de racemato quer dos seus enantiómeros (Esquema 1).

Principais níveis tróficos
Produtores
Consumidores

Fig.2 Chlorella vulgaris

Fig.3 Daphnia magna

Fig.4 Artemia salina

Fig.5 Tetrahymena thermophila

Organismo — Exposição — Antidepressivos / Estimulantes
S-fluoxetina / R-fluoxetina / R,S-fluoxetina
S-anfetamina / R-anfetamina / R,S-anfetamina
Concentração — Concentrações ambientais relevantes — EFEITOS

Alguns estudos têm descrito os efeitos nefastos de alguns antidepressivos em organismos aquáticos, como o crustáceo Daphnia magna e Hyalella azteca e no peixe Pimephales promelas [2,3]. Stanley et al [3] demonstrou que a mistura racémica de fluoxetina é mais tóxica para o P. promelas do que os seus enantiómeros separados e o enantiómero S é mais tóxico do que o R.

Fernando et al [4] demonstrou que para a Daphnia magna o enantiómero S da fluoxetina é mais tóxico (maior percentagem de mortalidade) do que o R. Uma possível explicação para que o enantiómero S da fluoxetina seja mais tóxico reside no facto de esta ser mais potente nos mamíferos [5].

No entanto, ainda são poucos os estudos que avaliam a ecotoxicidade do racemato e dos enantiómeros de fármacos e drogas psicoativas.

RESULTADOS

Os dados obtidos neste estudo permitirão avaliar a toxicidade seletiva em diferentes organismos e avaliar o impacto destes poluentes no ambiente, assim como determinar qual o modelo mais adequado para este tipo de estudo.

CONCLUSÕES

A avaliação do impacto de fármacos e drogas psicoativas quirais no ambiente é essencial para uma correta avaliação do risco e para a implementação de medidas de controlo destas substâncias no ambiente.

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Annex 2

Annex 2. F. Teles, A. Pereira, M. E. Tiritan, C. Ribeiro, Ecotoxicidade Enantiosseletiva de Substâncias Psicotrópicas, XIII Jornadas Científicas IUCS and IV Congresso APCF, Porto, Portugal, 11-12 April 2019 (Abstract and poster communication)

ECOTOXICIDADE ENANTIOSSELETIVA DE SUBSTÂNCIAS PSICOTRÓPICAS

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Introdução: O consumo excessivo de substâncias psicotrópicas (SP) tem vindo a aumentar na sociedade constituindo um problema grave de saúde pública. As SP são um grupo heterogéneo de compostos que incluem fármacos e drogas ilícitas. No último ano cerca de 1,7 milhões e 2,6 milhões de adultos, na casa dos 15-64 anos, consumiram anfetaminas (AM) e metilenodioximetanfetamina (MDMA) [1]. A cetamina (K) é um fármaco com ação sedativa comumente utilizado na veterinária e também encontra-se entre as drogas recreativas alucinogénicas mais utilizadas [1, 2]. Por descarte direto ou após consumo, SP assim como os seus metabolitos têm sido detetados em águas residuais, águas de superfície, águas de consumo e inclusivamente em organismos fazendo parte do "pool" de poluentes ambientais [3, 4]. Estas substâncias são biologicamente ativas podendo causar efeitos adversos em organismos não alvo e no Homem. Para além disso, a maioria das SP são quirais. De facto, estes poluentes têm sido encontrados em amostras ambientais, quer na forma de racemato quer em diferentes proporções enantioméricas [3]. É bem conhecido que os enantiómeros podem apresentar propriedades biológicas diferentes. Stanley et al 2007 demonstrou que a mistura racémica de fluoxetina é mais tóxica para o *Pimephales promela* do que os seus enantiómeros separados e o enantiómero S é mais tóxico do que o R [5]. Desta forma, os ensaios de ecotoxicidade enantiosseletiva são de extrema importância para uma correta avaliação do impacto destes poluentes em organismos não alvo. Até a data são poucos os estudos realizados para avaliação dos efeitos enantiosseletivos ecotoxicológicos de SP quirais nos organismos não alvo.

Objetivos: Este estudo tem como objetivo avaliar a toxicidade enantiosseletiva de várias classes de SP como os estimulantes (AM, metanfetamina (MA) e MDMA) e alucinogénicas (K e o seu metabolito norcetamina (NK)) em organismos aquáticos ambientalmente relevantes, o crustáceo *Daphnia magna* e o protozoário *Tetrahymena thermophila*.

Material e métodos: Foram realizados ensaios agudos e crónicos expondo os organismos a diferentes gamas de concentrações tendo em conta concentrações ambientais reportadas e ensaios para determinação de concentração letal (EC50) na forma de racemato e dos seus enantiómeros.

Resultados: Nos ensaios realizados com a K foi possível observar toxicidade enantiosseletiva uma vez que o enantiómero 1 (K1) da K é o mais tóxico para a *D.magno* e para o *T. thermophila* comparativamente com o enantiómero 2 (K2). Para a NK, o enantiómero 2 apresentou maior toxicidade para ambos os organismos. Foi possível observar também diferente suscetibilidade dos organismos para a K e NK. Assim, a K demonstrou maior toxicidade para a *D. magno* enquanto que a NK é mais tóxica para o *T. thermophila*. Nos ensaios realizados com as SP estimulantes determinou-se o valor de EC50 para os racematos de AM, MA e MDMA. Verificou-se menor toxicidade para a *D. magna*, com EC50 de 34.6 e 31.8 mg/L para a AM e o MDMA, respetivamente. Para os *T. thermophila* os valores de EC50 são 28.3, 26.8

e 20.2 mg/L para a MA, AM e MDMA, respetivamente.

