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Toxicity of butylone and its enantiomers to Daphnia magna and its degradation by advanced oxidation technologies

Ana Rita Teixeira Carvalho

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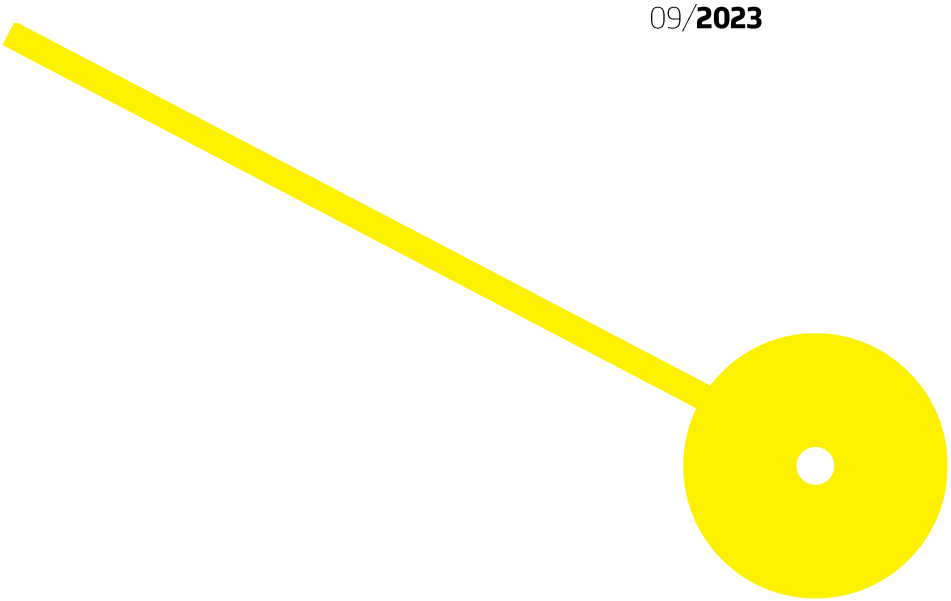

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Toxicity of butylone and its enantiomers to Daphnia magna and its degradation by advanced oxidation technologies

Autor

Ana Rita Teixeira Carvalho

Orientador(es)

Professora Doutora/Cláudia Ribeiro/ Instituto Universitário de Ciências da Saúde

Doutora/Ana Rita Lado Ribeiro/Faculdade de Engenharia da Universidade do Porto

Professora Doutora/Maria Manuela Amorim/Escola Superior de Saúde – Instituto Politécnico do Porto

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Resumo

A butilona (BTL) é uma catinona sintética quiral disponível na forma de racemato e relatada em águas residuais. Contudo, não há estudos sobre o seu impacto nos ecossistemas e possível enantioseletividade. O objetivo deste trabalho foi avaliar: (i) os possíveis efeitos ecotoxicológicos em *Daphnia magna* da BTL, na forma de racemato ou dos seus enantiómeros puros (*R*)- e (*S*)-, e (ii) a eficiência das tecnologias avançadas de oxidação (TAO) na remoção e toxicidade da BTL e produtos de degradação em águas residuais.

Os enantiómeros foram separados por cromatografia líquida utilizando uma coluna semipreparativa quiral, sendo obtidos com elevada pureza enantiomérica (> 97%). Para os ensaios de toxicidade dáfnias foram expostas a concentrações de 0,1; 1 ou 10 µg L^{-1} do racemato ou 0,1 ou 1 µg L^{-1} de cada enantiómero. Verificou-se significativamente alterações morfofisiológicas, bioquímicas e reprodutivas dependentes da forma da substância e desenvolvimento do organismo (juvenil ou adulto). Diferentes TAO (ozonização, O_3; ultravioleta, UV; e UV/O_3) foram aplicadas a águas residuais dopadas com BTL e os ensaios de toxicidade aguda (24 e 48h) demonstraram que as TAO reduziram a toxicidade do efluente contaminado com BTL, sendo que a maior redução de toxicidade foi observada após UV e O_3, respetivamente após 24 e 48 h de exposição.

Palavras-chave: contaminantes emergentes; substâncias psicoativas; catinonas sintéticas; ecotoxicidade; organismos aquáticos; água residuais

Abstract

Butylone (BTL) is a chiral synthetic cathinone available as a racemate and reported in wastewaters. However, there are no studies on its impact on ecosystems and possible enantioselectivity. The objective of this work was to evaluate: (i) the possible ecotoxicological effects on *Daphnia magna* of BTL in the form of racemate or its (*R*)- and (*S*)- enantiomers; and (ii) the efficiency of advanced oxidation technologies (AOT) in the removal and toxicity of BTL and its by-products in wastewaters.

Enantiomers were separated by liquid chromatography using a chiral semi-preparative column, being obtained with high enantiomeric purity (> 97%). For toxicological studies, daphnia were exposed to concentrations of 0.1; 1 or 10 μg L^{-1} of the racemate or 0.1 or 1 μg L^{-1} of each enantiomer. There were a significant morphophysiological, biochemical and reproductive changes depending on the form of the substance and the development of the organism (juvenile or adult). Different AOTs (ozonation, O_3; ultraviolet, UV; and UV/O_3) were applied to wastewater spiked with BTL and acute toxicity tests (24 and 48h) demonstrated a higher toxicity reduction of BTL-spiked effluents, with the highest reduction of toxicity observed after UV and O_3, respectively after 24 and 48 h of exposure.

