CESPU
COOPERATIVA DE ENSINO SUPERIOR POLITÉCNICO E UNIVERSITÁRIO

Enantioselective ecotoxicity of psychoactive substances in *Daphnia magna*

IUCS - 2022

CESPU
INSTITUTO UNIVERSITÁRIO DE CIÊNCIAS DA SAÚDE

Enantioselective ecotoxicity of psychoactive substances in *Daphnia magna*

Cristiano Manuel Araújo Gomes

Dissertation for the Master's Degree in Forensic Sciences and Laboratory Techniques

Gandra, **26 de Abril** de 2022

Enantioselective ecotoxicity of psychoactive substances in *Daphnia magna*

Cristiano Manuel Araújo Gomes

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

Under guidance of Professor Doctor Cláudia Ribeiro, Professor Doctor João Soares Carrola and Professor Doctor Maria Elizabeth Tiritan

DECLARATION OF INTEGRITY

CRISTIANO MANUEL ARAÚJO GOMES, student of the Master's Degree in *Forensic Sciences and Laboratory Techniques* of the University Institute of Health Sciences, declare that I have acted with absolute integrity in the preparation of this Dissertation. I confirm that in all the work leading to its elaboration I did not use any form of falsification of data or the practice of plagiarism (an act by which an individual, even by omission, assumes the authorship of the intellectual work belonging to another, in its entirety or in parts of it). I further declare that all the sentences that I have taken from previous works belonging to other authors have been referenced or written with new words, in which case I have cited the respective bibliographic source.

AGRADECIMENTOS

A realização deste trabalho só foi possível devido ao vasto número de pessoas envolvidas, bem como ao apoio proporcionado por várias instituições. Assim, pretendo desde já agradecer de uma forma geral a todos pela disponibilidade e pelo auxílio prestado.

Quero agradecer em especial à minha orientadora, a Professora Doutora Cláudia Ribeiro, por todo o seu esforço durante a realização deste projeto, por toda a sua disponibilidade, pelo facto de puxar por mim todos os dias, pelos conhecimentos transmitidos, pelo seu apoio não só durante a realização deste projeto como também durante todos os anos em que nos acompanhou como Professora e por toda a sua disponibilidade para tirar dúvidas. Quero desde já agradecer o acompanhamento intenso e por todo o apoio que proporcionou durante todo este último ano. Muito obrigada por me deixar fazer parte desta equipa de investigação. Foi um prazer trabalhar com a Professora, muito obrigado por ter aceitado a orientação e por ter tornado esta experiência possível.

Ao Professor Doutor João Soares Carrola por toda partilha dos seus conhecimentos transmitidos ao longo dos ensaios, e por toda ajuda e explicações dos programas informáticos para o tratamento dos dados das morfometrias e medição dos batimentos cardíacos, desenho vectorial no powerpoint, e para a realização das referências bibliográficas, e também as novas técnicas implementadas com as dáfnias, e também gostaria de lhe agradecer todo entusiasmo contagioso.

À Professora Doutora Maria Elizabeth Tiritan por todo apoio e conhecimentos transmitidos desde o meu primeiro semestre de universidade, por toda a sua disponibilidade e ajuda.

Ao Professor Bruno Castro pela partilha de todo o seu conhecimento do organismo e ajuda na melhor maneira da realização dos ensaios e na interpretação de resultados.

Quero agradecer a Ariana Pereira por todo o apoio, por ter me introduzido ao laboratório, por toda a ajuda prestada, por toda a sua partilha de conhecimento comigo, sem ela não teria sido possível qualquer desenvolvimento de trabalho.

AGRADECIMENTOS

Queria também agradecer a todo os trabalhadores da CESPU, por todo o apoio dado desde 2016, serão sempre como uma família para mim. Agradecer à TOXRUN-Unidade de Toxicologia do Instituto Universitário de Ciências da Saúde, por todo o suporte em equipamento e instalações para a execução deste trabalho.

Este trabalho foi financiado por fundos nacionais através da FCT/MCTES (PIDDAC), no âmbito do projeto PTDC/CTA-AMB/6686/2020.

Part of the results presented in this dissertation were used in the following scientific communication:

Poster communications:

1. Pereira A., **Gomes C.,** Carrola J., Tiritan M.E., Ribeiro C. (2021). Physiological behavioural and genotoxic effects of psychoactive drugs in aquatic organisms using daphnia as model. XIII Jornadas de Genética e Biotecnologia, Universidade de Trás-os-Montes e Alto Douro, 14 de abril de 2021, Vila Real, Portugal.

2. **Gomes C**., Pereira A., Carrola J., Castro B., Couto C., Tiritan M.E., Ribeiro C. (2021). Enantioselective ecotoxicity of psychoactive substances in *Daphnia magna*. 4th Doctoral Congress in Engineering - Symposium on Environmental Engineering, 26 June 2021, Porto, Portugal.

ABSTRACT

Psychoactive substances (PAS) are emergent contaminants frequently detected in aquatic ecosystems that may pose environmental risks in aquatic organisms even at low concentrations (ng to µg/L). Many PAS are chiral substances commercialized as racemate. Amphetamine (AMP) is a central nervous system stimulant used in the treatment of attention deficit hyperactivity disorder narcolepsy, and obesity. AMP is a chiral substance that exhibits enantioselective in its pharmacological activity being the (*S*)-AMP more potent and clinically effective than (*R*)-AMP. On the other hand, AMP is frequently used as a recreative drug. Due to its high consume and low biodegradability AMP has been detected in wastewaters and surface waters and can occur as enantiomeric mixtures or pure enantiomers. In this context, the evaluation of its enantioselectivity in eco-toxicity is crucial for a better understanding of AMP environmental risk on non-target organisms in freshwater ecosystems. Therefore, the present study aimed to investigate the enantioselectivity of AMP in toxicity on the aquatic invertebrate daphnia (*Daphnia magna*), used as a model organism to assess different biomarkers of toxicity. For that, neonates (less than 24h old) were exposed to 0.1; 1, and 10 µg/L of the racemate (*rac*-AMP) and to 0.1 and 1 µg/L of pure enantiomers, (*R*)-AMP and (*S*)-AMP, for 8 days. At selected 3, 5, and 8-days of exposure, different parameters were determined as morphophysiology (on days 3 and 8, as body size and heart rate, area, and length); swimming behaviour (on day 5, as swimming speed, active time, and total distance); reproduction (on day 8, number of eggs per daphnia, number of daphnia with eggs and number of neonates) and biochemical parameters (on day 8, like oxidative stress, catalase (CAT) and acetylcholinesterase (AChE) enzymatic activities).

Data showed a significant decrease in body size found for organisms exposed to (*S*)-AMP comparatively to (*R*)-enantiomer and to the racemate. Effects on the development and functioning of heart were observed with a significant decrease in heart rate for both racemate and enantiomers though a decrease was observed in the organisms exposed to the racemate at days 3 and 8, while for the enantiomers a decrease was observed at day 3 with an enantioselective effect at 0.1 ug/L (lower decrease for (*R*)-AMP) but on

day 8 no differences were found. A significant decrease in the heart area was observed for both enantiomers.

Regarding swimming behaviour, different results were observed between racemate and enantiomers. (*rac*)-AMP caused a reduction of the total travelled distance while for both enantiomers an increase in total travelled distance was noted though no enantioselective effects were observed. No changes were observed in active time in the organism exposed to (*rac*)-AMP while a reduction of the active time was observed for both enantiomers.

No changes in the number of eggs per daphnia or the number of daphnia with eggs were observed for the racemate, however, a tendency to increase of the number of neonates at 0.1 and 1 µg/L was observed while a significant increase was found at 10 µg/L. Regarding enantiomers, a significant difference was found between enantiomers with a decrease in the organisms exposed to (*S*)-AMP in contrast to the increase in the organisms exposed to (*R*)-AMP at 1 µg/L. Though no significant differences were observed for the number of daphnia with eggs, a tendency to increase was observed in the organisms exposed to (*R*)-AMP. These results show that AMP affects reproductive performance of daphnia, and these effects are enantiomer dependent. Changes in biochemical parameters were also observed with a significant decrease in reactive oxygen species (ROS) and CAT activity for enantiomers.

These results showed that AMP can interfere with different biomarkers of toxicity and these effects can be enantioselective demonstrating the relevance and providing evidence for the need for this kind of study for an accurate environmental risk assessment. Additionally, some of the effects were observed at environmental reported concentrations (0.1 and 1 µg/L) AMP both racemate and enantiomers can cause adverse effects on *D. magna* reinforcing the concern of invertebrate medium- and long-term exposure to AMP.

Keywords: Environmental risk assessment, chiral drugs, oxidative stress, enantioselectivity, contaminants.

RESUMO

As substâncias psicoativas (SPA) são contaminantes emergentes frequentemente detetados em ecossistemas aquáticos e podem apresentar riscos ambientais mesmo quando presentes em baixas concentrações (ng a µg/L). Muitas SPA são substância quirais comercializadas como racemato. A anfetamina (AMP) é um estimulante do sistema nervoso central usado no tratamento do transtorno do déficit de atenção e hiperatividade, narcolepsia e obesidade. A AMP é uma SPA quiral em que a sua atividade farmacológica apresenta enantiosseletividade sendo a (*S*)-AMP mais potente e clinicamente mais eficaz do que a (*R*)-AMP. Por outro lado, a AMP é frequentemente usada como substância recreativa. Devido ao seu elevado consumo e baixa biodegradabilidade a AMP tem sido detetada em águas residuais e águas superficiais como misturas ou na forma enantiomericamente pura. Nesse contexto, o estudo de sua enantiosseletividade na eco-toxicidade é fundamental para uma melhor compreensão do risco ambiental em organismos não-alvo em ecossistemas fluviais.

O objetivo do presente estudo foi investigar a toxicidade enantiosseletiva da AMP para a dáfnia (*Daphnia magna*) usada como organismo modelo visando avaliar diferentes biomarcadores de toxicidade. Para isso, dáfnias (recém-nascidas com < 24 h de vida) foram expostas a 0,1; 1 e 10 µg/L do racemato (*rac*-AMP) e a 0,1 e 1 µg/L de cada enantiómero (*R*)-AMP e (*S*)-AMP durante 8 dias. Em dias selecionados 3º, 5º e 8º dias de exposição, diferentes parâmetros foram determinados como os morfofisiológicos (nos dias 3 e 8, como tamanho corporal e frequência cardíaca, área e comprimento do coração); comportamento de natação (no dia 5, como velocidade de natação, tempo ativo e distância percorrida); parâmetros reprodutivos (no dia 8, número de ovos por dáfnia, número de dáfnias com ovos e número de neonatos) e parâmetros bioquímicos (no dia 8, como stress oxidativo e atividades enzimáticas da catalase (CAT) e acetilcolinesterase (AChE).

Foram observadas alterações morfofisiológicas, e para alguns parâmetros estudados foram observados efeitos enantiosseletivos ou diferenças entre os enantiómeros e o racemato. Por exemplo, foi observada uma diminuição significativa no tamanho corporal em organismos expostos à (*S*)-AMP comparativamente ao (*R*)-enantiómero e ao racemato. Foram observados efeitos sobre o desenvolvimento e

funcionamento do coração com uma diminuição significativa na frequência cardíaca tanto para o racemato quanto para os enantiómeros, embora para o racemato tenha ocorrido em ambos os dias (3 e 8), enquanto para os enantiómeros foi observada uma diminuição no dia 3 com um efeito enantiosseletivo na concentração de 0,1 ug/L (decréscimo para a (*R*)-AMP), mas no dia 8 não foram encontradas diferenças.

Em relação ao comportamento de natação, foram observados resultados diferentes entre racemato e os enantiómeros. A (*rac*)-AMP causou uma redução da distância total percorrida, enquanto para ambos os enantiómeros observou-se um aumento na distância total percorrida, embora não se tenham observado efeitos enantiosseletivos. Não foram observadas alterações no tempo ativo nos organismos expostos ao (*rac*)-AMP enquanto uma redução do tempo ativo foi observada para ambos os enantiómeros.

Não foram observadas alterações no número de ovos por dáfnia e no número de dáfnias com ovos para o racemato, porém houve uma tendência ao aumento do número de neonatos a 0,1 e 1 µg/L enquanto na concentração de 10 µg/L verificou-se um aumento significativo. Em relação aos enantiómeros, foram observadas diferenças significativas entre os enantiómeros com uma diminuição significativa nos organismos expostos à (*S*)-AMP em contraste com o aumento nos organismos expostos à (*R*)-AMP a 1 µg/L. Embora não tenham sido observadas diferenças significativas para o número de dáfnias com ovos nos organismos expostos à (*R*)-AMP foi observada uma tendência para o aumento. Esses resultados mostram que a AMP afeta o desempenho reprodutivo da dáfnia e que os efeitos são dependentes dos enantiómeros. Mudanças nos parâmetros bioquímicos também foram observadas com uma diminuição significativa nas espécies reativas de oxigénio (EROs) e atividade da CAT para enantiómeros.

Esses resultados mostram que a AMP pode interferir em diferentes biomarcadores de toxicidade e que os efeitos podem ser enantiosseletivos, demonstrando a importância deste tipo de estudos para uma fazer uma avaliação mais correta do risco ambiental. Além disso, considerando que alguns dos efeitos foram observados em concentrações ambientais reportadas (1 e 1 µg/L), a AMP tanto racemato quanto os seus enantiómeros podem causar efeitos prejudiciais na *D. magna*,

reforçando ainda a preocupação com os efeitos da exposição à AMP a médio e longo prazo.

Palavras-chave: Avaliação de risco ambiental, substâncias quirais, stress oxidativo, enantiosseletividade, contaminantes ambientais.

INDEX

INDEX OF FIGURES

INDEX OF TABLE

LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

AChE	Acetylcholinesterase
ADHD	Attention Deficit Hyperactivity Disorder
AMP	Amphetamine
AMT	Metatartaric acid
ASTM Hard water	Hard Reconstituted Water
ATCI	Acetylthiocholine Iodide
BHT	Butylated Hydroxytoluene
CAT	Catalase
CEC	Contaminants Of Emerging Concern
DCFH	Dichlorofluorescin
DTNB	5,5'-Dithiobis (2-nitrobenzoic acid)
EF	Enantiomeric Fraction
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
FDA	Food and Drug Administration
FLX	Fluoxetine
H2DCFDA	2,7-Dichlorofluorescin diacetate
ISO	International Organization Of Standardization
K	Ketamine
MAO	Monoamine Oxidase Enzymes
MDA	Malondialdehyde
MHRW	Moderately Hard Reconstituted Water
NK	Norketamine
OECD	Organization For Economic Cooperation and Development
PAS	Psychoactive Substances
ROS	Reactive Oxygen Species

SOD	Superoxide Dismutase
SPB	Sodium Phosphate Buffer
TBAR	Thiobarbituric Acid
TNB	5-thio-2-nitrobenzoic acid
WWTP	Wastewater Treatment Plant

1. Introduction

1.1. Contaminants of emerging concern

Contaminants of emerging concern (CEC) are a general term used to describe a broad class of chemicals of environmental contaminants, known or suspected to cause adverse health effects in wildlife and/or humans and are not yet regulated under current environmental laws (Su et al., 2020; Yadav et al., 2021).

Among CEC, psychoactive substances (PAS) are a serious environmental issue since they can cause biological responses at low concentrations due to their pseudo-persistence. PAS includes pharmaceuticals and illicit drugs. These substances are designed to change nervous system function and have been shown to interfere with biochemical, physiological, and behavioural processes of exposed non-target species (Fontes et al., 2020; Pérez-Pereira et al., 2020).

Like other CEC, PAS and their metabolites, are released into aquatic environments through multiple pathways, including industrial and urban wastewater treatment plant (WWTP) effluents, hospital WWTP discharges, urban direct discharges, improper manufacturers disposal, illicit production, and veterinary use (Ribeiro et al., 2016; Ribeiro et al., 2017; Fernández-Rubio et al., 2019; Ribeiro et al., 2020). **Figure 1** shows the different sources of pharmaceuticals and illicit drugs that reach water ecosystems.