Conclusões: A avaliação do impacto de SP quirais no ambiente é essencial para uma correta avaliação do risco e para a implementação de medidas de controlo destas substâncias no ambiente. É necessário realizar estudos de avaliação da toxicidade enantiosseletiva para os enantiómeros das SP estimulantes e avaliar a suscetibilidade de organismos de outros níveis tróficos.

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POSTER 37

AFOGAMENTO: CONSIDERAÇÕES MÉDICO-LEGAIS

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Introdução: O afogamento é definido como a insuficiência respiratória causada pela imersão ou submersão em um líquido extracorporal e a morte por afogamento dá-se seguida ao desenvolvimento da condição de hipoxia cerebral, progredindo, assim, para danos cerebrais irreversíveis.

Atualmente, é a terceira maior causa de morte no mundo por lesão não intencional (OMS), o que pode ser agravado devido a fatores de risco, nomeadamente idade, gênero, acesso e viagens por água e inundações. Dessa maneira, o afogamento constitui um dos maiores problemas mundiais de saúde pública.

Além disso, a possível ocorrência de lesões ante-mortem e lesões post-mortem, classifica o afogamento como um dos diagnósticos mais difíceis no campo da medicina legal, já que é necessária a distinção entre as ações e circunstâncias que envolveram a morte.

Objetivo: O objetivo deste trabalho é a explicação da fisiologia do afogamento e a sua relação para com a medicina legal.

Materiais e Métodos: Foram feitas pesquisas realizadas com artigos retirados da base PubMed e de comunicados publicados pela Organização Mundial da Saúde (OMS).

Resultados: O processo de afogamento é descrito em fases que têm duração variada: a retenção da respiração, a inspiração involuntária, a falta de ar e a perda de consciência, a qual geralmente ocorre após três minutos de submersão. Entretanto, as diferentes fases ocorrem durante dois eventos que são relacionados à fisiologia do afogamento: a imersão e a submersão. A imersão, quando as vias aéreas do indivíduo estão acima da água, envolve respostas cardiorrespiratórias integradas à pele e à temperatura corporal profunda, incluindo choque frio, incapacitação física e hipovolemia. Já a submersão, quando as vias aéreas se encontram embaixo da água, relaciona-se ao medo de afogamento, resposta ao mergulho, reflexo das vias aéreas superiores, conflito autonômico, aspiração e
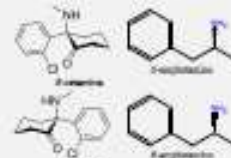
ECOTOXICIDADE ENANTIOSSELETIVA DE SUBSTÂNCIAS PSICOTRÓPICAS

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INTRODUÇÃO

Fig.1 Enantiómeros da cetamina e norcetamina

OBJETIVOS

MATERIAL E MÉTODOS

Fig.2 Daphnia magna

Fig.3 Tetrahymena thermophila

RESULTADOS e DISCUSSÃO

T. thermophila

Fig.4 Percentagem de inibição do crescimento da T. thermophila para os racematos de AM, MA e MDMA.

D. magna

Fig.5 Percentagem de imobilização da D. magna para os racematos de AM, MDMA.

Tabela 1: Resultados dos valores de EC50 de AM, MA e MDMA determinados para a D. magna e T. thermophila.
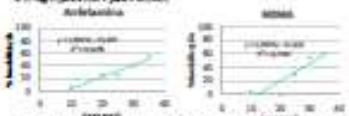
Tabela 2: Resultados das percentagens de imobilização para a D. magna e inibição do crescimento T. thermophila expostos aos enantiómeros da K e NK.

CONCLUSÕES

Referências

Annex 3

Annex 3. F. Teles, JS Carrola, M. E. Tiritan, O. Ribeiro, L. Felix, C. Ribeiro, Ecotoxicidade Enantiosseletiva da Cetamina e Norcetamina no Peixe Zebra (*Danio rerio*) - Dados Preliminares, 3ª Reunião Internacional Rede Académica das Ciências da Saúde da Lusofonia, Braga, Portugal, 26-28 March 2020, abstrat submitted (Abstract)

ECOTOXICIDADE ENANTIOSSELETIVA DA CETAMINA E NORCETAMINA NO PEIXE ZEBRA (*DANIO RERIO*) - DADOS PRELIMINARES

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Introdução: As substâncias psicotrópicas (SP) têm atraído cada vez mais consumidores aumentando assim a sua presença, incluindo os seus metabólitos nos ecossistemas, nomeadamente em águas residuais e de superfície [1]. A maioria das SP são quirais, e apesar de serem maioritariamente comercializadas na forma de racemato, têm sido encontradas em amostras ambientais em diferentes proporções enantioméricas [1,2]. A cetamina (K) é um fármaco com ação sedativa utilizado na medicina veterinária e pediátrica. Por outro lado, induz alucinações e delírios o que tem fomentado o seu uso recreativo [3]. Todavia, Félix et al (2014) verificaram que os embriões de peixe zebra (*Danio rerio*) expostos 20 minutos à K (racemato) apresentavam alterações no desenvolvimento, aumento de anomalias morfológicas e maior mortalidade [4]. Até a data não existem estudos que avaliem os efeitos enantiosseletivos da K e do seu metabolito, a norcetamina (NK) em peixe zebra (PZ).

Objetivos: Avaliar a enantiosseletividade da K e NK ao longo do desenvolvimento embrionário do PZ.

Materiais e Métodos: Embriões de PZ com 2 a 3 horas pós fertilização foram expostos durante 96h a K e NK, racematos e enantiómeros isolados, numa gama de concentrações de 10-2000 µg/L. Foram avaliadas as mortalidades, malformações e o comprimento total dos alevins.