Keywords: emerging contaminants; psychoactive substances; synthetic cathinones; ecotoxicity; aquatic organisms; wastewaters

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List of abbreviations, symbols and acronyms

AChE Acetylcholinesterase

AMT Ammonium molydate tetragydrate

ATCI Acetylthiocholine iodide

AOT Advanced oxidation technologies

BTL Butylone

CAT Catalase

CNS Central nervous system

CSP Chiral stationary phase

DA Dopamine

DCF 2,7-dichlorofluorescein

DCFH Dichlorofluorescin

DTNB 5,5′-dithio-bis-(2-nitrobenzoic acid)

EF Enantiomeric fraction

EMCDDA European Monitoring Centre for Drugs and Drug Addiction

GC Gas chromatography

H_2DCFDA Dichlorofluorescein diacetate

HPLC-DAD High-performance liquid chromatographic with a diode array detector

ISO International Organization for Standardization

LC Liquid chromatography

MPs Micropollutants

MHRW Moderately hard reconstituted water

MDA Malondialdehyde

MDMA 3,4-methylenedioxymethamphetamine

NPS New psychoactive substances

NA Noradrenaline

OCDE Organisation for Economic Cooperation and Development

PAS Psychoactive substances

PBS Phosphate buffer solution

ROS Reactive oxygen species

SC Synthetic cathinones

SERT Serotonin

TBA Thiobarbituric acid

TBARS Thiobarbituric acid reactive substances

UPW Ultra–pure water

WWTP Wastewater treatment plant

1. INTRODUCTION

1.1. Chiral psychoactive drugs as environmental contaminants

Psychoactive substances (PAS) include pharmaceuticals and illicit drugs (1,2). These substances are designed to change nervous system function and have been shown to interfere with biochemical, physiological, and behavioural parameters of non-target organisms exposed to them (3–5). Like other contaminants, PAS and their metabolites enter the water systems through different routes as urban, hospital and industrial effluents discharges, since they are not completely eliminated during wastewater treatment (2) (**Figure 1**). Other sources include direct disposal, improper manufacturer discharges, illegal production, and veterinary use (**Figure 1**) (2,4). Therefore, PAS are frequently detected in environmental samples due to their widespread use and recalcitrance in wastewater treatment plants (WWTPs), posing risks to wildlife and human health (6–8).

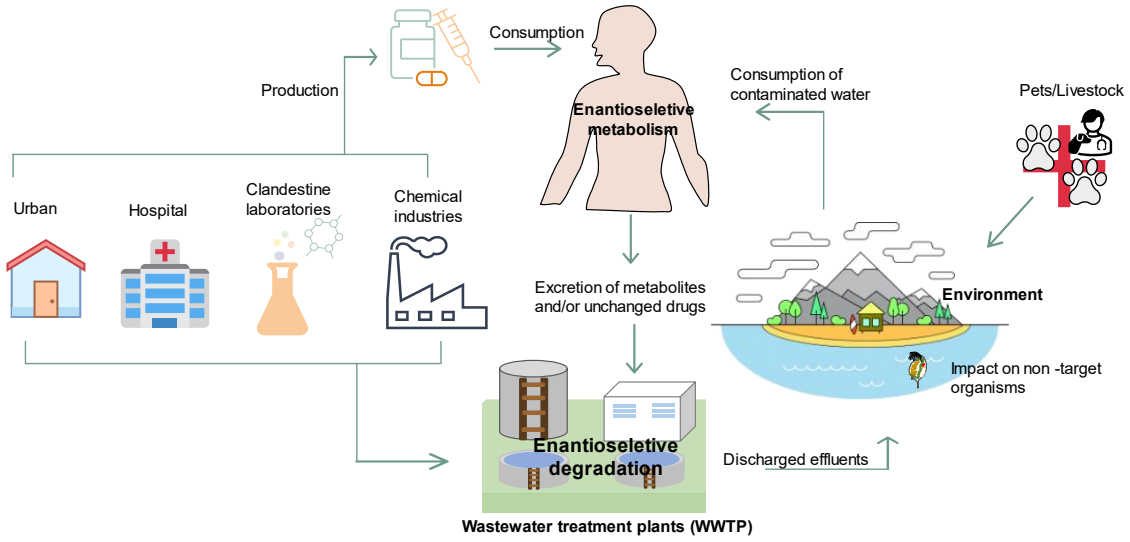

Figure 1| Sources of pharmaceuticals and illicit drugs as environmental contaminants.

In fact, PAS have been reported in sewage, rivers, seawater, drinking water and even biota at concentrations ranging from ppt to ppb(8–10) and various studies have shown that PAS induce acute and chronic toxicity in both aquatic and terrestrial organisms. For instance, changes in swimming behaviour were reported in several invertebrate species due to exposure to antidepressants such as fluoxetine and citalopram (11). Moreover, it has been demonstrated that exposure to methamphetamine alters the earthworm's neurotransmitter system and the physiological characteristics of the medaka fish larvae (12–14). Another study showed that oxidative stress in daphnia was caused by exposure to cocaine (3), whereas the exposure to its

metabolite benzoylecgonine at concentrations comparable to those found in the aquatic environment was shown to cause inhibition of acetylcholinesterase (AChE) activity and to alter swimming behaviour and reproduction (15). Even though, there is still a lack of information about the ecotoxicological effects of PAS. The knowledge on the impact of these pollutants on aquatic organisms is of high importance for risk assessment and further establishment of measures for environmental protection. Additionally, most PAS are chiral and it is known that enantiomers may behave differently in biological systems. After consumption, chiral PAS may undergo enantioselective metabolism and both parent and metabolites can be excreted in different enantiomeric fractions. The non-racemic presence of chiral PAS in wastewaters and the receiving waters has been reported in many studies due to enantioselective human metabolism and biodegradation (5,16–29). The ecotoxicological effects of single enantiomers *vs* their racemate on non-target species are still largely unknown and commonly neglected despite the recognition of the possible different behaviour of pairs of enantiomers (30). Thus, the possible enantioselective ecotoxicity of chiral PAS should be considered for an accurate environmental risk assessment of their presence in the environment.

Indeed, enantioselective ecotoxicity in non-target species has been observed for some PAS. For instance, changes in the morphophysiological parameters of *D. magna* during the first development stages were observed after exposure to 3,4-methylenedioxymethamphetamine (MDMA) racemate and (*S*)- enantiomer, whereas no changes were found in the organisms exposed to the (*R*)-enantiomer (11). Enantioselective effects were also observed in *Tetrahymena termophila* growth inhibition after exposure to ketamine (16). Indeed, a higher growth inhibition of *Tetrahymena termophila* was observed in the organisms exposed to (*S*)-ketamine (18). In another study, both amphetamine (AMP) racemate and (*S*)-enantiomer originated changes in morphophysiological parameters on *D. magna*, whereas no changes were found in the organisms exposed to (*R*)-enantiomer(17).