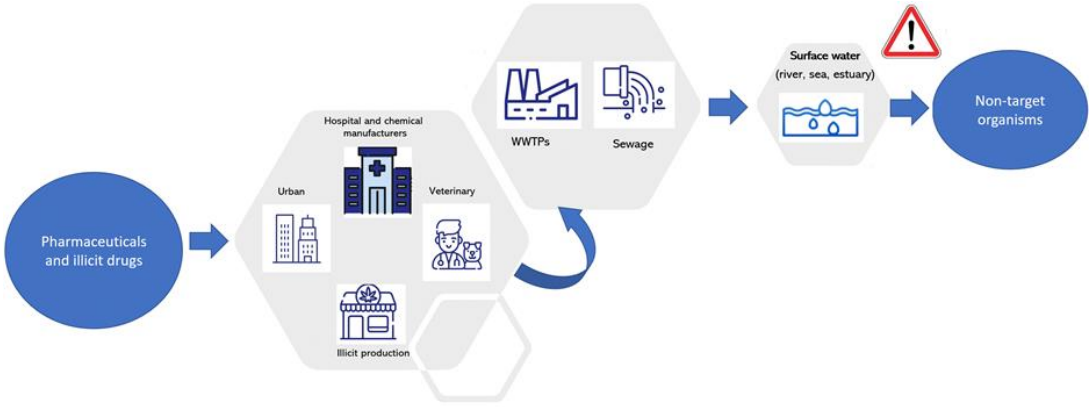


Figure 2 - Sources of pharmaceuticals and illicit drugs in water resources.

The increasing consumption of PAS and the limited efficiency of classical sewage treatment processes contribute to their extensive spread in the aquatic environment. In fact, PAS has been reported in sewage, river, seawaters, drinking water and even biota at concentrations ranging from ng/L to μg/L (Ribeiro et al., 2016; Ribeiro et al., 2017; Fontes et al., 2020). Additionally, the "pool of substances" that can affect non-target species, even in low doses of exposure, can be even higher due to transformation products resulting from environment structural changes in both parent compounds and metabolites by a variety of biotic and abiotic transformations (Subedi and Kannan, 2015; Ribeiro et al., 2020).

Several studies have shown that PAS induce acute and chronic toxicity in aquatic organisms at trace levels (Xie et al., 2015; Guo et al., 2016; Yang et al., 2018; Wang et al., 2020a). For instance, exposure to methamphetamine (MAMP) has been shown to cause changes in the bacterial community in sediments, alter the neurotransmitter system of the earthworm and cause physiological changes in the larvae of medaka fish, *Oryzias latipes* (Liao et al., 2015; Wang et al., 2019; Wang et al., 2020b). Exposure to cocaine at environmental levels (50 ng/L and 500 ng/L) induced the production of reactive oxygen species (ROS) and modulated antioxidant defence system in daphnids under laboratory studies (De Felice et al., 2019). Exposure to the cocaine metabolite benzoylecgnonine at concentrations similar to those found in aquatic ecosystems inhibited acetylcholinesterase activity causing changes in swimming behaviour and reproduction in *Daphnia magna* (Parolini et al., 2018b).

Even though, there is still a lack of information about the real effects of PAS. Knowledge about the impact of these pollutants on aquatic organisms is of high importance for risk assessment and further establishment of measures for environmental protection.

1.2. Chiral psychoactive drugs

Most PAS are chiral, and their enantiomers may behave differently in biological systems. Despite the current understanding concerning the distinct behaviour of the enantiomers in biological systems, environmental toxicological impacts of single

enantiomers on non-target organisms are still largely unknown and have frequently been neglected in most studies. Accurate risk assessment of chiral PAS in the environment therefore must consider the enantioselectivity in fate and ecotoxicity.

The enantioselectivity in non-target toxicity has been observed. For instance, in standardized growth inhibition and acute immobilization tests, enantiotoxicity and different susceptibility of the organisms protozoan *Tetrahymena thermophila* and the microcrustacea *D. magna* exposed to ketamine (K) and its metabolite norketamine (NK) were observed. (*S*)-K enantiomer presented higher toxicity than the (*R*)-K. No toxicity of NK enantiomers was found for *D. magna* while the protozoan growth inhibition was observed after exposure to both enantiomers. In sublethal and behavioral endpoints studies, (*S*)-fluoxetine (FLX) was found to be more toxic than (*R*)-FLX to fathead minnow, *Pimephales promelas* (Stanley et al., 2007).

The enantioselectivity in toxicity of dopa, FLX and atenolol was also reported in three organisms: *Pseudokirchneriella subcapitata*, *D. magna* and *T. thermophila*. The (*S)*-enantiomer was the most toxic and *T. thermophila* was the most sensitive species to the enantiomers of FLX and atenolol (De Andres et al., 2009).

1.2.1 Amphetamine

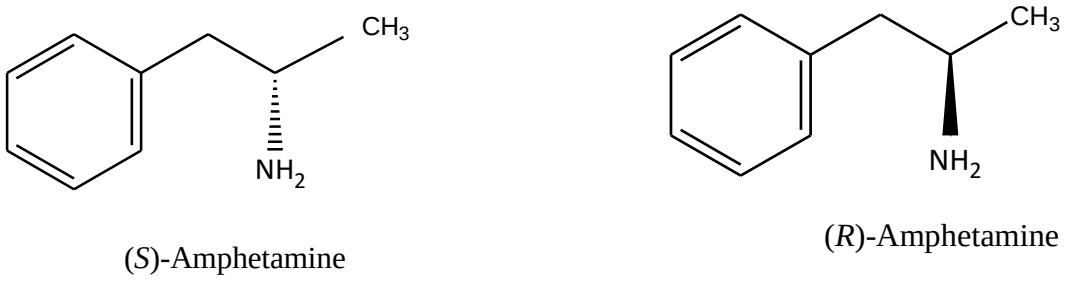
Amphetamine (AMP) was first synthesized from ephedrine which possesses a phenylethylamine structure. AMP use peaked during World War II, when it was administered to German and American soldiers to eliminate fatigue, enhance morale, build endurance, and maintain alertness (ROBINS et al., 1974).

Nevertheless, due to its psychoactive activity, AMP is mostly recognized as a recreational drug (EMCDDA, 2020, 2021). It remains one of the most frequently abused drugs however, it is also used in clinical context for the treatment of Parkinson disease, attention deficit hyperactivity disorder (ADHD) and obesity (Coutts and Baker, 1989; de la Torre et al., 2004).

According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), cannabis is the most used illicit drug followed by amphetamines in the in northern and eastern Europe, as opposed to cocaine in western and southern Europe (EMCDDA, 2020, 2021).

AMP has one stereogenic center (**Figure 2**) and the enantiomers present different pharmacodynamic response. The (S) enantiomer ((+)-AMP, dexamphetamine) shows stimulant and anorexia properties. Due to these properties, it is used as a psychomotor stimulant, in hyperkinetic syndrome and narcolepsy (Coutts and Baker, 1989) and the (*R)* enantiomer ((-)-AMP, levoamphetamine) with similar properties but lower potency. The majority of prescribed AMP consists of the pure (*S*)-enantiomer, but therapeutic formulations containing the (*R*)-enantiomer in various proportions are also available (Faraone, 2018). Illegal amphetamine remains available mainly as a racemate.

In Portugal, AMP was added in 2019-02-06 to the list of drugs in Decree-Law no. 15/93,"Law to Combat Drugs". Medications that contained the two enantiomers of AMP and AMP like substances were banned from circulation in “Deliberation nº 431/2001” due to their danger of addiction, and therefore, formulations with only the (*S*)-*enantiomer* are available such as Elvanse®



(*S*)-Amphetamine (*R*)-Amphetamine

Figure 3 - Chemical structure of amphetamine enantiomers (*S* and *R*).

AMP is a neuromodulator of the central nervous system. It stimulates the release of catecholamines such as adrenaline, norepinephrine, and dopamine from the presynaptic neurons, blocks their reuptake, and inhibits degradations by the monoamine oxidase enzymes (MAO) (Coutts and Baker, 1989; Attafi et al., 2021).

In general, catecholamines have a short half-life, and AMP half-life is also considerably low compared to other psychoactive substances (≈6-12 hours). After consumption AMP is mainly metabolized in the hepatic system. Despite this being quite extensive there is a large percentage of AMP that is not metabolized, being eliminated in the urine (de la Torre et al., 2004). Studies further demonstrate enantioselective metabolism of AMP, with preferential elimination of the (*S*)-enantiomer (Gunne and Galland, 1967; Caras and Sharpe, 2019). The biodegradation in WWTP can also be enantioselective due to microbiological processes during the treatment of effluents, which leads to changes in their enantiomeric fraction (EF). Nevertheless, AMP has been found in effluents and aquatic systems in different EF but also as a racemate (Goncalves et al., 2019; Langa et al., 2021a; Langa et al., 2021b). Concentrations ranging from 7 to 14 ng/L in surface waters and up to 700 ng/L in effluent samples have been reported (Coelho et al., 2019). Though some studies have been describing the adverse effects on non-target organisms of illicit drugs, studies regarding the potential toxicity effects of AMP are scarce and enantioselective studies are not reported.

1.3. Toxicity assays

Standardized protocols developed by international organizations, such as the - *Organization for Economic Cooperation and Development* (OECD) or *International Organization of Standardization* (ISO) are used for the assessment of the potential effects of contaminants (OECD, 2004; ISO, 2012a, b; OCDE, 2012). These protocols use keystone species and describe the procedure and endpoints that should be evaluated.

European Union directive n. º 2010/63/EU recommend avoiding unnecessary tests on vertebrates and cephalopods, and following the 3R principle (refinement, replacement and reduction). The concern about animal used in research increased the pressure to use *in silico*, *in vitro* test but also to use, when possible, organisms like microcrustacean as *D. magna* as a keystone invertebrate organism of freshwater systems. *D. magna* is a recommended test species for the OECD and ISO in their chemical testing guidelines (**Figure 3**).

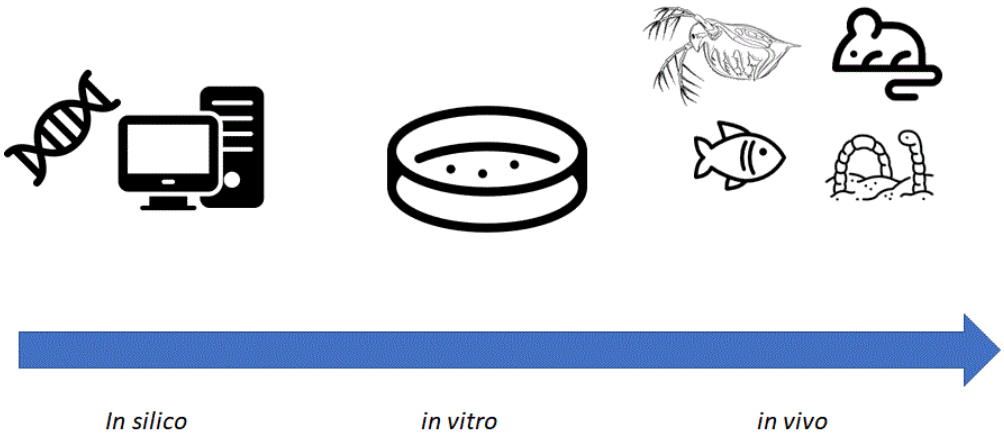


Figure 4 - Schematic representation the different methods that can be used to estimate or evaluate ecotoxicological effects: *in silico* studies using computational techniques; *in vitro* using cell or embryo cultures, and *in vivo* techniques using diverse living organisms such as microcrustaceans, fish, earthworms, and rodents adapted from Ribeiro et al., 2021.

Nevertheless, some limitations have been pointed as these ecotoxicity tests are mainly focused on standard endpoints (e.g., reproduction, immobility or mortality) however ecological risk assessment requires other endpoints namely at sublethal or environmental levels.

Therefore, standardized protocols have been adjusted to include morphophysiological, biochemical, genetical and swimming behavioural endpoints that can be important biomarkers of toxicity.

1.3.1 Aquatic animal model, *Daphnia magna*

D. magna is one of the most used model organisms in aquatic ecotoxicological studies (OECD, 2004; OCDE, 2012). *Daphnia* sp also known as "water flea" is an invertebrate planktonic microcrustacean commonly found in freshwater ecosystems (Mergeay et al., 2006; Loureiro et al., 2011; Serra et al., 2019). They are filter-feeding organisms (e.g., bacteria, algae, cyanobacteria, protozoans and other small particles suspended in the water) playing a key role as a primary consumer in the aquatic food chain and as a key food source contributing to secondary consumers and for

maintenance of freshwater systems (Tkaczyk et al., 2021). Therefore, *Daphnia* is an ecologically relevant organism and there is extensive literature regarding its characteristics, life cycle, ecological role, ecotoxicity and genomic (Shaw et al., 2008; Loureiro et al., 2011; Harris et al., 2012; Bownik, 2017; Castro et al., 2018; Ribeiro et al., 2021).

D. magna presents several advantages as a model organism: it is relatively easy to maintain in laboratory; has a short life cycle; easy handling and low cost of maintenance. Easy reproduction and high fecundity are important to obtain many organisms in a short period. Another practical advantage is the possibility to analyse numerous parameters (immobility, behaviour, reproduction, heartbeat) with non-invasive methods such as microscope or loupe (including image and video processing systems), because of its semi-transparent body (Tkaczyk et al., 2021).

Standard acute assays are based on the observation of mortality or immobilization and standard chronic assays are mainly based on reproductive endpoints (Nunes et al., 2014; Nevesa et al., 2015). Nevertheless, some studies include other endpoints as the assessment of behaviour, morphophysiology, biochemical parameters and even transgenerational effects are important biomarkers to assess environmental stress at sublethal and environmental concentrations (Masteling et al., 2016; Castro et al., 2018; Parolini et al., 2018a; Bownik, 2020). **Figure 4** shows morphological and anatomic characteristics of *D. magna*.

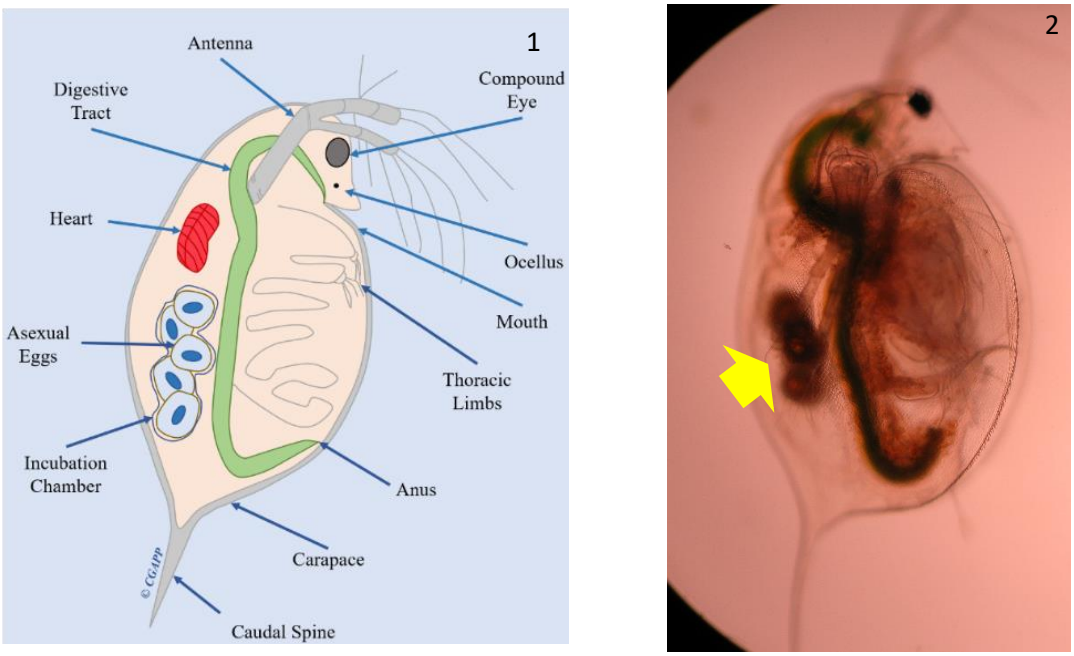


Figure 5 – Vectorial draw done in powerpoint showing the morphology and anatomy of *D. magna* (1); Photography of an adult female *D. magna* with eggs (yellow arrow) (2) (magnification 5.0x; scale 1000 µm).

1.3.2. Life Cycle and Reproductive Endpoints

Daphnia reproduce either sexually and asexually depending on the environmental conditions such as photoperiod, temperature, overpopulation among others (**Figure 5**) (Antunes et al., 2017; Ribeiro et al., 2021).

When the conditions are non-favourable, reproduction occurs by fertilization of haploid oocytes in which males contribute to the genetic variability of the neonates. Sexually produced eggs are typically in pairs and modified to a resistant structure called the ephippium, which allows the eggs to survive in unfavourable environmental conditions (**Figure 5**).