Resultados: Observou-se uma maior mortalidade e mais malformações em embriões expostos à K do que ao seu metabolito NK. Para a mesma gama de concentrações observou-se para os enantiómeros isolados da K maior número de malformações comparativamente com o racemato. O enantiómero (*R*)-K apresentou maior toxicidade em quase todas as concentrações comparativamente com o enantiómero (*S*)-K. Verificou-se menor comprimento total nos alevins na sequência da exposição aos enantiómeros da NK comparativamente com os enantiómeros da K. Constatou-se ainda que os alevins expostos ao enantiómero (*R*)-NK apresentaram menor comprimento total.

Conclusões: Verificou-se uma maior toxicidade dos enantiómeros (*R*)-NK e (*R*)-K nos vários parâmetros avaliados em embriões de PZ, o que indica enantiosseletividade. Este parâmetro deve ser considerado para uma correta avaliação do risco ambiental de fármacos quirais, em particular nos ecossistemas aquáticos.

keypoints (learning objectives):

Avaliação da toxicidade enantiosseletiva da K e NK no desenvolvimento embrionário do peixe zebra;

Resultados demonstraram diferente toxicidade da K e NK nos parâmetros avaliados e possível enantiosseletividade.

Estudos ecotoxicológicos enantiosseletivos são fundamentais para uma correta avaliação do impacto dos poluentes em organismos não alvo.

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Annex 4

       Annex 4. Statistical analysis data considering the differences between compounds and between concentrations.

Table 1. Characterization and comparison of **length** between concentrations and between compounds.

Concen-tration (µg/L)	Length (mean ± DP)							Kruskal-Wallis Test [2]
	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	
TOTAL		$3.6\pm0.1_{12}$	$3.6\pm0.1_{12}$	$3.5\pm0.2_{23}$	$3.4\pm0.1_{3}$	$3.7\pm0.1_{1}$	$3.7\pm0.2_{1}$	**p < 0.001**
Control	3.6±0.2	3.6±0.0	3.7±0.0	3.7±0.3	3.5±0.1	3.7±0.1	3.6±0.3	p = 0.427
10	3.6±0.2	3.6±0.1	3.5±0.1	3.5±0.1	3.4±0.2	3.6±0.0	3.7±0.2	p = 0.115
100	3.6±0.2	3.5±0.2	3.6±0.1	3.6±0.1	3.5±0.1	3.7±0.1	3.7±0.2	p = 0.066
500	3.6±0.1	3.6±0.1	3.5±0.1	3.4±0.1	3.5±0.1	3.7±0.1	3.6±0.3	p = 0.154
1000	3.6±0.2	3.6±0.1	3.6±0.0	3.5±0.1	3.4±0.2	3.7±0.0	3.6±0.3	p = 0.174
2000	3.6±0.2	3.7±0.1	3.6±0.1	3.5±0.1	3.5±0.1	3.7±0.0	3.6±0.3	p = 0.192
Kruskal-Wallis Test [1]	p = 0.735	p = 0.489	p = 0.336	p = 0.653	p = 0.936	p = 0.529	p = 0.984	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 2. Characterization and comparison of **mortality** between concentrations and between compounds.

Concen-tration (µg/L)	Mortality (mean ± DP)							Kruskal-Wallis Test [2]
	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	
TOTAL		$20.8\pm3.5_{12}$	$23.2\pm6.1_{1}$	$17.2\pm4.8_{12}$	$17.8\pm6.9_{12}$	$16.1\pm5.7_{2}$	$15.8\pm6.1_{2}$	**p = 0.001**
Control	16.2±3.3	19.0±0.0	16.1±2.5	16.5±2.3	16.1±2.5	10.6±1.9	18.8±0.4	p = 0.053
10	18.9±7.7	22.7±6.0	26.3±0.9	18.6±10.2	19.4±8.5	15.6±7.5	10.8±3.9	p = 0.235
100	19.5±6.5	18.9±5.8	25.6±10.9	18.6±4.0	18.6±6.8	21.1±6.3	14.3±1.4	p = 0.410
500	18.3±7.7	21.6±2.0	23.8±3.0	13.7±4.1	22.1±12.6	12.8±6.7	15.6±9.9	p = 0.349
1000	19.8±4.7	22.1±1.5	23.2±3.1	16.3±2.7	19.4±3.0	18.3±2.9	19.4±10.3	p = 0.206
2000	18.3±5.6	20.7±2.7	24.2±8.3	19.7±3.1	11.2±3.3	18.3±1.7	15.8±4.4	p = 0.125
Kruskal-Wallis Test [1]	p = 0.397	p = 0.617	p = 0.221	p = 0.591	p = 0.457	p = 0.247	p = 0.497	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 3. Characterization and comparison of **malformations** between concentrations and between compounds.

Concen-tration (µg/L)	Malformations (mean ± DP)							Kruskal-Wallis Test [2]
	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	
TOTAL		$9.7\pm3.9_{1}$	$13.1\pm6.6_{1}$	$2.4\pm2.1_{2}$	$2.0\pm2.4_{2}$	$2.7\pm3.4_{2}$	$3.3\pm3.0_{2}$	**p < 0.001**
Control	3.2±3.6	4.4±2.9	6.1±5.4	1.1±1.9	1.5±2.6	3.9±5.4	2.1±2.6	p = 0.522
10	5.1±5.6	$8.3\pm4.1_{12}$	$14.9\pm3.8_{1}$	$3.7\pm1.1_{23}$	$1.9\pm1.7_{3}$	$1.1\pm1.0_{3}$	$0.6\pm1.0_{3}$	**p = 0.013**
100	6.7±7.8	$13.5\pm4.9_{1}$	$18.1\pm7.5_{1}$	$3.2\pm3.3_{2}$	$0.4\pm0.7_{2}$	$2.8\pm4.8_{2}$	$2.0\pm2.1_{2}$	**p = 0.038**
500	5.8±5.2	$10.8\pm1.2_{1}$	$13.3\pm3.7_{1}$	$1.4\pm0.2_{2}$	$3.0\pm3.4_{2}$	$1.1\pm1.0_{2}$	$5.3\pm1.7_{12}$	**p = 0.017**
1000	5.6±4.3	$10.5\pm2.4_{12}$	$10.3\pm3.9_{1}$	$4.0\pm2.7_{23}$	$2.6\pm2.8_{3}$	$2.8\pm2.5_{3}$	$3.5\pm3.1_{3}$	**p = 0.047**
2000	6.8±6.9	$10.8\pm2.2_{1}$	$15.9\pm10.3_{1}$	$1.0\pm0.9_{2}$	$2.2\pm3.8_{2}$	$4.4\pm4.8_{2}$	$6.4\pm4.0_{12}$	**p = 0.044**
Kruskal-Wallis Test [1]	p = 0.463	p = 0.160	p = 0.273	p = 0.218	p = 0.850	p = 0.749	p = 0.156	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 4. Characterization and comparison of **SOD** between concentrations and between compounds.