1.2. Butylone

Synthetic cathinones (SC) are derivatives of the naturally occurring cathinone, found in the plant *Catha edulis* Forsk. (*khat*). These substances are sold on the internet and frequently used for recreational purposes due to their stimulant properties, similar to amphetamine and MDMA (31). SC are chiral drugs available as racemate although they exist in two stereoisomeric forms, which may differ in their potencies (32).

Butylone (1-(1,3-benzodioxol-5-yl)-2-(methylamino) butan-1-one) is a chiral SC that emerged in the European drug market as a new designer drug in 2008 (**Figure 2**). Studies regarding the mechanisms of action of BTL are scarce; however, some studies have been shown that SC inhibit dopamine (DA) serotonin (SERT) and noradrenaline (NA) reuptake, increase their release and induce tolerance and dependence (33–35).

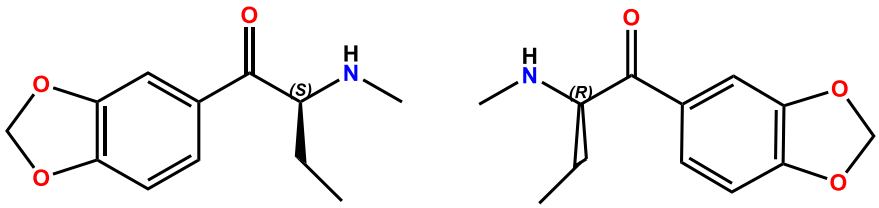

Figure 2| Chemical structure of BTL enantiomers. (*S*)-BTL on the left and (*R*)-BTL on the right.

Only few studies reported the environmental occurrence and ecotoxicity of BTL although its occurrence in wastewaters has already been reported (5,16–30,36). **Table 1** shows the reported PAS levels in surface waters and wastewaters, their EF and toxicity.

The predicted acute toxicity of 44 synthetic cathinones including BTL was determined by an *in silico* study (16). BTL median lethal concentration (LC_{50}) values were 23.2 mg L^{-1} for fish and 2.9 mg L^{-1} for daphnia, whereas chronic toxic concentrations were 1.2 mg L^{-1} for fish, 0.25 mg L^{-1} for daphnia and 2.2 mg L^{-1} for green algae (16). However, the same study showed changes in the morphophysiological endpoints of *D. magna* in both juveniles and adults exposed to BTL at a concentration of 10 µg L^{-1} and a significant increase of reactive oxygen species (ROS) levels at the same concentration (16).

Table 1| Environmental occurrence and ecotoxicity of psychoactive substances (PAS).

PAS	Surface water (ng L^{-1})	Wastewater (ng L^{-1})	EF (effluent)	Toxicity effects
AMP	0.5–1.4 (19) 5.10±2.6 (20) 3.3 (21)	21.8±2.6 (20) 7.7 (21) 22.0 (22)	0.6 (22) (*R*)-AMP	Enantioselectively effects were reported at environmental reported levels (0.1 µg L^{-1}) on morphophysiological parameters of *D. magna* (17).
METH	0.5–58.2 (19) 86.4±64.3 (20)	125±32.8 (20) 1.0 (21) 28.0 (22) 22 (23)	0.5 (22) (*R,S*)-METH	Low concentrations of METH (50 and 500 ng L^{-1}) affected the oxidative status and the development of *D. magna* (24).
K	0.1–20.7 (19)	75±1.9 (25)	n.d.	K led to daphnia reproduction inhibition at environmental concentrations (26).
NK	0.3–6.5 (19)	110±3.0 (25)	n.d.	No toxicity was observed for Daphnia acute immobilization test and *T. thermophila* growth inhibition test (18).
MDMA	0.4–1.3 (19) 8.7 (21)	6.1±0.3 (20) 37.7 (21) 45.3±0.5 (22) 7 (23)	0.9 (22) 0.71 (27) (*R*)-MDMA	Exposure to MDMA can interfere with the morphophysiological and swimming behaviour of *D. magna* (5).
MDPV	1.4–1.6 (28)	2.8–25.0 (28)	n.d	MDPV at 10 µg L^{-1} increases the number of eggs per daphnia and the TBARS levels (16).
BTL		2.51 (29)	n.d.	BTL at 10 µg L^{-1} significant increase of body size on *D. magna* (16).

AMP: amphetamine; BTL: butylone; MDMA: 3,4-methylenedioxymethamphetamine; MDPV: 3,4-methylenedioxypyrovalerone; METH: methamphetamine; k: ketamine; NK: norketamine; TBARs: thiobarbituric acid reactive substances.

1.3. Enantioseparation of chiral drugs

Chromatographic methods such as liquid chromatography (LC), gas chromatography (GC), capillary electrophoresis and supercritical fluid chromatography are used to separate enantiomers, either for analytical or preparative purposes. Nevertheless, LC methods have been the most commonly use in preparative studies due to some advantages such as speed, sensitivity, and easier procedures to isolate/collect enantiomers (1,37).

The racemate resolution can be achieved by direct and indirect methods (1,38). The indirect method is based on the reaction of the enantiomers with an enantiomerically pure chiral derivatization reagent. This approach leads to the formation of diastereomers that have different physical-chemical properties and thus, they can be separated by non-chiral separation methods. In this case, the enantiomers can be recovered by overturning the derivatization procedure. Direct enantioresolution by LC is achieved using a chiral stationary phase (CSP), consisting of a chiral selector attached chemically or adsorbed on a solid support, which are designed to preferentially interact with one of the enantiomers, causing transitory diastereomeric complexes (1,38). Consequently, the enantiomer that forms the least stable complex elutes first, whereas the one forming the most stable complex will elute later. The selection of stationary phases is based on experience, literature knowledge, the analyte, the selector characteristics, and trial and error attempts (1,38). The direct method is preferred over the indirect since it offers some advantages for both preparative and analytical separations. In fact, direct methods do not require derivatization, require less sample handling, and allow use after the preparative chromatography (1,38). LC applying CSPs has been useful as a method for determining the enantiomeric fractions of numerous drugs in a variety of matrices.

There are four different elution modes in chiral chromatography. The normal elution mode uses nonpolar solvents such as hexane with polar organic solvents (*e.g.*, isopropanol or ethanol). Polar organic elution mode uses only polar organic solvents (acetonitrile, methanol, ethanol, propanol, and their mixtures). In reversed elution, polar phases, such as water and polar organic solvents, are used as mobile phases. Polar ionic mode involves the mixture of polar organic solvents with acids, bases, or soluble volatile salts (*e.g.*, ammonium acetate). The polar ionic elution mode is useful when the target analyte has an ionizable group (39,40).