Under favourable conditions, the reproduction occurs by parthenogenesis which eggs complete embryogenesis in the brood pouch and latter are released as miniature versions of the adult, with no genetic contribution from males and genetically identical to the progenitor (Ebert, 2005; Antunes et al., 2017; Ribeiro et al., 2021). This type of

reproduction under optimal conditions is the most important to ecotoxicological studies because there is no genetic variability (**Figure 5**).

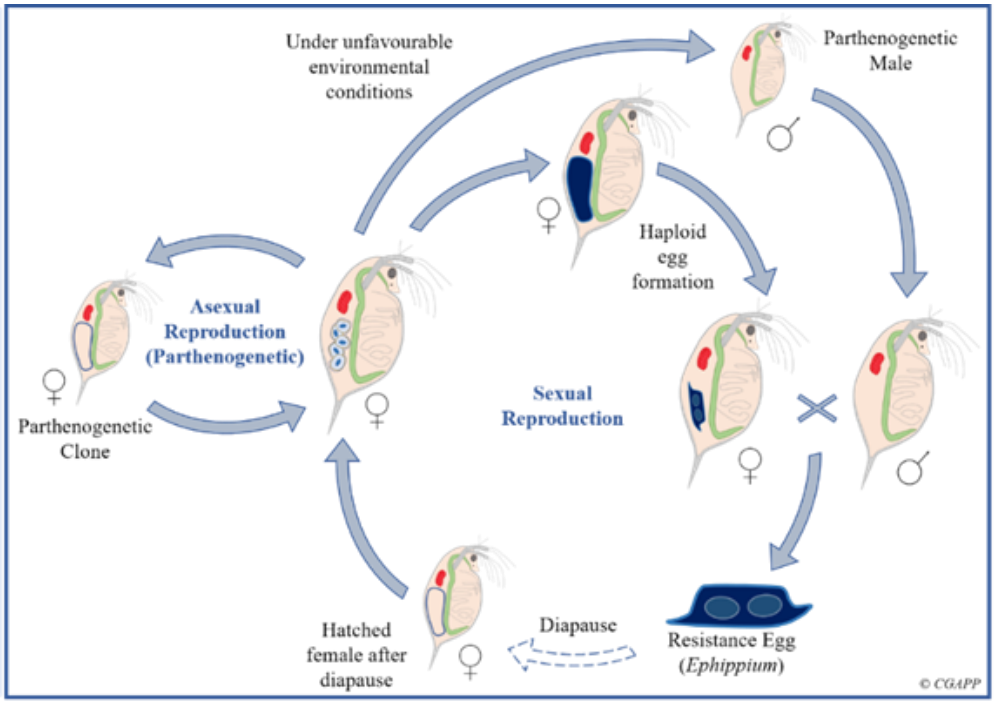


Figure 5 – Scheme representing *D. magna* life cycle, showing sexual and asexual reproduction.

Daphnia prefers temperature of 20 ± 2ºC, can attain a length of 5-6 mm when mature and the expected lifetime is of about 40 to 56 days depending on water temperature.

Life cycle is divided into 4 stages, (1) egg; (2) neonate; (3) juvenile; and (4) adult. The neonates attain maturation within 6-14 days, and the time needed to change from the first stage to the second is usually 2-3 days and occurs when the progenitor is detaching the external carapace.

The number of neonates depends on different factors such as food availability and environmental conditions, mainly temperature. Neonates from the first two broods are less resistant than the subsequent broods. The highest number of *D. magna* neonates born occurs during the fifth adult larval stage (Jonczyk and Gilron, 2005).

1.3.3. Morphophysiological endpoints

Several morphophysiological endpoints such as body size, heart activity, thoracic limb movements, post-abdominal claw activity have also been used in many studies as sensitive and reliable indicators of toxicity since they may be observed earlier than mortality or immobilization (Bownik, 2020; Szabelak and Bownik, 2021). Further, changes in physiological parameters can be linked to cellular and molecular responses (Rivetti et al., 2016; Magni et al., 2017; Yang et al., 2018). Different methodologies may be used for the determination of morphophysiological endpoints however, due to daphnid transparent body and small size, even subtle alterations of organs morphology and physiology may be measured by microscopic observation supported by digital video analysis (Bownik and Stępniewska, 2015; Bownik, 2020; Szabelak and Bownik, 2021).

1.3.4. Swimming behavioural endpoints

Daphnid behavioural endpoint is also a sensitive biomarker to predict the effects of various toxicants at environmental concentrations (Szabelak and Bownik, 2021). Some studies have shown that swimming behaviour may be altered by exposure to contaminants such as PAS and analysis of the various swimming parameters (e.g., swimming speed, active time or distance travel) is an important tool for toxicity assessment. Also, different methodologies have been used for digital video analysis, and advantages and limitations of swimming behaviour methodologies as a tool have been discussed (Bownik, 2017).

1.3.5. Biochemical endpoints

Reactive oxygen species

ROS is a frequently use term for defining oxygen-containing reactive species such as superoxide radicals, hydrogen peroxide, hydroxyl radicals and oxygen atoms that occur when the reduction of oxygen is incomplete (Li et al., 2016; Yang et al., 2019).

ROS play a key role in the immunological defence, the most know is the production of reactive species from phagocytic cells in killing pathogenic microorganisms and performing an essential role in regulating various physiological functions of living organisms. (Li et al., 2016; Yang et al., 2019).

In moderate concentration, ROS species can be second messengers for physiological regulation, although excessive ROS can cause oxidative damage to the cells or even induce necrosis or apoptosis. Cellular integrity is guaranteed by maintaining the balance between ROS generation and elimination by the antioxidant defence system (Yang et al., 2019; Yang and Lian, 2020).

ROS can be generated at different locations, being the major producer the mitochondria (Yang and Lian, 2020).

ROS are present in the organisms not only by the endogenous production but also can be induced by exogenous sources, such as radiation, air pollutants, and xenobiotics that undertake constant reduction and oxidation cycles. So the levels of the ROS in the organism not only depend in the production by the organism but also the cellular antioxidant defences levels because of the exogenous induction of ROS production (Li et al., 2016).

Catalase

Organisms developed a variety of mechanisms for protection against ROS. Catalase (CAT) are enzymes that play an important role in protecting cells against the toxic effects of ROS such as hydrogen peroxide (Goyal and Basak, 2010; Mhamdi et al., 2010).

The CAT prevent cell oxidative damage by degrading hydrogen peroxide to water and oxygen (Alfonso-Prieto et al., 2009).

Lipoperoxidation - Tiobarbituric acid reactive substances

The formation of ROS can lead to the development of lipid peroxidation of cell membranes. Thiobarbituric acid reactive substances (TBARS) are widely used to measure lipid peroxidation (Wenjiao et al., 2014; Ghani et al., 2017; Tsikas, 2017).

TBARS assays are deemed as a good indicator, of the overall levels of oxidative stress because the lipid peroxidation is downward of oxidative stress that is harmful to health (Tsai and Huang, 2015). Oxidative stress is the disorder in the balance between the ROS species and the antioxidant defences (Betteridge, 2000).

Lipid peroxidation is a process in which free radicals, ROS, attack carbon-carbon double bonds in lipids (Aguilar Diaz De Leon and Borges, 2020).

Acetylcholinesterase

Acetylcholinesterase (AChE) is an enzyme from the nervous system that belongs to the family of cholinesterases. This enzyme terminates the action of the neurotransmitter acetylcholine by hydrolysing it into acetic acid and choline (Silman and Sussman, 2008; Lionetto et al., 2013).

AChE is most often found in the neuromuscular junctions and cholinergic synapses in the central nervous system where the enzyme ceases the synaptic transmission being essential to normal functioning. But AChE is also found in the red blood cells (Lionetto et al., 2013).

In the last few years, AChE has been used as a biomarker of effect of contaminants in the nervous system (Lionetto et al., 2013).

2. Aims

This study aimed to investigate the enantioselectivity in ecotoxicity effects of AMP using an ecological relevant aquatic organism, *D. magna.*

For that, a sub-chronic toxicity assay at different concentrations (environmental reported levels and higher) was performed using neonates. Organisms were exposed to 0.1; 1 and 10 µg/L of the racemate (*rac*-AMP) and to 0.1 and 1 µg/L of isolated enantiomers for 8 days [(*R*)-AMP and (*S*)-AMP].

At selected 3, 5 and 8-days of exposure morphophysiological (body size, heart rate, area and length), swimming behaviour (swimming speed, active time and travel distance), biochemical and first reproductive events parameters were determined (**Figure 6**).

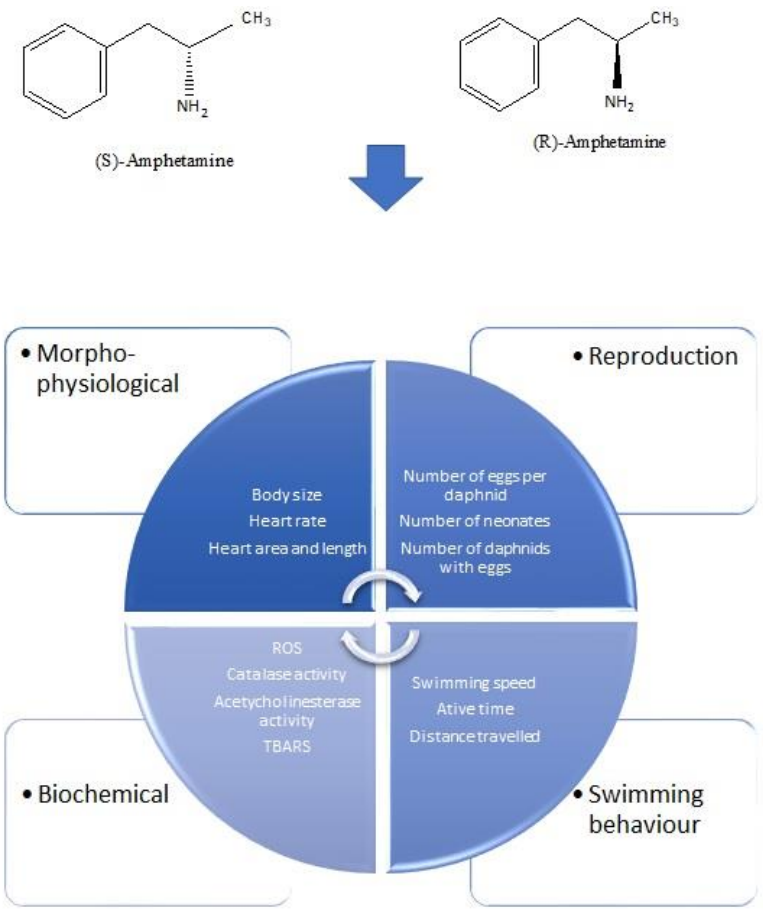


Figure 6 - Aims and parameters considered for toxicity evaluation.

3. Materials and Methods

3.1. Chemicals and materials

AMP racemate (*rac*-AMP) and isolated enantiomers standards [(*R*)-AMP and (*S*)-AMP] (purity > 98%) were purchased from Lipomed (Arlesheim, Switzerland). Ultra-pure water was obtained from an Ultrapure Water System (SG Ultra Clear UV plus). Individual stock solutions of standards were prepared in ultra-pure water at concentration of 1 mg mL^{-1} and stored at -20 ºC in amber vials. Working solutions were prepared by dilution of stock solutions in ultra-pure water.

For the ecotoxicity assays, the kit DAPHTOXKIT F MAGNA and small beads of the microalgae *Raphidocelis subcapitata* were acquired from MicroBioTests Inc., (Gent, Belgium).

For the preparation of the daphnia culture medium the following substances were used: $NaHCO_3$ (≥ 99,7 %) acquired from Sigma-Aldrich (Missouri, USA), $CaSO_4.2H_2O$ (>99%) and $MgSO_4.7H_2O$ (>99%) were obtained from Merck (Darmstadt, Germany). KCl (>99%) was acquired from Panreac (Barcelona, Spain). *Ascophyllum nodosum* was purchased from Alltech (Kentuchy, USA), biotin (H, ≥ 99 %) acquired from Panreac AppliChem ITW Reagents (Darmstadt, Germany), cyanocobalamin (B12, > 98,9%) acquired from Fragon Iberian (Terassa, Spain) while thiamine. HCl (B1) was purchased from Couto pharmacy manipulation laboratory (Oporto, Portugal) and yeast was purchased from Pura Vida, Pingo Doce (Lisbon, Portugal).

For the preparation of the algae culture medium the following substances were used: $NaNO_3$ (> 99%), $NaHCO_3$ (≥ 99,7 %), $Na_2MoO_4.2H_2O$ (≥ 98%) and $Na_2EDTA.2H_2O$ (≥ 98.5%) were acquired from Sigma-Aldrich (Missouri, USA), $MgCl_2.6H_2O$ (> 98%), $ZnCl_2$ (> 97%) and K_2HPO_4 (> 99%) were acquired from Panreac AppliChem ITW Reagents (Darmstadt, Germany), $CaCl_2.2H_2O$ (≥ 99%) acquired from Riedel-de-Haën (North Caroline, USA), $MgSO_4.7H_2O$ (> 99%) and H_3BO_3 (≥ 99.8%) were both acquired from Merk (Darmstadt, Germany), $MnCl_2.4H_2O$ (≥ 98%) and $CoCl_2.6H_2O$ (> 99%) were both acquired from PA Panreac (Barcelona, Spain) and $FeCl_3.6H_2O$ (≥ 97%) was purchased from PRS Panreac (Barcelona, Spain).

For the biochemical assays the following substances were used: Bovine serum albumin (BSA, ≥ 96%), Coomassie Plus (The Better Bradford AssayTM Reagent), 5,5'-dithiobis-2-nitrobenzoic acid (DNB, ≥ 98%), acetylthiocholine iodide (ATCI, ≥ 99%), 2,7-dichlorofluorescin diacetate (H_2DCFDA, ≥ 97%), 2,7-dichlorofluorescein (DCF, 90%), dimethyl sulfoxide (DMSO, ≥ 99.9%), NaH_2PO_4 (≥ 99%), butylated hydroxytoluene (BHT, ≥ 99%), thiobarbituric acid (TBA, ≥ 98%), sodium dodecyl sulphate (SDS, ≥ 98.5%) and malondialdehyde (MDA, ≥ 96%) were acquired from Sigma-Aldrich (Missouri, USA).

Sterile syringe PTFE filters with 0.22 µm pore size were purchased from Teknokroma (Barcelona, Spain) and sterile cellulose filters with 0.45 µm pore size were purchased CruzLab (Vila do Conde, Portugal).

3.2. Equipments

Organisms, both daphnia and microalgae were incubated in an Infors HT Ecotron incubator (Fisher Scientific, Portugal) for hatching daphnia ephippia and beginning the optimization cultures conditions. Absorbance was measured using an UV/Vis spectrometer (ATI Unicam, Leeds, England). An autoclave from PBI (South Carolina, USA) and a laminar flow chamber SC4 from Allentown (New Jersey, USA) were used for the preparation and manipulation of solutions and mediums. The Multiparameter HI98194 and the multiparameter analyser HANNA Consort C863 (Turnhout, Belgium) instruments were used to measure the physical-chemical parameters [pH, conductivity, percentage of dissolved oxygen (%OD), temperature]. A Heraeus Biofuge Primo R (Heraeus, Germany) and a Heraeus Biofuge 1.0R (Heraeus, Germany) centrifuges were used for centrifugation of algae suspensions. An Inverse Microscope of ZEISS (Jena, Germany) with a Neubauer chamber were used for microalgae cell counting. The microplate reader, BioTek Synergy 2 (Vermont, USA) and an ultrasonic of VWR USC-TH (Pennsylvania, USA) were used for biochemical analysis and preparation of the daphnia homogenates, respectively. A microscope Axiostar plus ZEISS (Jena, Germany) coupled to a digital camera (Canon PowerShot G9) was used for photo and videorecording of the morphophysiological parameters and a Canon Legria HF R506 was used for videorecording for the swimming behaviour parameters.