Concentration (µg/L)	SOD (mean ± DP)							Kruskal-Wallis Test [2]
	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	
TOTAL		139.4±23.9[2,3]	154.8±22.5[2]	163.6±41.3[2]	164.6±21.9[2]	113.6±16.8[3]	235.9±51.2[1]	**p < 0.001**
Control	124.2±19.2[b]	112.4±13.2[1,2][c]	118.7±3.6[1,2][b]	118.7±3.7[1,2][b]	132.4±3.0[1,2]	101.6±13.7[2][b]	162.9±8.0[1][c]	**p = 0.042**
10	168.7±42.3[a]	165.2±18.6[1,2][a]	180.9±11.9[1][a]	180.5±5.1[1][a]	172.0±14.6[1,2]	93.1±15.8[2][b]	219.5±24.4[1][bc]	**p = 0.039**
100	167.2±48.3[a]	125.9±10.3[2][bc]	157.5±9.9[1,2][a]	235.6±29.3[1][a]	153.4±21.4[1,2]	114.0±5.3[2][ab]	216.9±18.1[1][bc]	**p = 0.008**
500	174.7±38.0[a]	164.1±2.6[1,2][a]	161.3±8.7[1,2][a]	154.8±9.0[2][b]	176.8±27.5[1,2]	130.8±10.2[2][a]	245.9±4.2[1][ab]	**p = 0.048**
1000	174.8±72.8[a]	154.2±9.0[2][ab]	149.3±11.4[2][ab]	128.8±10.7[2][b]	179.1±10.0[1,2]	113.6±6.0[2][ab]	324.1±27.6[1][a]	**p = 0.008**
2000	160.7±38.2[a]	114.5±1.9[2][c]	161.2±26.6[1,2][a]	163.0±16.2[1,2][a]	173.7±7.1[1,2]	130.4±12.7[2][a]	221.6±28.7[1][bc]	**p = 0.014**
Kruskal-Wallis Test [1]	**p = 0.004**	**p = 0.028**	**p = 0.042**	**p = 0.007**	p = 0.107	**p = 0.047**	**p = 0.027**	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).
[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 5. Characterization and comparison of **CAT** between concentrations and between compounds.

Concentration (µg/L)	CAT (mean ± DP)							Kruskal-Wallis Test [2]
	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	
TOTAL		38.7±33.7[1]	35.9±52.0[1]	20.0±4.8[1]	1.2±0.7[2]	40.0±32.4[1]	1.0±0.8[2]	**p = 0.001**
Control	10.0±16.0	42.3±3.5	0.3±0.1	20.0±0.1	1.0±0.5	0.5±0.3	0.7±0.2	p = 0.069
10	15.7±29.1	78.9±8.4	0.7±0.4	20.0±3.3	0.9±0.3	0.3±0.3	0.6±0.4	p = 0.075
100	31.7±36.1	56.2±12.3[1]	102.1±0.0[1]	20.0±8.5[1,2]	0.5±0.3[2]	48.2±4.6[1]	0.5±0.3[2]	**p = 0.025**
500	39.9±42.9	72.1±1.7[1]	110.2±10.6[1]	20.0±2.1[1,2]	1.9±1.1[2]	81.9±14.7[1]	1.7±1.6[2]	**p = 0.025**
1000	12.3±20.6	0.6±0.1[2]	0.7±0.6[2]	20.0±8.5[1,2]	1.4±0.5[2]	57.0±18.0[1]	1.2±0.9[2]	**p = 0.050**
2000	11.1±18.1	0.7±0.4[2]	1.3±0.3[2]	20.0±4.4[1,2]	1.6±0.1[2]	52.1±14.0[1]	1.1±0.4[2]	**p = 0.020**
Kruskal-Wallis Test [1]	p = 0.061	p = 0.051	p = 0.087	p = 0.973	p = 0.119	p = 0.095	p = 0.474	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).
[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 6. Characterization and comparison of **ROS** between concentrations and between compounds.