1.4. Ecotoxicity assays

There are several assays that can be performed for evaluation of the toxicity of environmental contaminants. Indeed, some standardised protocols have been developed by international organizations (*e.g.*, Organisation for Economic Cooperation and Development (OECD) and International Organization for Standardization (ISO)) for the assessment of acute or chronic toxicity effects of contaminants, aiming to comply with the need of acceptable data to predicting the environmental effects, hazard, and risk of chemical substances (41). Nevertheless, various toxicity assays have also been performed considering other endpoints such as morphophysiological, biochemical and swimming behaviour (5,16,17).

1.4.1. Daphnia as an invertebrate model in ecotoxicity

Daphnia is a primary consumer in the aquatic trophic chain, by filter-feeding small suspended particles, particularly unicellular algae, thus contributing to improve water quality. *D. magna* is one of the species of the genus *Daphnia* belonging to the order Cladocera (42). Daphnids are approximately 5 mm long and colourless due to their transparent carapace. They have two compound eyes with ommatidia for light detection, a small simple eye (ocellus), two pairs of antennae (the first with a sensory function and the second with a swimming function) and 4-6 pairs of thoracic limbs. Males are smaller than females and have larger first antennae (41,43,44). The morphological and anatomical characteristics of *D. magna* are represented in **Figure 3**.

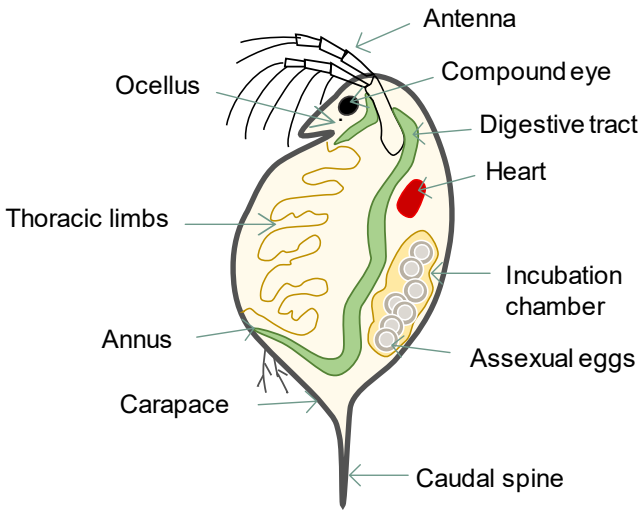

Figure 3| Morphology and anatomy of an adult *D. magna* with eggs.

The life cycle of daphnia is dependent on environmental conditions (**Figure 4**). In favourable conditions, daphniids reproduce asexually, producing diploid eggs that will originate juvenile females genetically identical to the parent (cyclic parthenogenetic). This type of reproduction is essential in ecotoxicity assessment since it decreases the variability. Sexually reproduction occurs when conditions are unfavourable such as low temperature, overpopulation, presence of predators, and decrease in water quality. These changes may induce production of males with subsequent sexual reproduction and production of eggs that have a resistant protective membrane – ephippia/resistance eggs – that do not develop until environmental conditions are favourable. Adults can produce 3 to 5 broods, being the first and second broods less resistant, whereas the third and fourth broods are more resistant and ideally used in ecotoxicity assays (41–44). **Figure 4** shows the sexual and asexual life cycle of *D. magna*.

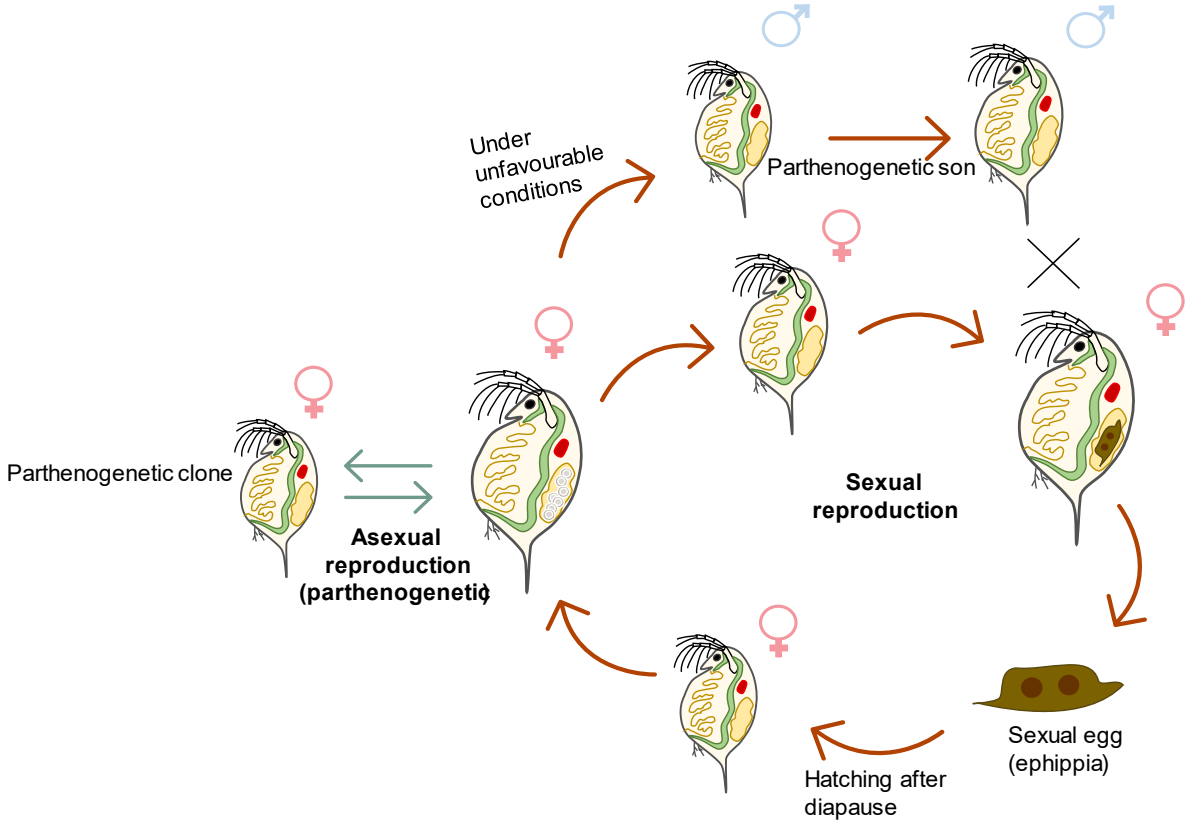

Figure 4| Illustration of *D. magna* life cycle. Adapted from D. Ebert (43)