3.3. Optimization and culture of *Daphnia magna*

3.3.1. Ephippia hatching and culture mediums

Organisms were obtained from the MicroBioTests DaphtoxKit F magna Kit which uses the dormant eggs (ephippia) of the microcrustacean *D. magna* clone that can be stored for long periods, in darkness at 5ºC (± 2º C), without losing their viability. The content of one tube was poured into the micro sieve and then carefully washed to eliminate all storage medium. After washing, the ephippia were transferred into the hatching petri dish, containing 15 mL of standard freshwater culture medium prepared by dissolving, in distilled water, solutions of concentrated salts [$NaHCO_3$ at 67.75 mg/L, $CaCl_2.2H_2O$ at 294 mg/L; $MgSO_4.7\ H_2O$ at 123.25 mg/L and KCl at 5.75 mg/L]. The medium was aerated for about 20 minutes with the aid of an aeration pump and a magnetic stirrer, before use. The petri dish with the ephippia was covered and incubated for 72 h at a temperature between 20 and 22ºC and with constant lighting of 6000 lux an Infors HT Ecotron incubator. The neonates were fed with a suspension of spirulina microalgae 2 h and then distributed for two different culture mediums:

A) Moderately hard reconstituted water (MHRW). This medium was prepared as followed: 123 mg of $MgSO_4.7H_2O$, 96 mg of $NaHCO_3$, 60 mg of $CaSO_4.2H_2O$ and 4 mg of KCl in 1 L of distilled H_2O.

B) Hard reconstituted water (ASTM). This medium was prepared in accordance with ISO 10706. For that, 10 mL of each of the following stock solutions was added into 1 L of distilled H_2O: $MgSO_4.7H_2O$ (245.7 mg/mL), $NaHCO_3$ (192 mg/mL), $CaSO_4.2H_2O$ (24 mg/mL) and KCl 8 mg/mL). Stock solutions were stored in amber flasks at 4ºC.

Each culture medium was supplemented with the following substances:

3.3.2. Mixture of vitamins

A mixture of vitamins consisting of B1 at 1.5 mg/mL, B12 at 0.02 mg/L and H at 0.015 mg/L were prepared in ultra-pure H_2O, filtered through a 0.22 µm PTFE filter in a laminar air flow chamber to 1 mL Eppendorf's and storage at -20°C.

3.3.3. Yeast Solution

Yeast solution was prepared by dissolving 10 mg of yeast in 100 mL of ultrapure H_2O, filtered through a 0.45 µm cellulose filter and stored in an amber vial at 4ºC for 15 days.

3.3.4. *Ascophyllum nodosum* extract

Ascophyllum nodosum extract was prepared by measuring 900 µL of the algal extract to 100 mL of ultra-pure H_2O. The OD was measured at λ of 400 nm and should be between ~ 0.6. The final solution was filtered through a 0.45 µm cellulose filter and stored in an amber bottle at 4°C.

3.3.5. Culture and maintenance of *Raphidocelis subcapitata*

Small beads of *R. subcapitata* were used to start the microalgae culture. For that, beads were clean with different chemical compounds from the kit and transferred to a culture medium prepared based on OECD guideline nº 201 (OECD, 2011) and ISO Standard 8692 (ISO, 2012), consisting of macronutrients and micronutrients to support microalgae growth according to the following:

A) Macronutrients stock solutions were prepared individually consisting in 2.55 g of $NaNO_3$; 1.22 g of $MgCl_2.6H_2O$; 0.441 g of $CaCl_2.2H_2O$; 1.47 g of $MgSO_4.7H_2O$; 0.1044 g of K_2HPO_4 and 1.500 g of $NaHCO_3$ solubilized in 100 mL of distilled H_2O and stored in amber glass bottles at 4°C.

B) Micronutrient stock solution containing 46.38 mg of H_3BO_3; 103.85 mg of $MnCl_2.4H_2O$; 0.818 mg of $ZnCl_2$; 39.94 mg of $FeCl_3.6H_2O$; 0.357 mg of $CoCl_2.6H_2O$; 1.815 mg of $Na_2MoO_4.2H_2O$; 75.0 mg of $Na_2EDTA.2H_2O$ solubilized in 250 mL of distilled H_2O and stored in an amber glass bottle at 4°C.

For the preparation of the microalgae medium, 1 mL of each macronutrient stock solution was added to 1 L of distilled H_2O and autoclaved. Afterward, 1mL of micronutrient solution was added and pH adjusted to 7.5 ± 0.1. At the beginning of the microalgae culture, the medium was inoculated with the beads of *Raphidocelis subcapitata.* In the subsequent cultures, a fraction of the culture is collected into an autoclaved Erlenmeyer in a laminar air flow chamber and used to start a new culture .

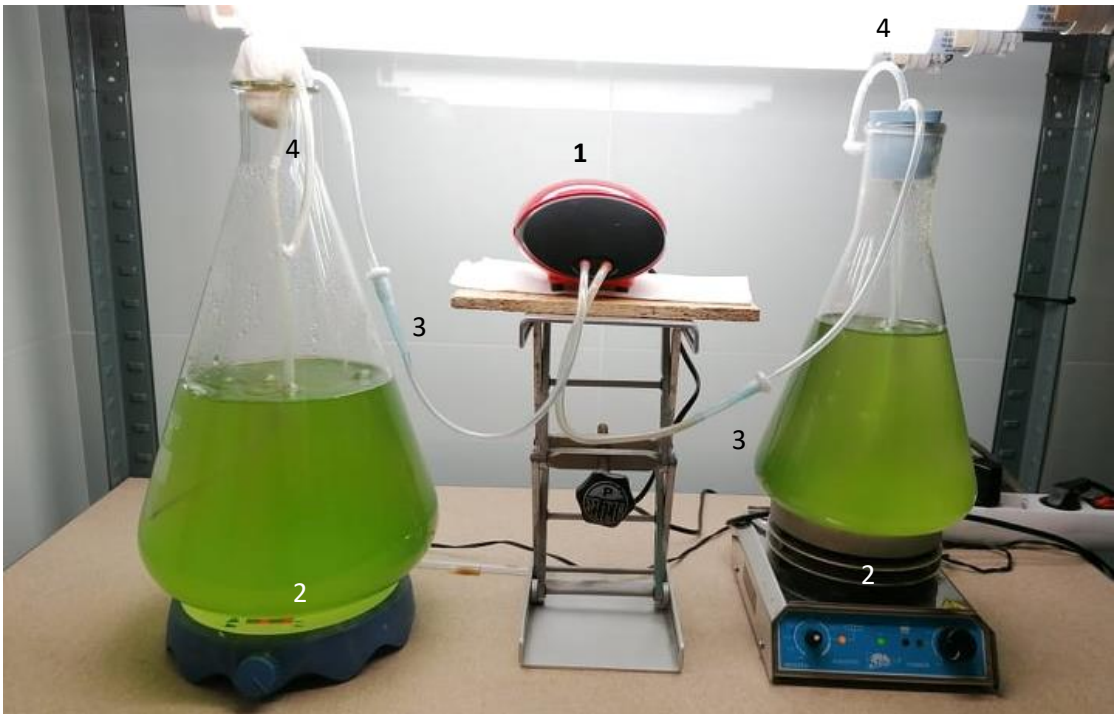


Figure 7 – Microalgae culture: Erlenmeyer containing the microalgae culture (1 - air pump system;2 - magnetic agitation;3 - filtered air supply with a 0.22 µm filter;4 - tube for air flow out).

Microalgae culture is carried out for 8 days in the bioterium at a constant temperature of 21°C with a lighting of 10,000 lux and a photoperiod of 16 h light: 8 h dark. During this time, the culture medium is in continuous agitation and connected to an aeration system (**Figure 7**).

After 8 days of growth, the culture of the microalgae is transferred to 50 mL falcons previously autoclaved and centrifuged at 3000 rpm for 10 minutes. After

centrifugation, the supernatant is discarded, and the pellet is resuspended in 2 mL of MHRW. The suspension is centrifuged again at 3000 rpm for 10 minutes, the supernatant discarded, and the pellet resuspended in 2mL of MHRW. Subsequently grouping all in a falcon and storing at 4°C. The OD of the microalgae culture is measure at λ of 440 nm and should be between 0.6 and 0.8. The number of cells is also counted using a Neubauer chamber in an Inverse Microscope and is usually above $1.8x10^7$ cells/mL.

3.4. Maintenance and feeding of *Daphnia magna*

A preliminary study to select the best conditions for culture and maintenance of daphnia were performed. For that, 20 daphniids were maintained in both ASTM and MHRW mediums and mortality and fecundity were recorded for selection of the medium. MHRW was selected as the best medium for culture and maintenance of organisms.

Therefore, organisms were cultured in an acclimatized room in the bioterium at 20-21ºC with a 16h ligth:8 dark h period. Mediums were prepared every week, and changed three times a week according to the following procedure.

The culture medium was aerated using a pump and a magnetic stirrer for 30-40 minutes. The medium is then supplemented with 50 µL of stock vitamin mix solution, 9 mL of the algae extract stock solution and 500 µL yeast extract per L. Afterwards, the culture medium was aerated for 30 - 40 minutes and physical-chemical parameters pH, conductivity, temperature, and dissolved oxygen measured.

Organisms were fed when every culture medium was change with the microalgae at $3.0x10^5$ cells/mL for the neonates while for adults at $6.0x10^5$ cells/mL.

3.5. Ecotoxicity assay

For the ecotoxicity assays, groups of 25 daphnids were isolated in 800 mL of medium and maintained as previously referred (3.4.). The daphnids, less than 24 h old originated from 3rd – 5th brood females from stock cultures were used for the experiments (**Figure 8**).

Figure 8 - Isolated daphnids culture for the ecotoxicity assays.

3.5.1. Experimental design and standards preparation

A sub-chronic experimental ecotoxicity assay was designed to include different endpoints as morphophysiological parameters (body size, heart rate, heart area and length), swimming behaviour parameters (swimming speed, total distance and active time), biochemical parameters (ROS, CAT, TBARS and AChE) and first reproductive events parameters (number of eggs per daphnia, number of daphnia with eggs and number of neonates) (**Figure 9 and 10**). Concentrations of racemate and isolated enantiomers were selected to include environmental reported levels and higher concentrations. Therefore, three concentration levels were selected for the racemate, 0.1, 1 and 10 µg/L and two concentrations levels for the enantiomers 0.1 and 1 µg/L (**Figure 9)**. Each experimental unit consisted of a batch of 20 daphnia and five replicates per test concentration and control. Sub-chronic exposures were from day 0 (starting with < 24h old neonates) until day 8 (with medium changes and feeding at every 48 h intervals) to follow ontogenetic period, initial life stages and first reproductive events.

During exposure, organisms were checked for mortality. On day 3 and 8, three random daphnids per replicate were collected and used for the determination of morphophysiological parameters. On day 5, swimming behavioural were video recorded using three random daphnids per replicate, and total distance, swimming speed and active time determined. On day 8, three random daphnids were photographed for determination of the number of eggs per daphnia and the number of neonates and of daphnia with eggs were determined. After that, organisms of each replicate were collected into an Eppendorf, the culture medium removed and substitute by buffer (0.800 g NaCl, 0.020 g KCl, 0.144 g Na_2HPO_4 and 0.024 g KH_2PO_4 in 100 mL of H_2O_{UP}) and stored at -20ºC until biochemical analysis (**Figure 9**).

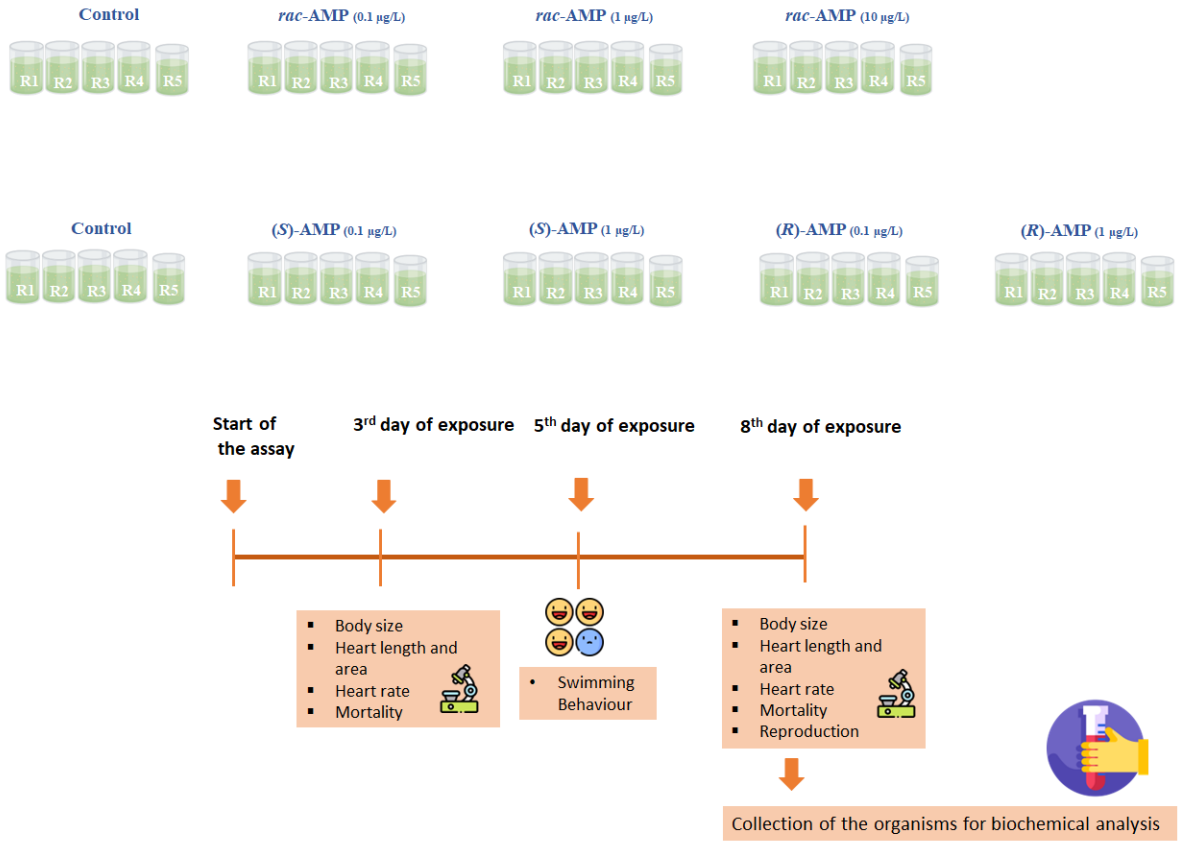


Figure 9 - Schematic representation of the experimental design.

Figure 10 - Toxicity assay of the enantiomers with the respective replicates.

3.5.2. Morphophysiological parameters

Three random daphnids from each replicate per experiment were collected on days 3 and 8 for morphophysiological parameters determination. A single daphnid was transferred to a microscope slide with a drop of the corresponding medium. The microscope slide was placed in a microscope coupled to the digital camera (Canon PowerShot G9, **Figure 11**) and the organism was photographed and video recorded for a minimum of 1 minute. After that, the daphnids were returned to the corresponding replicate and images and videos were processed using specific software (**Figure 12**).

Figure 11 - Optical microscope coupled to a digital camera for photograph and video recording.

A. Body Size

The photos were taken with the 2.0x zoom of the camera and the 5x objective lens of the microscope. The photos were analysed using the specific software (**Figure 12**).

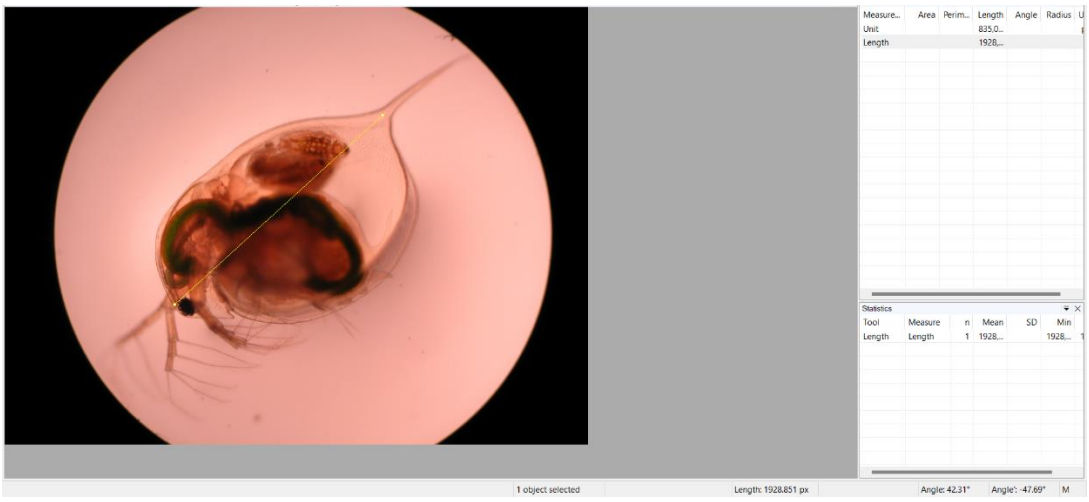


Figure 12 - Body size measurement of *D. magna*, using the specific software.