Concentration (µg/L)	TOTAL	ROS (mean ± DP)						Kruskal-Wallis Test [2]
		(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	
TOTAL		$67.1\pm51.0_{2}$	$105.5\pm148.7_{2}$	$141.6\pm86.1_{12}$	$184.3\pm69.9_{1}$	$124.0\pm101.8_{12}$	$97.9\pm115.5_{2}$	**p = 0.004**
Control	70.0 ± 48.0^{b}	50.2 ± 42.3	2.3 ± 3.3	85.6 ± 15.1	116.2 ± 9.3	39.0 ± 0.0^{b}	126.2 ± 0.0	p = 0.084
10	61.0 ± 53.8^{b}	68.3 ± 84.6	57.0 ± 1.3	44.4 ± 6.7	133.9 ± 17.8	44.2 ± 61.7^{b}	8.3 ± 14.3	p = 0.110
100	101.1 ± 96.6^{b}	23.7 ± 28.1	15.1 ± 21.4	118.9 ± 0.0	204.7 ± 50.2	123.6 ± 0.0^{ab}	257.4 ± 0.0	p = 0.159
500	84.2 ± 70.5^{b}	$78.7\pm57.2_{1}$	$66.5\pm4.0_{12}$	$100.4\pm0.8_{1}$	$181.0\pm40.8_{1}$	$73.5\pm96.0_{12}^{ab}$	$1.0\pm1.7_{2}$	**p = 0.049**
1000	176.3 ± 117.6^{a}	66.9 ± 51.0	214.6 ± 303.5	191.0 ± 17.1	285.3 ± 57.4	181.6 ± 29.4^{a}	155.0 ± 0.0	p = 0.267
2000	225.1 ± 98.3^{a}	114.9 ± 43.5	277.6 ± 148.8	273.3 ± 97.6	245.2 ± 90.6	239.6 ± 144.9^{a}	255.1 ± 41.8	p = 0.298
Kruskal-Wallis Test [1]	**p < 0.001**	p = 0.413	p = 0.263	p = 0.058	**p = 0.041**	p = 0.286	p = 0.125	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 7. Characterization and comparison of **GSH** between concentrations and between compounds.

Concentration (µg/L)	TOTAL	GSH (mean ± DP)						Kruskal-Wallis Test [2]
		(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	
TOTAL		$335.0\pm64.1_{2}$	$418.7\pm69.1_{12}$	$400.9\pm99.8_{1}$	$411.5\pm51.1_{1}$	$372.1\pm100.0_{12}$	$455.9\pm82.4_{1}$	**p = 0.003**
Control	380.1 ± 68.4	457.8 ± 24.8	440.9 ± 5.2^{ab}	324.3 ± 23.0^{b}	390.0 ± 9.9^{ab}	228.8 ± 0.0	386.2 ± 46.2^{b}	p = 0.052
10	364.7 ± 82.7	274.9 ± 16.4	358.4 ± 16.5^{b}	321.6 ± 4.9^{b}	364.0 ± 21.5^{b}	533.2 ± 2.1	336.6 ± 47.9^{b}	p = 0.097
100	395.2 ± 90.9	326.4 ± 3.3	350.3 ± 1.7^{b}	567.5 ± 45.6^{a}	345.8 ± 52.1^{b}	346.4 ± 28.6	457.7 ± 33.3^{ab}	p = 0.120
500	428.3 ± 75.9	$347.2\pm9.9_{2}$	$434.0\pm12.8_{12}^{ab}$	$523.8\pm25.8_{1}^{a}$	$429.7\pm39.3_{12}^{b}$	$306.8\pm32.3_{2}$	$492.6\pm32.0_{1}^{a}$	**p = 0.026**
1000	426.6 ± 98.6	$283.4\pm23.1_{3}$	$371.3\pm30.4_{23}^{b}$	$363.5\pm2.7_{23}^{ab}$	$449.3\pm38.0_{12}^{a}$	$434.4\pm17.4_{12}$	$573.1\pm17.1_{1}^{a}$	**p = 0.034**
2000	413.8 ± 82.0	$320.1\pm2.2_{2}$	$529.0\pm24.8_{1}^{a}$	$362.3\pm34.9_{2}^{ab}$	$468.1\pm12.5_{12}^{a}$	$311.5\pm20.4_{2}$	$426.7\pm20.2_{12}^{ab}$	**p = 0.013**
Kruskal-Wallis Test [1]	p = 0.226	p = 0.061	**p = 0.032**	**p = 0.046**	**p = 0.024**	p = 0.111	**p = 0.028**	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 8. Characterization and comparison of **GSSG** between concentrations and between compounds.

Concentration (µg/L)	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	Kruskal-Wallis Test [2]
TOTAL		$1226.4\pm370.6_2$	$1469.0\pm543.4_2$	$2023.2\pm610.5_1$	$2074.1\pm391.0_1$	$1364.7\pm502.9_2$	$2798.6\pm662.5_1$	**p < 0.001**
Control	1345.2 ± 326^{b}	1155.5 ± 23.3^{ab}	1062.9 ± 49.5	1749.4 ± 28.5	1624.7 ± 53.6	1010.5 ± 17.5	1780.2 ± 0.0	p = 0.058
10	1570.7 ± 613^{ab}	1753.9 ± 91.1^{a}	1394.1 ± 9.2	1486.1 ± 10.3	1513.9 ± 80.6	647.4 ± 23.1	2629.0 ± 173.3	p = 0.063
100	1899.6 ± 873^{ab}	1420.9 ± 106^{ab}	822.1 ± 5.9	3163.2 ± 24.5	2210.1 ± 20.0	1152.6 ± 11.4	2629.0 ± 173.3	p = 0.056
500	1733.3 ± 514^{ab}	857.9 ± 37.5^{b}	1465.7 ± 57.7	1589.1 ± 50.7	2223.8 ± 41.7	2027.2 ± 123.4	2235.8 ± 36.3	p = 0.061
1000	2066.9 ± 1026^{ab}	704.5 ± 24.7^{b}	2008.7 ± 882.5	1767.1 ± 19.8	2363.1 ± 61.5	1697.7 ± 25.6	3860.5 ± 152.2	p = 0.112
2000	2209.2 ± 591^{a}	1500.8 ± 106.5^{a}	2060.6 ± 51.7	2384.2 ± 20.3	2509.1 ± 84.8	1652.6 ± 360.9	3147.9 ± 141.7	p = 0.063
Kruskal-Wallis Test [1]	p = 0.043	**p = 0.042**	p = 0.112	p = 0.061	p = 0.063	p = 0.063	p = 0.091	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 9. Characterization and comparison of **TBARS** between concentrations and between compounds.