D. magna has many advantages such as the small body size, short life cycle, high fecundity rate, and parthenogenetic reproduction. Moreover, they are easily handled, and their maintenance is not expensive and relatively easy under laboratory conditions. Daphnia testing includes principles of the 3Rs (replacement, reduction, and refinement) (44).

1.4.2. Morphophysiological and reproduction endpoints

The transparent carapace of *D. magna* facilitates the observation of sensitive indicators to assess toxicity: morphophysiological parameters (*e.g.*, filtration rate, heart size, thoracic limb activity) and reproduction parameters (*e.g.*, presence of eggs in the chamber). In fact, the organisms may be sensitive to sublethal concentrations of contaminants and thus, changes on these parameters may manifest earlier than mortality (5,16,17). Furthermore, it is possible to measure individual parameters intravitally by non-invasive optical methods, such as a light microscope (44).

1.4.3. Biochemical endpoints

The assessment of daphniids biochemical parameters after exposure to PAS is a valuable tool to complement the observed alterations of morphophysiological or behavioural endpoints (44). The biochemical endpoints provide an overview of metabolic changes that may occur due to toxic effects on *D. magna* (44).

AChE is mainly found at neuromuscular junctions and cholinergic synapses in the central nervous system(45). The activity of this cholinergic enzyme may affect behavioural endpoints related to locomotion and feeding activity in aquatic species, including *D. magna*, which may result in reduced growth and reproduction (46). Thus, AChE is a good biomarker to provide an integrative measurement of the overall neurotoxic risk (45).

Another biochemical approach is the determination of free radicals (reactive oxygen species (ROS)) or the product of lipid peroxidation (malondialdehyde (MDA)), or the depleted antioxidant defences (catalase (CAT)). ROS are unstable molecules that include hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2) and superoxide (O_2^{-}) released by cells (47). ROS are known to induce cell damage (47). Lipid peroxidation is the reaction of ROS with lipids. When oxidative stress occurs, ROS attack lipids such as polyunsaturated fatty acids (PUFAs) that are peroxidized and form MDA (48). MDA is a biomarker of lipid peroxidation, which can reflect the degree of lipid peroxidation in organisms and indirectly reflect the degreed of cell injury, as an indicator of oxidative stress (48).

CAT is an antioxidant enzyme that catalyses the decomposition of H_2O_2 to H_2O and O_2, ubiquitously present in aerobic cells containing a cytochrome system(49).

1.5. Advanced oxidation technologies

WWTP treatments involve a combination of physical, chemical, and biological processes to remove insoluble particles and soluble contaminants from effluents (50). The conventional treatments usually applied at WWTPs are not sufficient to remove the micropollutants (MPs) and thus, the implementation of advanced treatments is required to overcome their limited removal efficiencies.

The advanced oxidation technologies (AOT) include chemical oxidation technologies and advanced oxidation processes (AOPs) based on the formation of hydroxyl radicals (HO•). AOTs include ultraviolet (UV)–based and ozone (O_3) based treatments, *e.g.*, by the combination of oxidants such as O_3 or H_2O_2 with UV or visible irradiation. They have been demonstrated to be effective in removing a wide range of MPs, including estrogens, pharmaceuticals, and pesticides (50–52).

UV radiation is quite used in WWTPs for disinfection purposes, but it can also remove MPs based on direct photolysis during absorption of UV-C protons at the wavelength of 254 nm. Moreover, indirect photolysis may also occur, due to the presence of some organic and inorganic species existing in the water matrix that act as photosensitizers and promote the production of ROS by UV irradiation (53). The effectiveness of UV irradiation is highly dependent on the applied dose (in mJ cm^{-2} or mWs cm^{-2}), calculated as the product of average UV intensity and contact time (54).

Ozonation degrades the pollutants through two ways: directly by attack from molecular O_3 and/or indirectly by reaction with HO•, depending on the water pH (55). In Switzerland and in other countries, because of the wide spectrum of MPs that can be removed by ozonation, this procedure was selected as the oxidation technology of choice for enhancing wastewater treatment (51).

The improvement on the ecotoxicological indicators in wastewater has been reported after application of AOT. For instance, Oropesa et al. performed a 48-h acute immobilization assay with *D. magna* to assess the toxicity of effluents previous spiked with pharmaceuticals (acetaminophen, antipyrine, caffeine, carbamazepine, diclofenac, hydrochlorothiazide, ketorolac, metoprolol, and sulfamethoxazole) after treatment with aerobic biological oxidation and AOTs (single ozonation, solar photolytic ozonation, solar photocatalytic ozonation, TiO_2, Fe_3O_4, and solar photocatalytic oxidation, TiO_2). Overall, lethal effects were not observed after all treatments (56).

1.6. AIMS

Studies about the possible adverse effects of BTL are scarce and there is no information about its enantioselectivity on non-target organisms. Moreover, no data is available for its degradation by AOTs and the possible toxicity of the treated water towards non-target organisms. The aim of this work was to evaluate the possible enantioselective ecotoxicological effects of BTL using *Daphnia magna* as aquatic model organism and its degradation by AOTs.

For this, the specific objectives of this work were to:

a. obtain the pure enantiomers of BTL by semipreparative LC for further toxicity assays with *D. magna*;
b. evaluate the possible toxicity of BTL and its isolated enantiomers using an ecologically relevant aquatic organism, *D. magna*. The assay was carried out for 9 days, at environmentally relevant concentration (0.1 μg L^{-1}) and higher sublethal concentrations (1 and 10 μg L^{-1}). Different parameters were selected (morphophysiological, reproductive and biochemical) and the critical stages of development of this organism, the transition juvenile to adult, were considered.
c. evaluate its degradation in wastewater by AOTs and ecotoxicity of treated effluents. For that, wastewater samples were spiked with BTL at 10 μg L^{-1} and different technologies (UV, ozonation (O_3), and O_3/UV) were applied. The removal rate of BTL was determined and the acute immobilisation test was performed to investigate toxicity before and after application of the AOTs.