B. Heart area and length, and heart rate

Heart area and length were determined using specific software. For heart rate, videos were cut to 1 minute long and edited to reduce the speed to 0.25x of the original video using Da Vinci free software program to improve the accuracy in the counting. Heart rate (beats per minute) were counted using a manual cell counter.

3.5.3. Swimming Behaviour parameters

Swimming behaviour parameters were determined by recording organisms in 6-well plates. For that, each well was filled with 5 mL of melted 1 % agarose. Once solidified, a circular portion was stamped out using a plastic ring of ~27 mm diameter creating a circular swimming area that improved the optics at the edge of each well preventing shadows and blind spots. Wells were filled with 5 mL of the respective exposure medium. Three organisms of each replicate were individually transferred to each of the 6-well plates and acclimatize for 5-10 minutes. The plates were placed on the top of a computer screen with a white background and swimming behaviour of organisms of exposed and controls were recorded for a 2 min with a digital camera Canon Legria HF R506 (with a 30 frames/s) mounted on a perpendicular position (**Figure 13**). After that, the daphnids were returned to the corresponding replicate. The videos were processed using DaVinci and Real Trackfish free software.

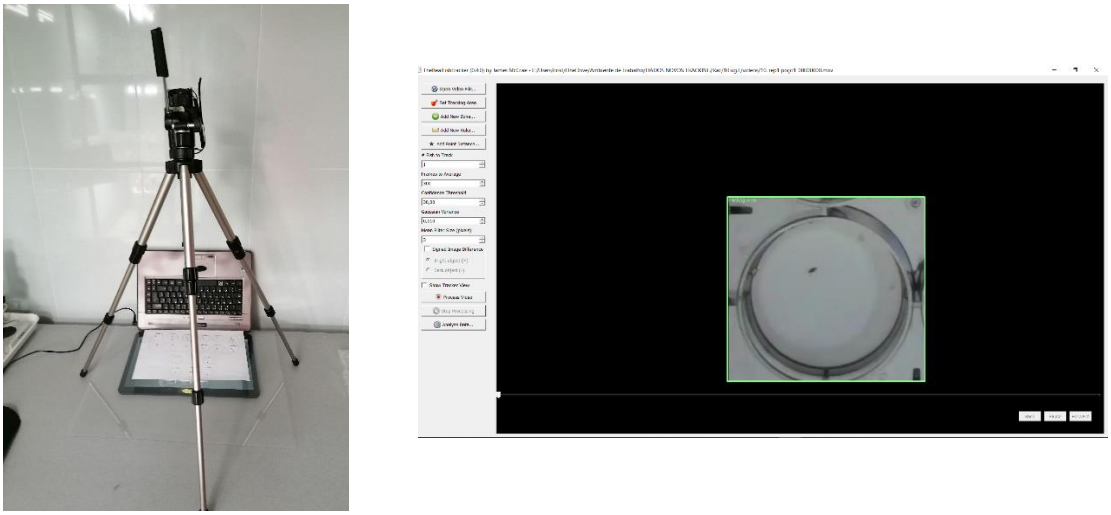


Figure 13 - Set up of equipment for swimming behaviour and Real Trackfish software program.

3.5.4. Reproduction parameters

On day 8, the number of eggs per daphnia, of daphnids with eggs and of neonates in each replicate was recorded. For determination of the number of eggs per daphnia, photographs used for determination of heart area and length were also used for counting the number of eggs per daphnia.

3.5.5. Biochemical parameters

The remaining daphnids were collected in a 2 mL Eppendorf, the medium removed and 250 µL of the buffer (0.800 g NaCl, 0.020 g KCl, 0.144 g Na_2HPO_4 and 0.024 g KH_2PO_4 in 100 mL of H_2O_{UP}) were added. Samples were stored at -80ºC until analysis.

Daphnid tissue was homogenized via ultrasonication, and then centrifuged at 13 000 g for 20 min at 0ºC. The supernatant of the homogenate was immediately collected to analyze enzymatic activity, ROS, and lipid peroxide content (TBARS).

A) Protein quantification

The protein concentration was measured following the Coomassie blue method. In this colorimetric assay, Bradford's reagent was used. For this assay 10 µL of sample, 150 µL of H_2O up and 40 µL Bradford reagent. The assay was performed in duplicate, and absorbance read at 595 nm using a microplate reader.

B) Acetylcholinesterase activity

The determination of AChE activity was calculated through the formation of 5-thio-2-nitrobenzoic acid (TNB), based on an improved Ellman method, in which thiocholine produced by the action of AChE forms a yellow colour with DTNB, generating TNB.

To determine the AChE activity, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide (ATCI) were used.

A duplicate was performed for each sample containing 20 µL of sample, 120 µL of 0.5 mM DNTB and 60 µL 20 mM ATCI and transferred into 96 well microplate. Absorbance was read at 412 nm with a microplate reader for 3 min at 25 °C.

C) Reactive oxygen species

For the determination of ROS, a fluorescent probe was used the 2,7-dichlorofluorescin diacetate (H2DCFDA), it can freely cross the membrane, it is a fluorogenic dye cell permeant reagent that measures hydroxyl, peroxyl and others ROS activities in the cell.

After it enters the cell, it is hydrolysed by intracellular esterase to form DCFH (dichlorofluorescin), a non-fluorescent compound. But in the presence of ROS, DCFH is oxidized to DCF, which is a green, fluorescent substance.

Duplicates of each sample were performed, containing 10 µL of sample and 110 µL of PBS and then 8.3 µl of H2DCFDA was added.

D) To read the fluorescence samples were transferred to a 96-well plate, and the fluorescence of the DCF read using a λ excitation of 485 nm and an emission of 528 nm using a microplate reader at 25 °CTBARS

The malondialdehyde (MDA) level was determined via the TBARS colorimetric method by measuring the absorbance of MDA and thiobarbituric acid–reactive products. Each sample was performed in duplicate containing 10 µL of sample, 70 µL of H_2O up. 50 µL of 50 mM phosphate buffer, 10 µL of 1 mM butylated hydroxytoluene (BHT), 75 µL of a stock solution containing 1.3% thiobarbituric acid (TBA) and 0.3% sodium hydroxide (NaOH). After, this mixture was incubated for 40 minutes at 60 ºC, then cooled for 15 minutes on ice and then, 10 µl of 20% Sodium Dodecyl Sulfate (SDS) was added.

To read absorbance, 200 µL of each sample was transferred to a 96-well microplate and read at 530 nm in a microplate reader.

E) Catalase activity

The enzymatic activity of CAT was determined using oxygen peroxide (H_2O_2) and metatartaric acid (AMT). CAT causes the degradation of H_2O_2. For each sample a duplicate was performed, with 50 µL of sample, 100 µL of a stock solution of 60 mM Sodium Phosphate Buffer (SPB) with 0.065 M H_2O_2 and then allowed to react for 1 min at 37°C, after 1 min, 250 µl of 32 mM AMT was added to stop the reaction.

Then 200 µL of each sample was transferred to a 96 well microplate and the absorbance was read at 415 nm with a microplate reader at 25°

3.6. Statistical analysis

Jamovi version 2.2.2 statistical software was used for statistical analysis of data. General linear models (GLMs) were used to investigate the effects of racemate and enantiomers (using concentration and enantiomers as categorical variable and source of variable as continuous variable) for morphophysiological, swimming behaviour and biochemical parameters. Reproductive parameters were analysed as count data using generalized linear models by negative binominal model.

4. Results

4.1. Morphophysiological Parameters

On day 3 and 8, morphophysiological parameters as body size, heart rate, heart area and size were determined for both racemate (**Figure 14**) and enantiomers (**Figure 15**. Results of statistical analysis are reported in **Table 1**.

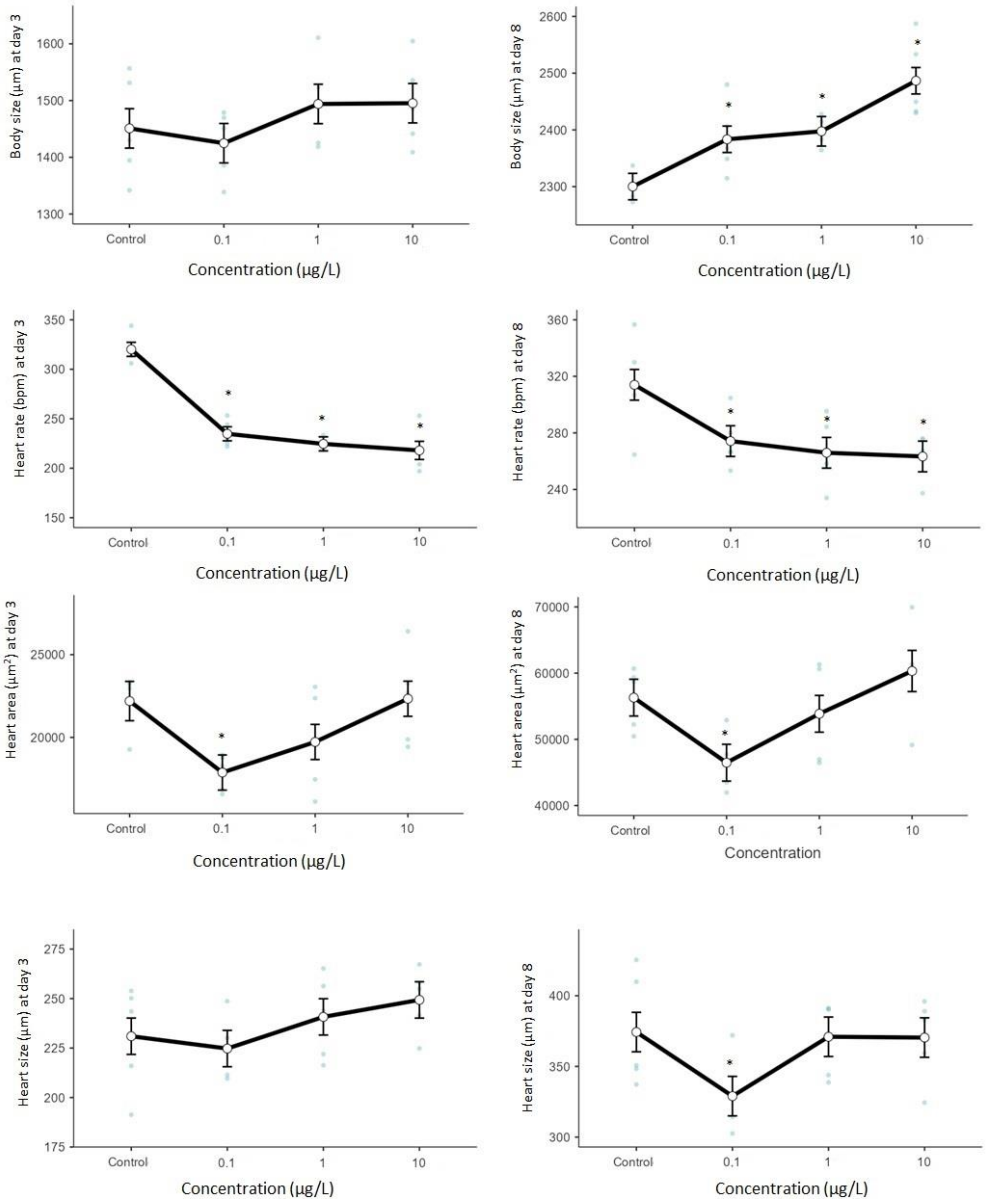


Figure 14 - Effects of *rac*-AMP on morphophysiological parameters determined at day 3 and 8 (asterisks (*) represent significant differences relatively to the control).

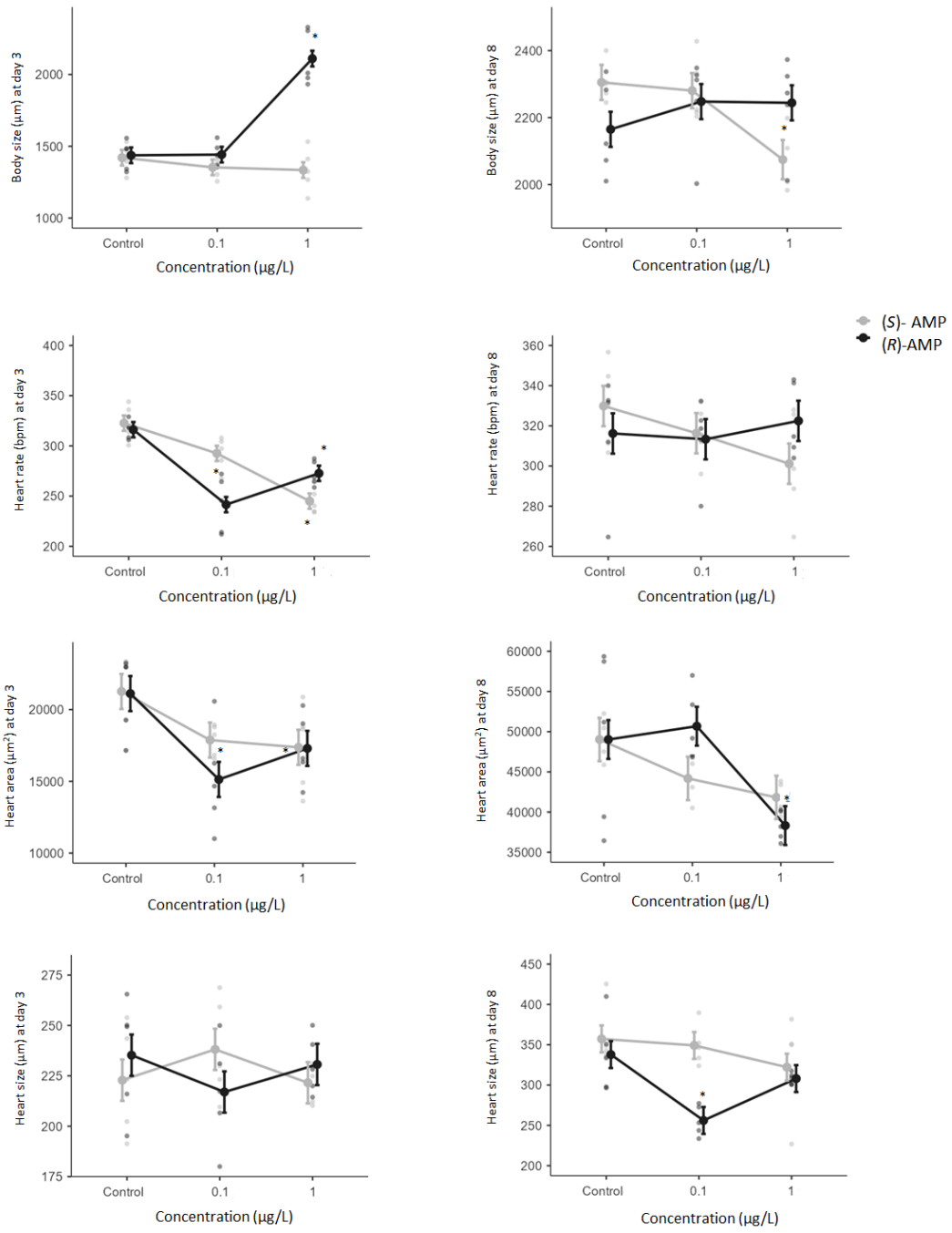


Figure 15 – Effects of the enantiomers on morphophysiological parameters determined at day 3 and 8. (asterisks (*) represent significant differences relatively to the control).

Table 1 – Effects of *rac*-AMP and its enantiomers on the morphophysiological parameters determined on day 3 and 8. Significant effects are reported in bold ($p < 0.05$).