Concentration (µg/L)	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	Kruskal-Wallis Test [2]
TOTAL		$25.5\pm10.1_2$	$25.5\pm15.6_2$	$33.5\pm11.5_2$	$28.5\pm12.1_2$	$24.8\pm11.8_2$	$112.2\pm84.0_1$	**p < 0.001**
Control	27.8 ± 8.3	35.9 ± 4.7^{a}	22.4 ± 5.5^{ab}	22.5 ± 6.2	27.3 ± 3.0	18.8 ± 0.0	37.0 ± 11.7	p = 0.115
10	29.7 ± 21.8	12.5 ± 2.6^{c}	16.4 ± 9.6^{b}	39.6 ± 12.7	36.4 ± 31.4	19.6 ± 11.5	61.0 ± 22.4	p = 0.091
100	47.4 ± 76.2	18.8 ± 8.5^{bc}	8.6 ± 4.8^{b}	47.5 ± 19.9	21.5 ± 2.8	13.9 ± 11.9	237.6 ± 38.0	p = 0.061
500	44.1 ± 46.3	22.4 ± 5.2^{bc}	30.8 ± 2.2^{a}	24.8 ± 4.2	28.5 ± 6.0	32.1 ± 11.4	161.3 ± 17.9	p = 0.099
1000	50.6 ± 53.0	30.7 ± 4.3^{ab}	46.6 ± 34.4^{a}	33.4 ± 2.0	29.0 ± 3.1	24.5 ± 9.4	181.5 ± 28.5	p = 0.240
2000	34.1 ± 8.9	32.8 ± 10.1^{ab}	35.4 ± 3.4^{a}	37.5 ± 8.8	28.2 ± 5.8	36.6 ± 7.8	34.0 ± 17.4	p = 0.634
Kruskal-Wallis Test [1]	p = 0.056	**p = 0.022**	**p = 0.027**	p = 0.074	p = 0.334	p = 0.223	p = 0.068	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 10. Characterization and comparison of **LDH** between concentrations and between compounds.

Concentration (µg/L)	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	Kruskal-Wallis Test [2]
TOTAL		$23.4\pm11.3_2$	$43.7\pm21.2_1$	$21.0\pm8.7_2$	$21.6\pm7.9_2$	$19.7\pm17.8_2$	$45.5\pm21.6_1$	**p < 0.001**
Control	25.2 ± 14.0	22.4 ± 7.6	37.5 ± 34.1	20.8 ± 10.3	18.7 ± 5.2	24.8 ± 0.0	42.7 ± 0.0	p = 0.707
10	35.8 ± 23.3	10.1 ± 5.1	62.6 ± 7.1	17.3 ± 5.7	28.0 ± 15.4	52.4 ± 25.9	52.4 ± 18.1	p = 0.060
100	26.2 ± 16.5	25.9 ± 10.0	40.6 ± 16.8	17.6 ± 8.6	20.9 ± 4.4	10.4 ± 1.9	42.1 ± 24.9	p = 0.089
500	28.1 ± 19.2	27.2 ± 8.9	58.8 ± 22.9	19.7 ± 1.8	19.3 ± 10.8	10.8 ± 3.9	26.0 ± 12.6	p = 0.073
1000	28.0 ± 14.3	25.3 ± 13.0	33.0 ± 14.2	30.8 ± 15.1	20.0 ± 6.3	20.7 ± 16.0	41.0 ± 19.4	p = 0.544
2000	29.1 ± 22.0	29.3 ± 17.3	30.3 ± 22.4	21.6 ± 11.1	22.8 ± 1.3	10.6 ± 6.4	60.3 ± 32.1	p = 0.139
Kruskal-Wallis Test [1]	p = 0.906	p = 0.281	p = 0.430	p = 0.831	p = 0.975	p = 0.243	p = 0.705	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 11. Characterization and comparison of **AcHE** between concentrations and between compounds.

Concentration (µg/L)	AcHE (mean ± DP)							Kruskal-Wallis Test [2]
	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	
TOTAL		$64.4\pm21.3_{23}$	$52.3\pm17.2_{3}$	$97.4\pm22.8_{1}$	$90.6\pm30.0_{12}$	$65.4\pm14.8_{23}$	$108.3\pm30.7_{1}$	**p < 0.001**
Control	66.3 ± 16.0	59.9 ± 16.3	60.5 ± 9.3	78.9 ± 12.5	77.2 ± 10.1	38.9 ± 0.0	83.7 ± 0.0^{ab}	p = 0.304
10	73.9 ± 28.6	57.7 ± 26.2	46.9 ± 13.8	82.2 ± 14.6	71.4 ± 31.2	71.7 ± 20.5	113.0 ± 18.9^{a}	p = 0.122
100	67.3 ± 23.9	57.2 ± 15.3	41.8 ± 15.4	88.7 ± 13.6	94.3 ± 17.3	50.3 ± 9.2	75.7 ± 11.0^{b}	p = 0.055
500	93.5 ± 40.7	$100.4\pm8.6_{12}$	$41.8\pm5.1_{3}$	$121.9\pm26.8_{12}$	$78.9\pm10.2_{23}$	$69.3\pm4.1_{23}$	$152.3\pm10.2_{1}{}^{a}$	**p = 0.021**
1000	78.3 ± 24.2	55.9 ± 23.3	70.4 ± 9.1	100.6 ± 35.0	91.1 ± 20.3	71.0 ± 13.7	77.8 ± 14.1^{b}	p = 0.311
2000	90.7 ± 34.7	$67.2\pm16.6_{23}$	$58.3\pm30.6_{3}$	$111.1\pm3.5_{12}$	$143.8\pm47.5_{1}$	$71.9\pm11.2_{23}$	$109.7\pm16.2_{12}{}^{ab}$	**p = 0.038**
Kruskal-Wallis Test [1]	p = 0.136	p = 0.336	p = 0.226	p = 0.172	p = 0.314	p = 0.255	**p = 0.044**	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 11. Characterization and comparison of **GST** between concentrations and between compounds.