2. MATERIALS AND METHODS

2.1. Enantioseparation of butylone

2.1.1. Chemicals, materials and equipments

Butylone [(*R,S*)-BTL.HCl; >98.8%] was acquired from Lipomed (Arlesheim, Switzerland). All solvents used were of chromatographic grade. Diethylamine (DEA, 99.5%) was acquired from Sigma-Aldrich (Co, Belgium), ethanol (EtOH, ≥99.8%) was acquired from Fisher Scientific UK (Leicestershire, United Kingdom), *n*-hexane (*n*-Hex, ≥97.0%) was acquired from VWR BDH Chemicals (Gliwice, Poland); and hydrogen chloride solution in diethyl ether was acquired from Alfa Aesar (Thermofisher, Kandel, Germany). For enantioseparation, a standard solution was prepared in a mixture of EtOH and *n*-Hex (7:3, *v/v*), at a concentration of 4 mg mL^{-1} and stored in amber vials at – 20 °C. Glass microfibers filters with 0.7 μm particle size, 47 mm diameter) were purchased from VWR®. A Büchi® Rotavapor® R-210 evaporator with vacuum controller (V-850) and water bath (B-491) acquired to Büchi Switzerland, and a centrifugal vacuum evaporator (CentriVap Concentrator) with a cold trap purchased from Labconco (Kansas City, Mo, USA), were used in the evaporation processes.

2.1.2. Equipment and chromatographic conditions

A LaChrom Merck Hitachi® high-performance liquid chromatographic equipment with a diode array detector (HPLC-DAD), equipped with a DAD (L-7455), an interface system (D-7000), an autosampler (L-7200), a pump (L-7100), and a data acquisition software (System Manager HSMP-7000, Version 3.0), was used for the semipreparative enantioresolution of (*R,S*)-BTL, and enantiomeric purity evaluation. The semi-preparative chromatographic enantioseparation was performed according to the methodology adapted by Spálovská et al.(33). The chiral stationary phase was a homemade 3,5-dimethylphenylcarbamate amylose column coated with APS-Nucleosil (500 A, 7 μm particle size, 20%, w/w; 20 x 0.7 cm internal diameter). The enantioseparation was performed under normal elution mode, at room temperature and under isocratic conditions, with a flow rate of 1.5 mL min^{-1} and the DAD detector was set at a wavelength of 230 nm. The mobile phase for the separation of the enantiomers was a mixture of 0.1% of DEA in n-Hex and 0.1% DEA in EtOH (70:30, v/v). Three fractions were collected into round-bottomed flasks corresponding to the first eluted enantiomer, an intermediate fraction (composed of both

enantiomers), and the second enantiomer eluted. The intermediate fraction containing the mixture of both enantiomers was reinjected in the semi-preparative column to obtain a better yield and purity of enantiomers. The fractions were evaporated using a Büchi® Rotavapor® R-210. The resulting extract was reconstituted in 1 mL of EtOH, followed by the dropwise addition of HCl in ether to promote precipitation, so the free-base form of the enantiomers was converted into the respective hydrochloride. The precipitate was collected and reconstituted in EtOH.

Enantiomeric ratio (e.r) was calculated according to our previous works (18,57) and the following formula: $\% \, e.r. = \frac{\mathrm{E}(S) \text{ (or } \mathrm{E}(R))}{(\mathrm{E}(S)+\mathrm{E}(R))} \times 100$.

For the quantification of both enantiomers, an analytical chromatography was performed using the same equipment and the following chromatographic conditions. The chiral stationary phase used was a Lux® 3 µm Amylose-1 column (LC Column 150 x 4.6 mm) and the mobile phase was a mixture of 0.1% DEA in *n*-Hex and 0.1% DEA in EtOH (65:35, *v/v*) at a flow rate of 0.7 mL min^{-1}; the sample injection volume was 10 µL, and DAD detector was set at a wavelength of 230 nm.

All mobile phases were previously filtered using the 0.7 µm glass microfibers filters.

2.2. Ecotoxicity assays

2.2.1. Chemicals and materials

A laminar flow chamber SC4 from Allentown (New Jersey, USA) and an autoclave from PBI (South Carolina, USA) were used for the preparation and manipulation of all solutions and media. Absorbance was measured using a UV/Vis spectrometer (GBC Cintra 10e, Australia). A microplate reader BioTek Synergy 2 (Vermont, USA) was used for biochemical analysis and an ultrasonicator Vibra-Cell™ model VCX750 (Sonics & Materials, Inc.) for preparation of the daphnia homogenates. A microscope Axiostar plus ZEISS (Jena, Germany) coupled to a digital camera (Canon PowerShot G9) was used for image and video recording for assessment of the morphophysiological and reproductive parameters and an Inverse Microscope of ZEISS (Jena, Germany) with a Neubauer chamber was used for microalgae cell counting.

For adjustment of pH, a 2M solution of sodium hydroxide (NaOH) acquired from Sigma-Aldrich (St. Louis, USA) and a 0.5 M solution of hydrochloric acid (HCl) acquired from Honeywell Fluka (Seelze, Germany), were used.

2.2.2. Subchronic toxicity assays with daphnia

2.2.2.1 *Daphnia magna* maintenance

Monoclonal cultures of *Daphnia magna* were maintained in a bioterium room at 20 C ±2 C and with a 16:8h light/dark cycle. They were suspended in moderately hard reconstituted water (MHRW) prepared using the following chemicals per litre: 96 mg of sodium bicarbonate ($NaHCO_3$, ≥99.7%) obtained from Sigma-Aldrich (Missouri, USA); 60 mg of calcium sulphate dihydrate ($CaSO_4.2H_2O$, >99%) and 123 mg magnesium sulphate heptahydrate ($MgSO_4.7H_2O$, >99%) purchased from Merck (Darmstadt, Germany); 4 mg of potassium chloride (KCl, >99%) acquired from Panreac (Barcelona, Spain). Ultrapure water (UPW) was obtained from an Ultrapure Water System (SG Ultra Clear UV plus).