		F	*p*
Body size at day 3 (µm)	*rac*	0.983	0.426
	Enantiomer	43.3	**< 0.001**
	Concentration	21.5	**< 0.001**
	Interaction	29.4	**< 0.001**
Body size at day 8 (µm)	*rac*	10.7	**< 0.001**
	Enantiomer	6.50e-4	0.980
	Concentration	1.98	0.161
	Interaction	4.19	**0.028**
Heart rate at day 3 (bpm)	*rac*	42.1	**< 0.001**
	Enantiomer	2.55	0.124
	Concentration	36.98	**< 0.001**
	Interaction	13.32	**< 0.001**
Heart rate at day 8 (bpm)	*rac*	4.70	**0.015**
	Enantiomer	0.0360	0.851
	Concentration	0.6701	0.521
	Interaction	1.5964	0.223
Heart area at day 3 (µm2)	*rac*	3.88	**0.031**
	Enantiomer	0.984	0.331
	Concentration	8.368	**0.002**
	Interaction	0.775	0.472
Heart area at day 8 (µm2)	*rac*	4.06	**0.027**
	Enantiomer	0.228	0.638
	Concentration	6.955	**0.005**
	Interaction	1.962	0.166
Heart size at day 3 (µm)	*rac*	1.39	0.283
	Enantiomer	0.000180	0.989
	Concentration	0.0410	0.960
	Interaction	1.6263	0.218
Heart size at day 8 (µm)	*rac*	2.38	0.108
	Enantiomer	9.60	**0.005**
	Concentration	3.85	**0.035**
	Interaction	3.49	**0.047**

On day 3, a tendency to the increase body size with increase of concentration was observed in the organisms exposed to *rac*-AMP compared to the control but no significant differences were observed (**Figure 14, Table 1**). However, on day 8, a significant increase in body size was observed for all tested concentrations compared to the control (**Figure 14, Table 1**).

Regarding the enantiomers, on day 3, no significant differences were observed among concentrations or enantiomers at the concentration of 0.1 µg/L (**Figure 15, Table 1**). However, a significant increase in body size at 1.0 µg/L was observed in the organisms exposed to (*R*)-AMP compared to the control and to (*S*)-enantiomer. In fact, an enantioselective effect was observed with organisms exposed (*R*)-AMP showing a much higher body size than those exposed to (*S*)-AMP.

On day 8, significant differences were still observed between concentrations and enantiomers. So, a significant decrease in body size was observed in the organisms exposed (*S*)-AMP at 1.0 µg/L in contrast to the organisms exposed to (*R*)-AMP with no significant differences in body size at both concentrations (**Figure 15, Table 1**).

Indeed, on day 3, a tendency to the increase in body size in the organisms exposed to *rac*-AMP and a significant increase in those exposed to (*R*)-enantiomer while on day 8, organisms exposed to the racemate still showed an increase in body side at all concentrations while a decrease of body size was observed in the organisms exposed to (*S*)-enantiomer with a significant decrease on day 8 (**Table 1**).

Regarding heart rate, a significant decrease was observed at all concentrations on both day 3 and 8 for the racemate with a tendency to decrease with the increase of concentration in comparison with the control (**Figure 14, Table 1**). For the enantiomers, on day 3, a significant decrease and an enantioselective effect was observed at 0.1 µg/L and was also observed with organisms exposed to (*R*)-AMP showing a higher decrease of heart rate. However, at 1 µg/L both enantiomers showed a significant decrease compared to the control but no differences between enantiomers were noted (**Table 1**). On day 8, no significant differences in heart rate were observed for both enantiomers though a tendency to the decrease was seen for (*S*)-AMP (**Figure 15**).

Comparing both days, while a decrease was observed for the *rac*-AMP and for the isolated enantiomers on day 3, on day 8 no significant differences were found for the isolated enantiomers while a decrease was still observed in those exposed to the *rac*-AMP.

A significant decrease was found in both heart area and size in the organisms exposed to *rac*-AMP at 0.1 µg/L on both days 3 and 8 however, no changes were found on both days at the higher concentrations (1 and 10 µg/L). For the enantiomers, also a significant decrease in heart area was observed on day 3 for both enantiomers and at both concentrations. On day 8, at the concentration of 1 µg/L a significant difference was still found heart area for both enantiomers compared to the control with a decrease in the heart area. However, no significant differences in heart size were found for both enantiomers on day 3 but on day 8 a significant decrease compared to the control and to (*S*)-AMP was noted for the organisms exposed to (*R*)-AMP at 0.1 µg/L.

4.2. Swimming Behaviour

Possible changes in swimming behaviour were investigated on day 5 considering speed, total distance, and active time for racemate (**Figure 16**) and enantiomers (**Figure 17**). **Table 2** shows the statistical analysis of swimming behaviour.

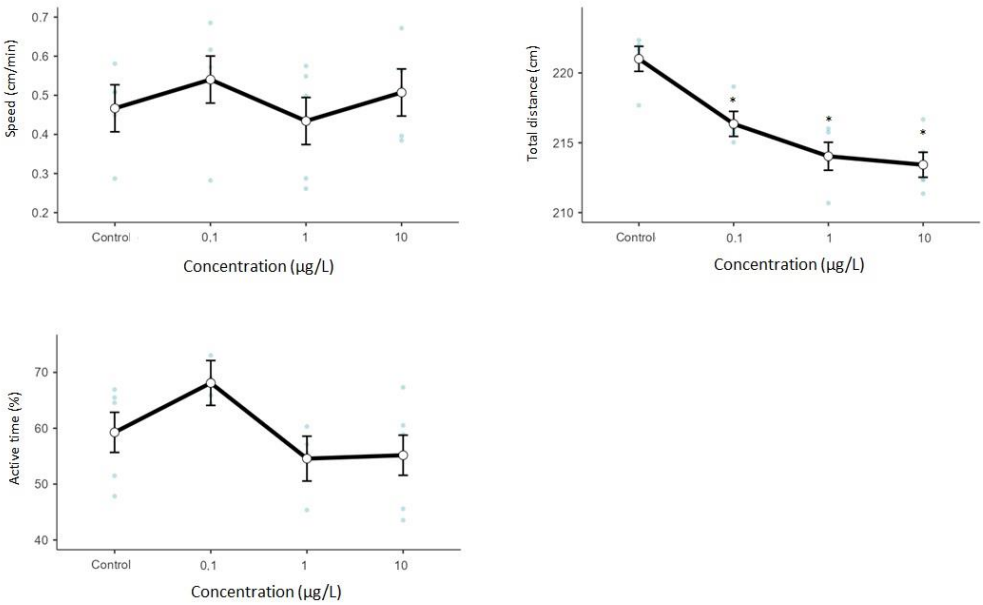


Figure 16 - Effects of *rac*-AMP on swimming behaviour determined at day 5 (asterisks (*) represent significant differences relatively to the control).

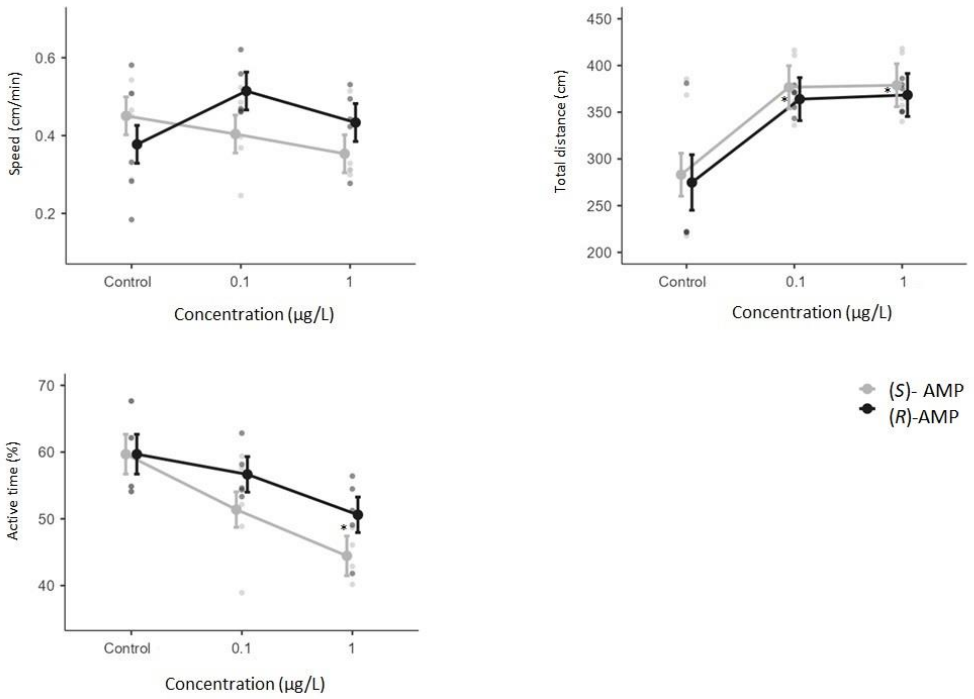


Figure 17 - Effects of the enantiomers on swimming behaviour determined on day 5 (asterisks (*) represent significant differences relatively to the control).

Table 2 – Effects of *rac*-AMP and its enantiomers on the swimming behaviour parameters determined on day 5. Significant effects are reported in bold ($p < 0.05$).

Source of variable		**F**	***p***
Speed (cm/min)	*rac*	0.593	0.629
	Enantiomer	0.971	0.334
	Concentration	0.954	0.399
	Interaction	2.043	0.152
Total distance (cm)	*rac*	14.4	**< 0.001**
	Enantiomer	0.28079	0.601
	Concentration	8.93174	**0.001**
	Interaction	0.00401	0.996
Active Time (%)	*rac*	2.50	0.102
	Enantiomer	2.733	0.113
	Concentration	8.840	**0.002**
	Interaction	0.655	0.530

No changes in speed were observed in the organisms exposed to racemate at all concentrations and the control as well as for the isolated enantiomers. However, a significant decrease in total distance was observed in all tested concentrations in the organisms exposed to the racemate, while on the contrary an increase in the total distance was observed with the isolated enantiomers. In fact, though no enantioselective effect was observed, a significant increase in total distance was observed for both enantiomers and at both concentrations. No changes were observed in active time for the racemate, while for both enantiomers a decrease was observed with a significant effect at 1 µg/L comparatively to the control but no differences between enantiomers.

4.3. Reproduction parameters

On day 8, reproduction parameters such as number of eggs per daphnia, number of daphnia with eggs and number of neonates were determined for racemate (**Figure 18**) and enantiomers (**Figure 19**). **Table 3** shows the statistical analysis of reproduction parameters.

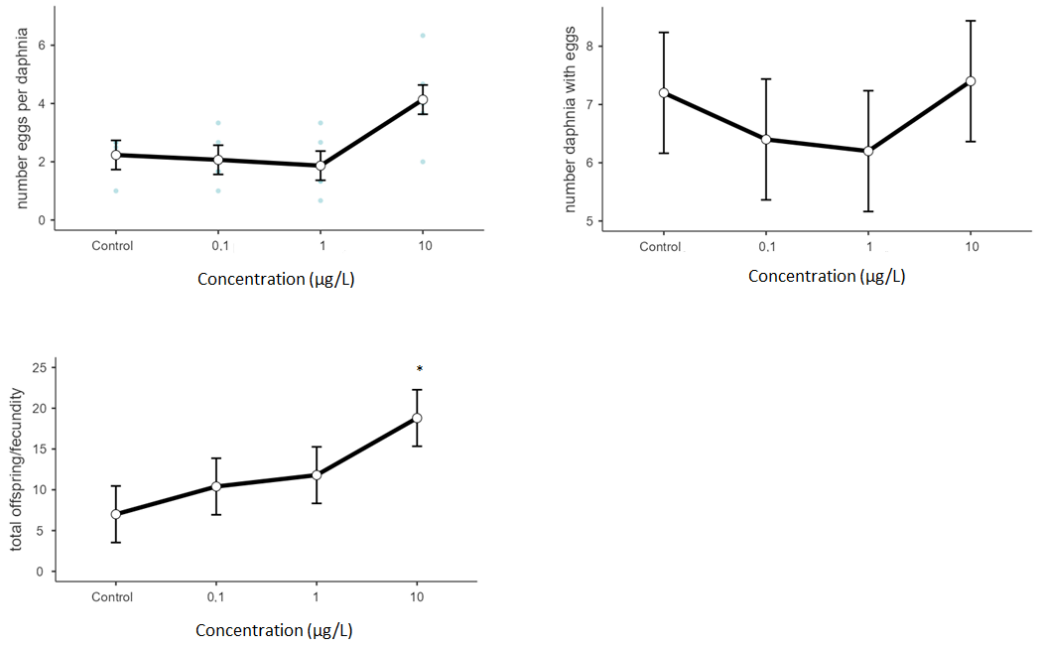


Figure 18 - Effects of *rac*-AMP on reproduction parameters (asterisks (*) represent significant differences relatively to the control).

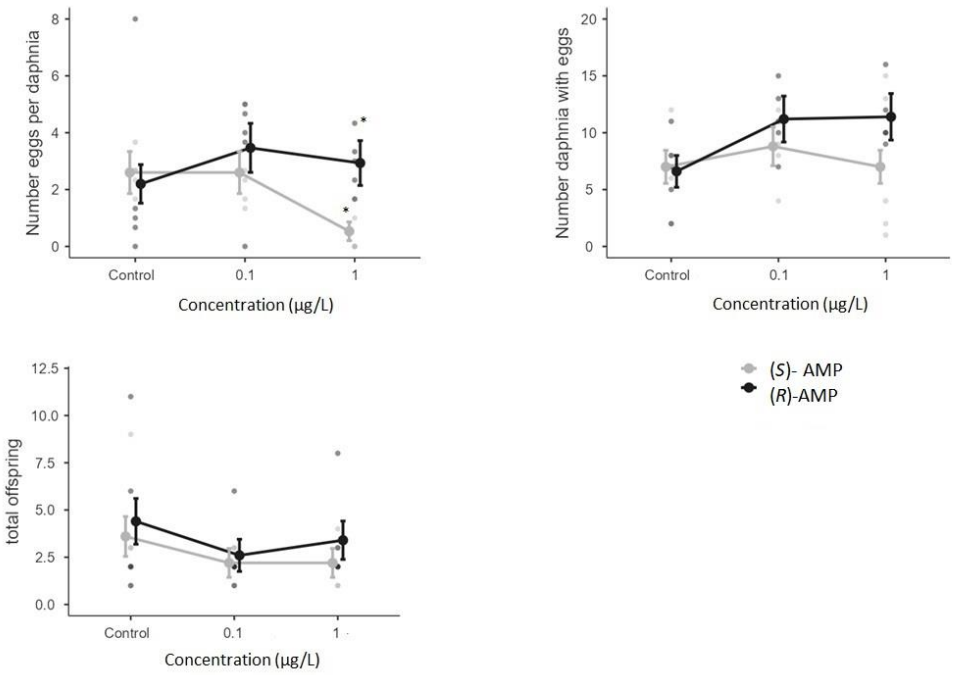


Figure 19 - Effects of (*R*)-AMP and (*S*)-AMP on reproduction parameters. (asterisks (*) represent significant differences relatively to the control).

Table 3 - Effects of *rac*-AMP and its enantiomers on reproduction parameters. Significant effects are reported in bold ($p < 0.05$).

Source of variable		**X^2**	***p***
Number eggs per Daphnia	*rac*	5.83	0.120
	Enantiomer	4.56	0.102
	Concentration	1.03	0.309
	Interaction	13.43	**0.001**
Number of Daphnia with eggs	*rac*	0.766	0.858
	Enantiomer	1.92	0.165
	Concentration	3.81	0.149
	Interaction	1.83	0.400
Total offspring	*rac*	9.67	**0.022**
	Enantiomer	1.080	0.299
	Concentration	3.014	0.222
	Interaction	0.206	0.902

No significant differences were found in *rac*-AMP for the number of eggs per daphnia though a tendency to increase was observed at the highest concentration (10 µg/L). Also, no significant differences were observed in the number of daphnia with eggs, however, an increase in the number of neonates was observed with significant differences in the organisms exposed to the highest concentration. Regarding enantiomers and the number of neonates, no significant differences at the concentration of 0.1 µg/L but significant differences were noted at 1.0 µg/L, a decrease in the number of neonates for the organisms exposed to (*S*)-AMP comparatively to the control, while an increase in the number of neonates was found for (*R*)-AMP. Also, a significant difference between enantiomers evidences an enantioselective effect on this parameter. Nevertheless, no significant differences were found for the number of daphnia with eggs and total offspring though a tendency to the decrease in the number of daphnia with eggs was observed for (*S*)-AMP.

4.4. Biochemical parameters

After exposure, organisms were collected for biochemical parameters determination for racemate (**Figure 20**) and enantiomers (**Figure 21**). **Table 4** shows the statistical analysis of biochemical parameters.