Concentration (µg/L)	GST (mean ± DP)							Kruskal-Wallis Test [2]
	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	
TOTAL		$97.3\pm28.6_{1}$	$84.8\pm23.2_{1}$	$7.1\pm8.1_{2}$	$2.0\pm5.0_{2}$	$63.7\pm18.0_{1}$	$5.3\pm10.3_{2}$	**p < 0.001**
Control	32.9 ± 35.5	79.9 ± 8.4	73.4 ± 15.2	6.2 ± 6.2	2.0 ± 3.2	40.3 ± 0.0	7.5 ± 3.1	p = 0.051
10	46.5 ± 54.1	$139.4\pm51.6_{1}$	$79.2\pm12.3_{1}$	$10.5\pm15.5_{2}$	$0.0\pm3.6_{2}$	$90.7\pm4.5_{1}$	$4.7\pm14.1_{2}$	**p = 0.029**
100	41.9 ± 44.9	$96.3\pm22.7_{1}$	$88.8\pm32.1_{1}$	$5.3\pm11.1_{2}$	$1.8\pm2.3_{2}$	$55.6\pm24.7_{1}$	$3.7\pm15.6_{2}$	**p = 0.017**
500	44.1 ± 44.8	$96.2\pm30.0_{1}$	$93.1\pm18.8_{1}$	$6.5\pm8.4_{2}$	$3.4\pm9.2_{2}$	$64.2\pm1.8_{1}$	$7.7\pm1.5_{2}$	**p = 0.022**
1000	44.5 ± 47.3	$102.6\pm18.2_{1}$	$88.1\pm56.0_{1}$	$5.8\pm5.2_{2}$	3.8 ± 11.5	$66.4\pm9.6_{1}$	$1.1\pm17.4_{2}$	**p = 0.036**
2000	40.2 ± 38.4	$77.8\pm18.4_{1}$	$87.3\pm24.0_{1}$	$8.6\pm5.1_{2}$	$1.5\pm2.0_{2}$	$58.4\pm15.4_{1}$	$7.7\pm6.8_{2}$	**p = 0.013**
Kruskal-Wallis Test [1]	p = 0.993	p = 0.404	p = 0.882	p = 0.993	p = 0.992	p = 0.218	p = 0.993	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 12. Characterization and comparison of **mean Speed (mm/min)** between concentrations and between compounds.

Concentration (µg/L)	Mean Speed (mm/min) (mean ± DP)							Kruskal-Wallis Test [2]
	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	
TOTAL		$13.7\pm6.2_1$	$12.6\pm5.9_{12}$	$10.2\pm2.8_{23}$	$12.8\pm5.5_{12}$	$7.7\pm5.1_4$	$9.9\pm4.7_3$	**p < 0.001**
Control	10.7±5.8	12.2±8.1	14.8±7.4	8.5±3.4	12.1±4.1	7.4±5.6	10.1±4.6	p = 0.101
10	11.4±6.3	10.8±4.4	14.1±7.4	11.1±2.0	14.4±8.6	6.5±3.7	12.5±8.0	p = 0.051
100	10.7±5.2	$15.5\pm6.3_1$	$10.5\pm3.5_{23}$	$10.4\pm2.5_{23}$	$11.3\pm3.6_{12}$	$6.3\pm6.5_3$	$10.0\pm4.5_{23}$	**p = 0.025**
500	12.0±5.9	$18.0\pm6.0_1$	$13.4\pm5.2_{12}$	$9.7\pm2.6_{23}$	$13.2\pm7.4_{123}$	$8.3\pm5.0_3$	$9.8\pm3.1_{23}$	**p = 0.017**
1000	11.4±5.0	13.3±3.2	12.5±7.5	10.8±2.7	13.1±5.0	8.7±5.4	9.5±3.8	p = 0.096
2000	10.4±4.9	11.6±7.8	10.3±3.5	10.8±3.4	12.9±4.5	8.7±5.1	8.0±3.2	p = 0.212
Kruskal-Wallis Test [1]	p = 0.715	p =0.106	p =0.773	p =0.507	p =0.924	p =0.415	p =0.607	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 13. Characterization and comparison of **travelled Distance (cm)** between concentrations and between compounds.

Concentration (µg/L)	Distance (cm) (mean ± DP)							Kruskal-Wallis Test [2]
	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	
TOTAL		$137.3\pm62.1_1$	$125.6\pm59.2_{12}$	$102.1\pm28.1_{23}$	$127.8\pm55.0_{12}$	$76.7\pm51.0_4$	$99.2\pm47.0_3$	**p < 0.001**
Control	107.0±57.7	122.1±81.1	147.7±74.3	84.5±34.1	121.1±41.0	73.9±56.0	100.9±46.3	p = 0.101
10	114.3±62.6	107.8±44.0	141.0±74.2	110.8±20.4	143.9±86.1	65.1±36.7	124.8±79.8	p = 0.051
100	107.4±52.2	$155.1\pm62.8_1$	$104.5\pm34.7_{23}$	$104.1\pm25.3_{23}$	$112.6\pm36.0_{12}$	$62.8\pm65.4_3$	$99.8\pm45.2_{23}$	**p = 0.025**
500	119.5±59.0	$179.9\pm60.4_1$	$133.9\pm52.2_{12}$	$96.7\pm26.4_{23}$	$131.6\pm74.2_{123}$	$82.9\pm49.7_3$	$97.8\pm31.5_{23}$	**p = 0.017**
1000	114.2±50.0	133.0±32.3	125.3±75.5	107.9±26.8	131.2±49.9	87.5±54.4	95.4±37.9	p = 0.096
2000	103.6±48.7	116.0±78.3	103.0±35.2	107.9±33.5	129.3±44.9	87.2±51.2	79.5±32.4	p = 0.212
Kruskal-Wallis Test [1]	p = 0.715	p =0.106	p =0.773	p =0.507	p =0.924	p =0.415	p =0.607	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 14. Characterization and comparison of **Distance to center** between concentrations and between compounds.