Before being used, the MHRW was aerated for about 30 minutes with an air pump and under continuous magnetic agitation and then, the pH and conductivity were measured. The MHRW was then supplemented with 9 mL of the *Ascophyllum nodosum* algae extract stock solution (acquired from SOL-PLEX® SIERRA|Alltech, Kentucky, USA), 500 µL of yeast extract stock solution (*Saccharomyces cerevisiae* yeast purchased from Pura Vida, Lisbon, Portugal) and 50 µL of stock vitamin mix solution (thiamine HCl (B1) purchased from Couto pharmacy manipulation laboratory (Oporto, Portugal); cyanocobalamin (B12, >98.9%) purchased from Fragon Iberian Laboratory (Oporto, Portugal); and biotin (H , ≥99%) purchased from Panreac AppliChem ITW Reagents (Darmstadt, Germany). Organisms were fed with microalgae suspension of *Raphidocelis subcapitata* at 3.0 x 10^5 cells mL^{-1} per day (neonates/juveniles) or 6.0 x 10^5 cells mL^{-1} per day (adults). Groups of 25 daphnids were isolated in 800 mL of medium and maintained as previously referred. Daphnids, less than 24h old originated from 3rd – 5th brood females from stock cultures were used for new cultures or experiments.

2.2.2.2 Preparation of *Raphidocelis subcapitata* microalgae culture medium

Microalgae were cultured in Woods Hole MBL medium, in a semicontinuous 4 L culture, with a 16:8 h light/dark cycle (at 20 °C ± 2 °C). Details about the culture maintenance of microalgae are described in Appendix A.

The medium was aerated for 30 minutes and inoculated with *R. subcapitata* collected from a previous culture and maintained for 7 days under uniform light (6000 lux for bottom illumination), with a cycle of 16:8h light/dark, at 20 °C ± 2 °C and aerated by an air pump system under continuous magnetic agitation. After 7 days of incubation, 250 mL of the microalgae culture is collected and used to start a new culture. The remainder of the microalgae culture was transferred to 50 mL falcon tubes and centrifuged at 3000 rpm for 10 minutes at 10 °C. The supernatant was discarded, and the microalgae pellet was reconstituted in 2 mL of MHRW medium. The algae suspension optical density (OD) was measured at a wavelength of 440 nm and adjusted to 0.6 and 0.8. The algae suspension was stored at 4 °C and used as food for the daphnia.

2.2.2.3 Experimental design

Each experimental unit consisted of a glass flask with 250 mL of MHRW medium with 20 daphnia from 3rd brood females (neonates less than 24h old) and 5 replicates per each concentration and control. Organisms were exposed to 0.1, 1, or 10 μg L^{-1} of BTL racemate or each enantiomer at 0.1 or 1 μg L^{-1} for 9 days, to follow the initial life stages and first reproductive events. The organisms were incubated in a bioterium at 20 °C ± 2 °C, with a cycle 16:8 h (light/dark). Every 48-hour intervals, the media was renewed, and organisms were fed with *R. subcapitata* ratio of 3.0 x 10^5 cells mL^{-1} (neonates/juveniles, until day 3) or 6.0 x 10^5 cells mL^{-1} (adults).

The ecotoxicity assay was designed to include endpoints as morphophysiological parameters (body size, heart area and size, and heart rate), reproductive parameters (number of daphniids with eggs, number of eggs per daphnia, and number of neonates) and biochemical parameters (determination of oxidative stress and enzymatic activity) (**Figure 5**).

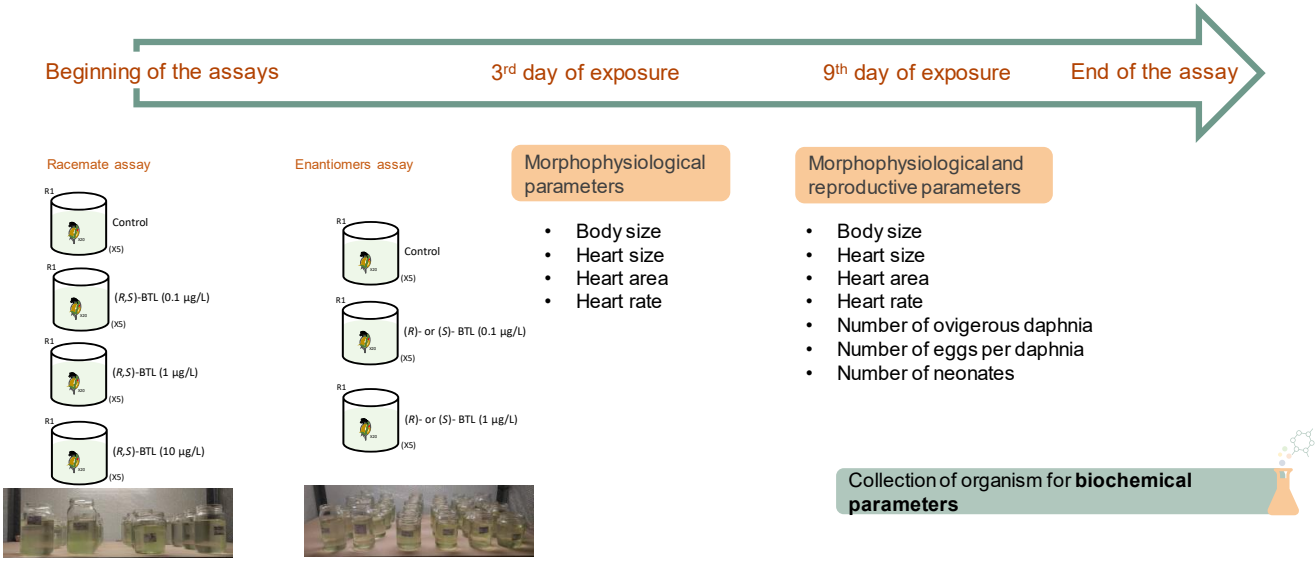

Figure 5| Schematic representation of the experimental design.