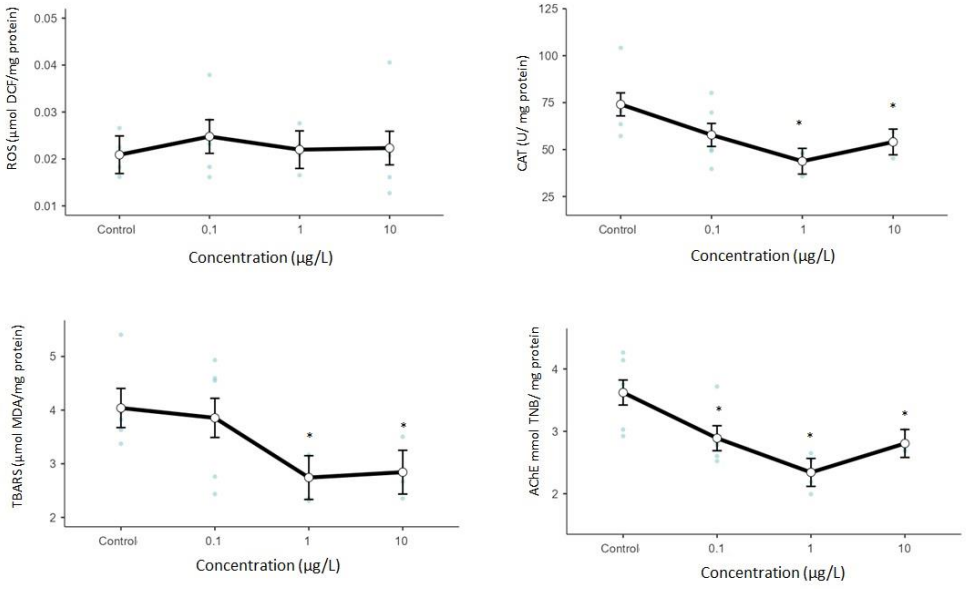


Figure 20 - Effects of *rac*-AMP on biochemical parameters (asterisks (*) represent significant differences relatively to the control).

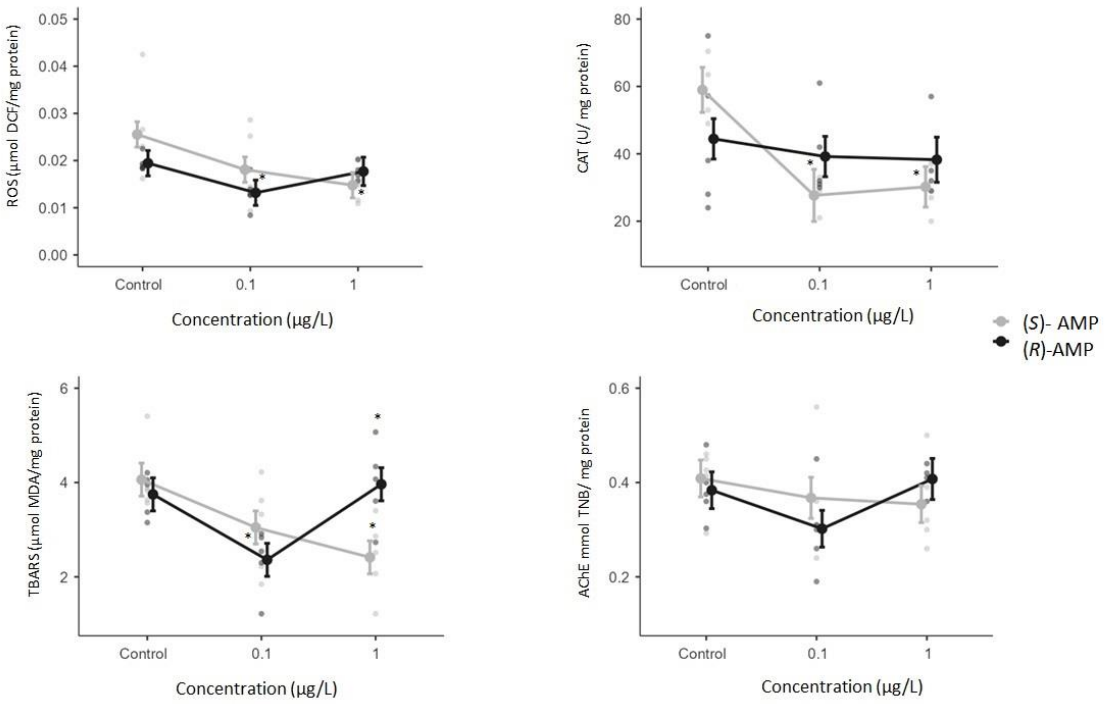


Figure 21 - Effects of (*R*)-AMP and (*S*)-AMP on biochemical parameters.

Table 4 – Effects of *rac*-AMP and its enantiomers on the biochemical parameters. Significant effects are reported in bold ($p < 0.05$).

Source of variable		F	*p*
ROS (µmol DCF/mg protein)	*rac*	0.193	0.900
	Enantiomer	1.45	0.241
	Concentration	3.94	**0.034**
	Interaction	1.54	0.236
AChE (mmol TNB/ mg protein)	*rac*	6.38	**0.006**
	Enantiomer	0.138	0.713
	Concentration	1.241	0.309
	Interaction	1.075	0.359
TBARS (µmol MDA/mg protein)	*rac*	3.02	0.065
	Enantiomer	0.413	0.526
	Concentration	5.959	**0.008**
	Interaction	5.852	**0.009**
CAT (U/ mg protein)	*rac*	3.83	**0.034**
	Enantiomer	0.0992	0.756
	Concentration	5.128	**0.016**
	Interaction	2.385	0.118

No significant differences were found in ROS levels at all concentration for *rac*-AMP while for the enantiomers a significant decrease in ROS levels was observed at both concentrations compared to the control however, no differences were found among enantiomers. Interesting to note a significant decrease in CAT activity at 1 and 10 µg/L for the *rac*-AMP and also for both enantiomers at 0.1 and 1 µg/L compared to the control but no differences among enantiomers. A significant decrease in TBARS was also noted for the racemate at the higher concentrations. Regarding enantiomers also a significant decrease was observed at 0.1 µg/L for both enantiomers but no enantioselective effects, however a significant decrease of TBARS was still found at 1 µg/L for (*S*)-AMP while for (*R*)-AMP a significant increase in TBARS levels were observed .

Regarding AChE activity a significant decrease was noted for the organisms exposed to the *rac*-AMP at the higher concentrations while no significant differences between control and both concentrations were found for the isolated enantiomers or among enantiomers.

5. Discussion

PAS are contaminants frequently detected in aquatic ecosystems due of their excessive use posing environmental risks (Fernández-Rubio et al., 2019; Fontes et al., 2020; Ribeiro et al., 2020). Even at low concentrations (ng to µg/L), their intrinsic biological properties may cause potential hazards to non-target organisms (Ding et al., 2017; Félix et al., 2017; De Felice et al., 2019). AMP is clinically used and exhibit enantioselective pharmacological activity being (*S*)-AMP more potent and clinically effective than (*R*)-AMP. On the other hand, AMP is frequently abused due to its euphoric and stimulant effects. Consequently, AMP is frequently detected in wastewaters and surface waters reaching concentrations up to 750 ng/L and 14 ng/L, respectively (Mercan et al., 2018; Coelho et al., 2019). Further, AMP human metabolism and biodegradation in WWTPs is stereoselective (Kasprzyk-Hordern and Baker, 2012). (*S*)-AMP metabolises faster than (*R*)-enantiomer. However, higher levels of (*S*)-AMP have been reported in environmental matrices that have been attributed to the metabolism of (*S*)-methamphetamine produced in the illicit market. Therefore, AMP has been found as both racemate and enantiomeric mixtures (Emke et al., 2014; Gonçalves et al., 2019; Langa et al., 2021a; Langa et al., 2021b).

In this study, different biomarkers oftoxicity were selected targeting possible enantioselective effects and to deeper current understanding about AMP environmental impacts.

Morphophysiological parameters have been determined because their are sensitive to sublethal effects (Bownik et al., 2015; Bownik, 2020). For Cladocera's such as daphnia, body size exhibits high plasticity in response to both biotic and abiotic factors (Bownik et al., 2015; Karpowicz et al., 2020). Larger bodies can be easily precepted by predators through daphnia body characteristics adaptations such as colourless and transparency to avoid predators (Karpowicz et al., 2020). Indeed, some studies have been showing that body size can be used as an ecological indicator of freshwater status (Bownik et al., 2015; Karpowicz et al., 2020).

In our study, exposure of *D. magna* to both racemate and pure enantiomers caused changes in body size. An enantioselective effect was noted with a significant

decrease in body size found for organisms exposed to (*S*)-AMP comparatively to (*R*)-enantiomer and the racemate. This smaller body size can be a result of the lower feeding rate. In fact, AMP has been therapeutically used as an appetite suppressant. On the contrary, both *rac*-AMP and (*R*)-AMP showed an increase in body size on day 3 though for (*R*)-AMP a significant increase was observed on day 3 but on day 8 no significant differences were found between (*R*)-AMP and the control. This can be due to an adaptation of the organisms to this enantiomer or to a better capacity for metabolization and excretion of this enantiomer. However, organisms exposed to *rac*-AMP showed at all concentrations a higher body size. Heart rate is a common physiological biomarker used to assess toxicity effects (Bownik and Stępniewska, 2015). In this study, heart rate, area and length also changed, showing that both racemate and enantiomers can influence the development and functioning of this organ. AMP is a sympathomimetic substance affecting cardiovascular organs. Both racemate and enantiomers caused a decrease in the heart rate, though on day 8 only racemate still continued to show a decrease.

Swimming speed is one of the most used parameters of daphnia activity and in this study it was expressed by velocity (cm/min). According to Hylander et al (2014), organisms with higher body sizes swim faster than smaller size organisms. Though organisms exposed to (*S*)-AMP showed lower body size while those exposed to *rac* and (*R*)-AMP showed higher body size, no changes in speed were observed at any of the tested concentrations. Total distance is also an important swimming parameter as it indicates locomotor activity (Bownik, 2017). Different results were observed with racemate that cause a reduction of the total travelled distance while for both enantiomers an increase in total distance was noted but no enantioselective effects Some studies have been shown altered swimming behaviour in daphnia exposed to psychoactive substances. For instance, exposure to benzoylecgonine at environmental reported concentrations (0.5 and 1 µg/L) caused a reduction in activity in *D. magna* organisms.

First reproductive events were also determined by targeting the number of eggs per daphnia, the number of daphnia with eggs, and the number of neonates. Most reproductive effects are investigated in chronic toxicity assays. In this study, no chronic

tests were performed, and only first reproductive events were possible to be reported on day 8. No changes in the number of eggs per daphnia or the number of daphnia with eggs were observed for the racemate however a tendency to increase the number of neonates at 0.1 and 1 µg/L was observed while a significant increase was found at 10 µg/L. Regarding enantiomers, a significant difference was found between enantiomers with a significant decrease in the number of eggs per daphnia in the organisms exposed to (*S*)-AMP in contrast to the increase in those exposed to (*R*)-AMP at 1 µg/L. Though no significant differences were observed for the number of daphnia with eggs also a tendency to increase was observed in the organisms exposed to (*R*)-AMP. These results show that AMP can affect the reproductive performance of daphnia, and that effects can be enantioselective however, the 21 days reproduction assays should be done to evaluate possible effects.

Assessment of daphnid biochemical parameters may give a valuable complement to observed previously alterations of swimming behavioral or morphophysiological parameters.

Oxidative stress induced by PAS have been reported. For instance, increase levels of ROS and oxidative damage were observed in *D. magna* exposed to citalopram and mirtazapine (Duan et al., 2022). Exposure to cocaine and its metabolite benzoylecgonine caused an increase of ROS production in daphnids and zebrafish embryos (Parolini et al., 2018a; Parolini et al., 2018b). In contrast, in this study, no changes were found on ROS levels in the organisms exposed to the racemate while a significant decrease in ROS levels was observed for both enantiomers. Further, in both racemate and enantiomers a reduction of CAT activity was observed at the higher concentrations. CAT is an antioxidant enzyme protecting cells from oxidation. Some studies have shown alterations of CAT activity induced PAS in various organisms such as fish, and daphnia among others. This could be a result of the lower capability of these organisms to produce more enzymes, nevertheless, considering the reduction of ROS also in the higher concentrations it seems that AMP can induce some protective capability against oxidation.

Levels of TBARs also decreased in racemate which corroborates the lower values observed for ROS and CAT and the same was seen for (*S*)-AMP. In addition, some studies

have shown the decrease of AChE activity by oxidative stress. In fact, the reduction of AChE has been reported in *D. magna* exposed to PAS such as benzoylecgonine, but also to other pollutants because of oxidative stress.

In this study, a significant decrease in AChE activity was observed for the racemate, however, no increase in ROS levels was observed or CAT activity. Therefore, maybe another mechanism of action can be related to the reduction of AChE activity. In contrast, no changes in the activity of AChE were found for both enantiomers that are in accordance with the reduction of ROS levels and CAT activity.

6. Conclusions and future perspectives

Currently, most ecotoxicological assays regard only racemates and do not consider the pure enantiomers. It is well known that enantiomers may have different biological activities, including toxicity. Further, after consumption, pharmaceuticals and illicit drugs can suffer enantioselective metabolism in humans and the biodegradation process during wastewater treatment can also change their EF. Thus, accurate risk assessment of their occurrence in the environment requires enantioselective toxicity assays for an adequate evaluation.

This study showed that both racemate and enantiomers can interfere with different parameters altering morphophysiological, swimming behaviour, reproduction and biochemical parameters. Some of the selected parameters showed enantioselective effects demonstrating the relevance of these studies for an accurate environmental risk assessment. Additionally, considering that some of the effects were observed at environmental reported concentrations (1 and 1 µg/L) ofAMP, both racemate and enantiomers can cause harmful effects on *D. magna.* The pure enantiomers showed to interfere with different parameters nonetheless we cannot discriminate each one is more toxic enantiomer.

Studies, regarding other toxicity biomarkers and chronic toxicity assays should be done to continue to deep the knowledge about AMP impacts on daphnia and assess its possible enantioselective effects. Additionally, it is important to stress, that chemicals do not occur alone in the environment, but as complex mixtures. Thus, the combined effect of AMP with other environmental contaminants should be evaluated due to possible adverse effects due to synergist/ addictive effects. On other hand, it can be interesting to study transgenerational effects to understand long-term exposure for low dose levels.

7. REFERENCES

Aguilar Diaz De Leon, J., Borges, C.R., 2020. Evaluation of Oxidative Stress in Biological Samples Using the Thiobarbituric Acid Reactive Substances Assay. JoVE, 159, e61122.

Alfonso-Prieto, M., Biarnés, X., Vidossich, P., Rovira, C., 2009. The molecular mechanism of the catalase reaction. Journal of the American Chemical Society 131, 11751-11761.

Antunes, S., Castro, B., , 2017. Pulgas-de-água (Daphnia spp.). Revista de Ciência Elementar 5, 4: 050.

Attafi, I.M., Tumayhi, M.M., Banji, D., Albeishy, M.Y., Khardali, I.A., Korashy, H.M., 2021. Analysis of fatalities involving amphetamine in Jazan, Saudi Arabia. Forensic Science International: Reports 4, 100237.

Betteridge, D.J., 2000. What is oxidative stress? Metabolism 49, 3-8.

Bownik, A., 2017. Daphnia swimming behaviour as a biomarker in toxicity assessment: A review. The Science of the Total Environment 601-602, 194-205.

Bownik, A., 2020. Physiological endpoints in daphnid acute toxicity tests. Science of The Total Environment 700, 134400.

Bownik, A., Stępniewska, Z., 2015. Protective effects of ectoine on behavioral, physiological and biochemical parameters of Daphnia magna subjected to hydrogen peroxide. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 170, 38-49.

Bownik, A., Stępniewska, Z., Skowroński, T., 2015. Effects of ectoine on behavioural, physiological and biochemical parameters of *Daphnia magna*. Comparative biochemistry and physiology. Toxicology & pharmacology : CBP 168, 2-10.

Caras, S., Sharpe, T., 2019. Pharmacokinetics of AR19, an Immediate-Release Amphetamine Sulfate Formulation Designed to Deter Manipulation for Administration Via Nonoral Routes: Bioequivalence to Reference Racemic Amphetamine Sulfate, Dose Proportionality, and Food Effect. Journal of Child and Adolescent Psychopharmacology, 30, 2: 69-80..

Castro, B.B., Freches, A.R., Rodrigues, M., Nunes, B., Antunes, S.C., 2018. Transgenerational Effects of Toxicants: An Extension of the Daphnia 21-day Chronic Assay? Archives of environmental Contamination and Toxicology 74, 616-626.