Concentration (µg/L)	Distance to center (cm) (mean ± DP)							Kruskal-Wallis Test [2]
	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	
TOTAL		1.38±0.15	1.32±0.15	1.43±0.20	1.39±0.26	1.35±0.26	1.40±0.16	p = 0.102
Control	1.37±0.16	1.27±0.33[2]	1.32±0.12[2]	1.50±0.09[1]	1.40±0.10[12]	1.27±0.14[2]	1.42±0.12[12][a]	p = 0.037
10	1.36±0.28	1.33±0.19	1.24±0.15	1.43±0.22	1.39±0.33	1.36±0.50	1.40±0.16[ab]	p = 0.472
100	1.41±0.19	1.44±0.10	1.34±0.24	1.36±0.23	1.42±0.24	1.39±0.18	1.50±0.12[a]	p = 0.528
500	1.36±0.21	1.39±0.09	1.28±0.12	1.43±0.17	1.36±0.36	1.31±0.21	1.40±0.21[ab]	p = 0.231
1000	1.35±0.19	1.38±0.12	1.37±0.08	1.44±0.29	1.38±0.23	1.28±0.21	1.26±0.15[b]	p = 0.482
2000	1.41±0.19	1.41±0.13	1.34±0.16	1.42±0.17	1.43±0.32	1.45±0.16	1.42±0.14[a]	p = 0.797
Kruskal-Wallis Test [1]	p = 0.102	p =0.719	p =0.552	p =0.557	p =0.486	p =0.317	**p =0.041**	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 15. Characterization and comparison of the **Activity** (% of moving time) between concentrations and between compounds.

Concentration (µg/L)	Activity (mean ± DP)							Kruskal-Wallis Test [2]
	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Kra10	NKrac	
TOTAL		45.0±21.9[2]	46.9±19.8[2]	49.8±19.9[2]	44.1±20.9[2]	64.1±22.7[1]	49.4±17.7[2]	**p < 0.001**
Control	49.9±22.1	54.1±26.0	45.1±19.2	43.3±19.5	41.2±24.7	59.8±24.2	56.8±20.0	p = 0.509
10	50.7±20.5	43.5±25.9	51.5±19.1	51.8±18.7	47.2±24.4	62.2±20.6	49.3±14.5	p = 0.426
100	45.5±20.3	38.8±19.4[2]	36.0±16.8[2]	45.4±15.9[2]	40.8±13.7[2]	72.1±23.5[1]	41.7±13.7[2]	**p = 0.013**
500	50.4±21.0	37.7±12.7	44.8±15.3	54.1±22.3	48.3±22.8	60.2±24.0	55.9±23.0	p = 0.333
1000	47.1±21.2	43.0±23.3	50.7±24.1	46.7±18.1	42.4±21.4	60.9±24.4	41.7±15.5	p = 0.587
2000	55.4±22.8	57.4±23.1	54.2±23.8	56.4±25.8	44.8±22.6	68.9±23.5	50.7±16.0	p = 0.396
Kruskal-Wallis Test [1]	p = 0.237	p =0.160	p =0.571	p =0.708	p =0.817	p =0.794	p =0.283	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

[2] Tests for comparison between compounds; [1,2,3] there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).

Table 16. Characterization and comparison of **Absolute Turn Angle** (inversions numbers) between concentrations and between compounds.

Concentration (µg/L)	Absolute Turn Angle (mean ± DP)							Kruskal-Wallis Test [2]
	TOTAL	(S)-K	(R)-K	(S)-NK	(R)-NK	Krac	NKrac	
TOTAL		7.88±2.84[12]	7.09±2.57[2]	8.09±2.39[12]	8.59±3.34[1]	5.48±2.24[3]	7.65±2.36[12]	**p < 0.001**
Control	6.93±2.16	6.44±3.39	8.15±2.71	6.27±1.78	7.39±1.63	5.68±1.79	7.36±1.71	p = 0.324
10	6.70±2.95	6.29±2.23	7.15±3.00	7.33±2.36	8.26±4.63	4.30±2.31	7.05±2.08	p = 0.173
100	7.56±2.94	9.25±3.78[1]	6.87±2.30[23]	8.41±1.43[12]	7.61±2.66[123]	4.93±2.78[3]	7.94±2.97[12]	**p = 0.038**
500	8.19±2.88	9.67±1.58[1]	6.58±2.98[23]	8.47±2.90[12]	9.62±3.53[1]	6.07±1.44[3]	8.77±2.74[12]	**p = 0.011**
1000	7.46±2.52	7.91±1.23	6.90±2.91	9.48±2.79	7.90±1.81	5.75±2.78	7.12±2.71	p = 0.258
2000	7.84±3.09	6.86±3.02[23]	6.88±2.00[23]	8.88±2.31[12]	10.57±4.47[1]	6.04±2.34[3]	7.60±1.87[12]	**p = 0.040**
Kruskal-Wallis Test [1]	p = 0.130	p =0.059	p =0.926	p =0.190	p =0.192	p =0.509	p =0.739	

[1] Tests for comparison between concentrations; [a,b,c] there are no statistically significant differences between concentrations with the same letter ($p > 0.05$ in the multiple comparison tests).

⁽²⁾ Tests for comparison between compounds; $_{1,2,3}$ there are no statistically significant differences between compounds with the same number ($p > 0.05$ in the multiple comparison tests).