Coelho, M.M., Lado Ribeiro, A.R., Sousa, J.C.G., Ribeiro, C., Fernandes, C., Silva, A.M.T., Tiritan, M.E., 2019. Dual enantioselective LC–MS/MS method to analyse chiral drugs in surface water: Monitoring in Douro River estuary. Journal of Pharmaceutical and Biomedical Analysis 170, 89-101.

Coutts, R.T., Baker, G.B., 1989. Implications of chirality and geometric isomerism in some psychoactive drugs and their metabolites. Chirality 1, 99-120.

De Andres, F., Castaneda, G., Rios, A., 2009. Use of toxicity assays for enantiomeric discrimination of pharmaceutical substances. Chirality 21, 751-759.

De Felice, B., Salgueiro-González, N., Castiglioni, S., Saino, N., Parolini, M., 2019. Biochemical and behavioral effects induced by cocaine exposure to *Daphnia magna*. The Science of the Total Environment 689, 141-148.

de la Torre, R., Farré, M., Navarro, M., Pacifici, R., Zuccaro, P., Pichini, S., 2004. Clinical pharmacokinetics of amfetamine and related substances: monitoring in conventional and non-conventional matrices. Clinical pharmacokinetics 43, 157-185.

Ding, J., Zou, H., Liu, Q., Zhang, S., Mamitiana Razanajatovo, R., 2017. Bioconcentration of the antidepressant fluoxetine and its effects on the physiological and biochemical status in *Daphnia magna*. Ecotoxicology and Environmental Safety 142, 102-109.

Duan, S., Fu, Y., Dong, S., Ma, Y., Meng, H., Guo, R., Chen, J., Liu, Y., Li, Y., 2022. Psychoactive drugs citalopram and mirtazapine caused oxidative stress and damage of feeding behavior in Daphnia magna. Ecotoxicology and Environmental Safety 230, 113147.

Ebert, D., 2005. Ecology, Epidemiology, and Evolution of Parasitism in Daphnia.

EMCDDA, 2020. *European Drug Report 2020: Trends and Developments,* European Monitoring Centre for Drugs and Drug Addiction.

EMCDDA, 2021. *European Drug Report 2020: Trends and Developments,* European Monitoring Centre for Drugs and Drug Addiction.

Emke, E., Evans, S., Kasprzyk-Hordern, B., de Voogt, P., 2014. Enantiomer profiling of high loads of amphetamine and MDMA in communal sewage: a Dutch perspective. The Science of the Total Environment 487, 666-672.

Faraone, S.V., 2018. The pharmacology of amphetamine and methylphenidate: Relevance to the neurobiology of attention-deficit/hyperactivity disorder and other psychiatric comorbidities. Neuroscience & Biobehavioral Reviews 87, 255-270.

Félix, L.M., Serafim, C., Martins, M.J., Valentim, A.M., Antunes, L.M., Matos, M., Coimbra, A.M., 2017. Morphological and behavioral responses of zebrafish after 24h of ketamine embryonic exposure. Toxicology and Applied Pharmacology 321, 27-36.

Fernández-Rubio, J., Rodríguez-Gil, J.L., Postigo, C., Mastroianni, N., López de Alda, M., Barceló, D., Valcárcel, Y., 2019. Psychoactive pharmaceuticals and illicit drugs in coastal waters of North-Western Spain: Environmental xposure and risk assessment. Chemosphere 224, 379-389.

Fontes, M.K., Maranho, L.A., Pereira, C.D.S., 2020. Review on the occurrence and biological effects of illicit drugs in aquatic ecosystems. Environmental Science and Pollution Research International 27, 30998-31034.

Ghani, M.A., Barril, C., Bedgood, D.R., Prenzler, P.D., 2017. Measurement of antioxidant activity with the thiobarbituric acid reactive substances assay. Food Chemistry 230, 195-207.

Goncalves, R., Ribeiro, C., Cravo, S., Cunha, S.C., Pereira, J.A., Fernandes, J.O., Afonso, C., Tiritan, M.E., 2019. Multi-residue method for enantioseparation of psychoactive substances and beta blockers by gas chromatography-mass spectrometry. Journal of Chromatography. B Anal. Technol. Biomed. Life Sci. 1125, 121731.

Gonçalves, R., Ribeiro, C., Cravo, S., Cunha, S.C., Pereira, J.A., Fernandes, J.O., Afonso, C., Tiritan, M.E., 2019. Multi-residue method for enantioseparation of psychoactive substances and beta blockers by gas chromatography–mass spectrometry. Journal of Chromatography B 1125, 121731.

Goyal, M.M., Basak, A., 2010. Human catalase: looking for complete identity. Protein & Cell 1, 888-897.

Gunne, L., Galland, L., 1967. Stereoselective metabolism of amphetamine. Biochemical Pharmacology 16, 1374-1377.

Guo, R., Liu, G., Du, M., Shi, Y., Jiang, P., Liu, X., Liu, L., Liu, J., Xu, Y., 2016. Early ketamine exposure results in cardiac enlargement and heart dysfunction in Xenopus embryos. BMC Anesthesiol 16, 23-23.

Harris, K.D.M., Bartlett, N.J., Lloyd, V.K., 2012. Daphnia as an Emerging Epigenetic Model Organism. Genetics Research International 2012, 147892.

Hylander, S., Ekvall, M.T., Bianco, G., Yang, X., Hansson, L.-A., 2014. Induced tolerance expressed as relaxed behavioural threat response in millimetre-sized aquatic organisms. Proceedings of the Royal Society B: Biological Sciences 281, 20140364.

ISO, 2012a. 6341:2012. Water quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) - Acute toxicity test. Fourth Edition.

ISO, 2012b. Water quality — Fresh water algal growth inhibition test with unicellular green algae.

Jonczyk, E., Gilron, G., 2005. Acute and Chronic Toxicity Testing with Daphnia Sp. in: Blaise, C., Férard, J.-F. (Eds.). Small-scale Freshwater Toxicity Investigations: Toxicity Test Methods. Springer Netherlands, Dordrecht, pp. 337-393.

Karpowicz, M., Sługocki, Ł., Kozłowska, J., Ochocka, A., López, C., 2020. Body size of Daphnia cucullata as an indicator of the ecological status of temperate lakes. Ecological Indicators 117, 106585.

Kasprzyk-Hordern, B., Baker, D.R., 2012. Enantiomeric profiling of chiral drugs in wastewater and receiving waters. Environmental Science & Technology 46, 1681-1691.

Langa, I., Gonçalves, R., Tiritan, M.E., Ribeiro, C., 2021a. Wastewater analysis of psychoactive drugs: Non-enantioselective vs enantioselective methods for estimation of consumption. Forensic Science International 325, 110873.

Langa, I., Tiritan, M.E., Silva, D., Ribeiro, C., 2021b. Gas Chromatography Multiresidue Method for Enantiomeric Fraction Determination of Psychoactive Substances in Effluents and River Surface Waters. Chemosensors 9, 224.

Li, R., Jia, Z., Trush, M.A., 2016. Defining ROS in Biology and Medicine. Reactive oxygen species (Apex, N.C.) 1, 9-21.

Liao, P.H., Hwang, C.C., Chen, T.H., Chen, P.J., 2015. Developmental exposures to waterborne abused drugs alter physiological function and larval locomotion in early life stages of medaka fish. Aquatic Toxicology (Amsterdam, Netherlands) 165, 84-92.

Lionetto, M.G., Caricato, R., Calisi, A., Giordano, M.E., Schettino, T., 2013. Acetylcholinesterase as a biomarker in environmental and occupational medicine: new insights and future perspectives. BioMed Research International 2013, 321213.

Loureiro, C., Castro, B.B., Pereira, J.L., Gonçalves, F., 2011. Performance of standard media in toxicological assessments with Daphnia magna: chelators and ionic composition versus metal toxicity. Ecotoxicology (London, England) 20, 139-148.

Magni, S., Parolini, M., Della Torre, C., de Oliveira, L.F., Catani, M., Guzzinati, R., Cavazzini, A., Binelli, A., 2017. Multi-biomarker investigation to assess toxicity induced by two antidepressants on Dreissena polymorpha. The Science of the Total Environment 578, 452-459.

Masteling, R.P., Castro, B.B., Antunes, S.C., Nunes, B., 2016. Whole-organism and biomarker endpoints in Daphnia magna show uncoupling of oxidative stress and endocrine disruption in phenolic derivatives. Ecotoxicology and Environmental Safety 134p1, 64-71.

Mercan, S., Kuloglu, M., Tekin, T., Turkmen, Z., Dogru, A.O., Safran, A.N., Acikkol, M., Asicioglu, F., 2018. Wastewater-based monitoring of illicit drug consumption in Istanbul: Preliminary results from two districts. The Science of the Total Environment 656, 231-238.

Mergeay, J., Declerck, S., Verschuren, D., Meester, L.D., 2006. Daphnia community analysis in shallow Kenyan lakes and ponds using dormant eggs in surface sediments. Freshwater Biology 51, 399-411.

Mhamdi, A., Queval, G., Chaouch, S., Vanderauwera, S., Van Breusegem, F., Noctor, G., 2010. Catalase function in plants: a focus on Arabidopsis mutants as stress-mimic models. Journal of Experimental Botany 61, 4197-4220.

Nevesa, M., Castroa, B.B., Vidala, T., Vieiraa, R., Marquesb, J.C., Coutinhod, J.A.P., alvesa, F.G., alvesa, A.G., 2015. Biochemical and populational responses of an aquatic bioindicator species , Daphnia longispina , to a commercial formulation of a herbicide (Primextra ® Gold TZ) and its active ingredient (S-metolachlor).

Nunes, B., Antunes, S.C., Santos, J., Martins, L., Castro, B.B., 2014. Toxic potential of paracetamol to freshwater organisms: a headache to environmental regulators? Ecotoxicology and Environmental Safety 107, 178-185.

OCDE, 2012. Test Nº211. Guidelines for testing of chemicals. Daphnia magna Reproduction Test. Adopted in 2 October 2012.

OECD, 2004. Test No. 202: Daphnia sp. Acute Immobilisation Test.

Parolini, M., Bini, L., Magni, S., Rizzo, A., Ghilardi, A., Landi, C., Armini, A., Del Giacco, L., Binelli, A., 2018a. Exposure to cocaine and its main metabolites altered the protein profile of zebrafish embryos. Environmental Pollution 232, 603-614.

Parolini, M., De Felice, B., Ferrario, C., Salgueiro-González, N., Castiglioni, S., Finizio, A., Tremolada, P., 2018b. Benzoylecgonine exposure induced oxidative stress and altered swimming behavior and reproduction in *Daphnia magna*. Environmental Pollution (Barking, Essex : 1987) 232, 236-244.

Pérez-Pereira, A., Ribeiro, C., Teles, F., Gonçalves, R., Gonçalves, V., Pereira, J.A., Carrola, J.S., Pires, C.A., Tiritan, M.E., 2020. Ketamine and norketamine: enantioresolution and enantioselective aquatic ecotoxicity studies. Environmental Toxicology and Chemistry, 41, 3: 569-579..

Ribeiro, A.R.L., Maia, A.S., Ribeiro, C., Tiritan, M.E., 2020. Analysis of chiral drugs in environmental matrices: Current knowledge and trends in environmental, biodegradation and forensic fields. TrAC Trends in Analytical Chemistry 124, 115783.

Ribeiro, C., Ribeiro, A.R., Maia, A.S., Tiritan, M.E., 2017. Occurrence of Chiral Bioactive Compounds in the Aquatic Environment: A Review. Symmetry 9, 215.

Ribeiro, C., Ribeiro, A.R., Tiritan, M.E., 2016. Priority Substances and Emerging Organic Pollutants in Portuguese Aquatic Environment: A Review. Reviews of Environmental Contamination and Toxicology 238, 1-44.

Ribeiro, O.M., Pinto, M.Q., Ribeiro, C., Tiritan, M.E., Carrola, J.S., 2021. A dáfnia como sensor da ecotoxidade. Revista Ciência Elementar 9, 044.

Rivetti, C., Campos, B., Barata, C., 2016. Low environmental levels of neuro-active pharmaceuticals alter phototactic behaviour and reproduction in *Daphnia magna*. Aquatic Toxicology (Amsterdam, Netherlands) 170, 289-296.

ROBINS, L.N., DAVIS, D.H., GOODWIN, D.W., 1974. DRUG USE BY U.S. ARMY ENLISTED MEN IN VIETNAM: A FOLLOW-UP ON THEIR RETURN HOM1. American Journal of Epidemiology 99, 235-249.

Serra, T., Müller, M.F., Barcelona, A., Salvadó, V., Pous, N., Colomer, J., 2019. Optimal light conditions for Daphnia filtration. The Science of the Total Environment 686, 151-157.

Shaw, J.R., Pfrender, M.E., Eads, B.D., Klaper, R., Callaghan, A., Sibly, R.M., Colson, I., Jansen, B., Gilbert, D., Colbourne, J.K., 2008. *Daphnia* as an emerging model for toxicological genomics. in: Hogstrand, C., Kille, P. (Eds.). Advances in Experimental Biology. Elsevier, pp. 165-328.

Silman, I., Sussman, J.L., 2008. Acetylcholinesterase: How is structure related to function? Chemico-Biological Interactions 175, 3-10.

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

Su, C., Cui, Y., Liu, D., Zhang, H., Baninla, Y., 2020. Endocrine disrupting compounds, pharmaceuticals and personal care products in the aquatic environment of China: Which chemicals are the prioritized ones? Science of The Total Environment 720, 137652.

Subedi, B., Kannan, K., 2015. Occurrence and fate of select psychoactive pharmaceuticals and antihypertensives in two wastewater treatment plants in New York State, USA. The Science of the Total Environment 514, 273-280.

Szabelak, A., Bownik, A., 2021. Behavioral and physiological responses of Daphnia magna to salicylic acid. Chemosphere 270, 128660.

Tkaczyk, A., Bownik, A., Dudka, J., Kowal, K., Ślaska, B., 2021. Daphnia magna model in the toxicity assessment of pharmaceuticals: A review. The Science of the Total Environment 763, 143038.

Tsai, M.-C., Huang, T.-L., 2015. Thiobarbituric acid reactive substances (TBARS) is a state biomarker of oxidative stress in bipolar patients in a manic phase. Journal of Affective Disorders 173, 22-26.

Tsikas, D., 2017. Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. Analytical Biochemistry 524, 13-30.

Wang, Z., Han, S., Cai, M., Du, P., Zhang, Z., Li, X., 2020a. Environmental behavior of methamphetamine and ketamine in aquatic ecosystem: Degradation, bioaccumulation, distribution, and associated shift in toxicity and bacterial community. Water research 174, 115585.

Wang, Z., Han, S., Cai, M., Du, P., Zhang, Z., Li, X., 2020b. Environmental behavior of methamphetamine and ketamine in aquatic ecosystem: Degradation, bioaccumulation, distribution, and associated shift in toxicity and bacterial community. Water Research 174, 115585.

Wang, Z., Xu, Z., Li, X., 2019. Impacts of methamphetamine and ketamine on *C.elegans's* physiological functions at environmentally relevant concentrations and eco-risk assessment in surface waters. Journal of Hazardous Materials 363, 268-276.

Wenjiao, F., Yongkui, Z., Yunchuan, C., Junxiu, S., Yuwen, Y., 2014. TBARS predictive models of pork sausages stored at different temperatures. Meat Science 96, 1-4.

Xie, Z., Lu, G., Li, S., Nie, Y., Ma, B., Liu, J., 2015. Behavioral and biochemical responses in freshwater fish Carassius auratus exposed to sertraline. Chemosphere 135, 146-155.

Yadav, D., Rangabhashiyam, S., Verma, P., Singh, P., Devi, P., Kumar, P., Hussain, C.M., Gaurav, G.K., Kumar, K.S., 2021. Environmental and health impacts of contaminants of emerging concerns: Recent treatment challenges and approaches. Chemosphere 272, 129492.

Yang, B., Chen, Y., Shi, J., 2019. Reactive Oxygen Species (ROS)-Based Nanomedicine. Chemical reviews 119, 4881-4985.

Yang, H., Lu, G., Yan, Z., Liu, J., Dong, H., 2018. Influence of suspended sediment characteristics on the bioaccumulation and biological effects of citalopram in *Daphnia magna*. Chemosphere 207, 293-302.

Yang, S., Lian, G., 2020. ROS and diseases: role in metabolism and energy supply. Molecular and Cellular Biochemistry 467, 1-12.