2.2.2.4 Morphophysiological parameters

The determination of morphophysiological parameters was performed after 3 and 9 days of exposure. Therefore, 3 random daphnids per replicate were collected. Each organism was transferred to a slide with 2/3 drops of culture medium and placed under a microscope coupled to a digital camera (Canon PowerShot G9, **Figure 6**). Each organism was photographed and video recorded for approximately 1 minute. After that, the organisms were placed back into the corresponding replica flask and the images and videos were analysed in specific software.

Figure 6| Optical microscope coupled to the digital camera for photograph and video recording to assess morphophysiological parameters using specific software.

For determining the body size and both heart area and size, the images were analysed using the software Digimizer® (Ver. 5.3.4).

For determination of heart rate (beat per minute, bpm), the video length was adjusted to 1 minute and to a speed of 0.25x of the original video, using the Microsoft Clipchamp® (Ver. 2.6.2.0). The number of heartbeats per daphnia was determined using the Handy Counter application.

2.2.2.5 Reproduction parameters

At the end of the assays (day 9), the number of eggs per daphnia was determined using the same images recorded for morphophysiological parameters. The number of ovigerous daphnia and the number of neonates were counted in each replicate.

2.2.2.6 Biochemical parameters

At the end of the assay all survival organisms were collected and stored in a 250 µL of phosphate buffer solution (PBS, pH 7.4, composed of 24.0 mg KH_2PO_4, 20 mg KCl, 144 mg Na_2HPO_4, and 800 mg NaCl in 100 mL of UPW). For biochemical determinations, daphnia tissues were homogenized via ultrasonication, centrifuged at 15000 g for 10 min at 4 °C, and the supernatant was immediately collected and stored at -20 °C until analysis.

All chemicals used for biochemical analysis were of highest purity available in the laboratory and can be found in Appendix B (Table 1A).

2.2.2.6.1 Protein quantification

The Bradford assay was used for protein determination, consisting in a colorimetric assay based on the addition of a dye that binds directly to the proteins (58).

The calibration curve of bovine serum albumin (BSA) was prepared using 9 standards and a blank according to Appendix B (Table 2A). Each sample was analysed in duplicate, using 2 µL of sample, 98 µL of PBS, and 100 µL of Bradford reagent and incubated for 5 min at room temperature. The samples (200 µL) were transferred into a 96-well microplate and the absorbance read at 585 nm using a microplate reader.

2.2.2.6.2 Acetylcholinesterase (AChE) activity

AChE activity was based on an improved *Ellman* method (59), in which thiocholine produced by the action of AChE forms a yellow colour with 5,5'-dithiobis (2- nitrobenzoic acid)

(DTNB). The assay uses DTNB to quantify the thiocholine produced from the hydrolysis of acetylthiocholine iodide (ATCI) by AChE (60).

All samples including the blanks were analysed in duplicate. For AChE activity assessment, all samples were prepared in 1.5 mL eppendorf tubes, by mixing 20 µL of sample, 120 µL of 0.5 µM DNTB, and 60 µL of 20 mM ATCI, and incubating for 5 min at room temperature. Then, 200 µL was transferred into a 96-well microplate, and the absorbance was read at 412 nm for 3 min at 25 °C. The AChE activity was calculated following the next formula:

$$\varepsilon = \frac{A}{c \times l}$$

where TNB extinction coefficient (ε_{412nm}) is 14,150 M^{-1} cm^{-1} and the optical path (l) was 0.8 cm. A denotes for absorbance of sample and c for molar concentration, being the results expressed as mmol TNB mg^{-1} protein.

2.2.2.6.3 Reactive oxygen species (ROS)

ROS determination uses dichlorofluorescein diacetate (H_2DCFDA), a fluorescent dye that measures hydroxyl, peroxyl and other reactive oxygen species. This substance can cross the cell membrane and is hydrolysed by intracellular esterase to form a non-fluorescent compound, dichlorofluorescin (DCFH). In the presence of ROS, DCFH is oxidized to 2,7-dichlorofluorescein (DCF) which is a strong green, fluorescent substance (61).

Standards solutions and blanks were prepared in 1.5 mL eppendorfs according to Appendix B (Table 3A). Samples for ROS analysis were prepared in duplicate in 1.5mL eppendorfs by adding 10 µL of sample and 110 µL of PBS, which were incubated at room temperature for 5 min, followed by addition of 8.3 µL of 21 mM H_2DCFDA. The fluorescence of DCF was measured at 25 °C with the excitation and emission wavelengths set at 485 nm and 528 nm, respectively. The results were expressed as nmol DCF mg^{-1} protein.

2.2.2.6.4 Catalase (CAT) activity

CAT activity was determined at 25 °C by a spectrophotometric method, which wavelength was set from 405 to 415 nm. The standards and samples for CAT analysis were incubated in H_2O_2/SPB at 37 °C for 1 min. The enzymatic reaction was stopped by the addition of ammonium molybdate tetrahydrate (AMT) and incubate for 5 min at room temperature. The residual H_2O_2 reacts with AMT to generate a yellowish complex (molybdate/H_2O_2 complex). CAT

activity is directly proportional to the rate of dissociation of H_2O_2, and the result was expressed as U CAT mg^{-1} protein (62). The CAT standards curves were prepared according to Appendix B (Table 4A). Samples for measurement of CAT activity were prepared in duplicate in a 96-well microplate and the absorbance was read at λ 415 nm.

2.2.2.6.5 Lipid peroxidation assay

The malondialdehyde (MDA) – thiobarbituric acid (TBA) is a pink complex formed by reacting thiobarbituric acid reactive substances (TBARS) with TBA at high temperature (for example, 2 hours at 60 °C) and under acid conditions, the concentration of which is linearly related to the concentration of TBARS in the sample. The results are expressed as µM MDA mg^{-1} protein (63).

The standards were prepared according to the Appendix B (Table 5A). In 1.5 eppendorfs, a 10 µL sample was added to 70 µL of UPW, 50 µL of 50 mM PBS, 10 µL of 1 mM BHT, 75 µL of 1.3%TBA/0.3%NaOH and 50 µL of 50% TCA, and incubated for 2 hours at 60 °C, then cooled for 15 min on ice and then, 10 µL of sodium Dodecyl Sulphate (SDS) pre-heated was added. The assay was performed in duplicate and 200 µL of each standard, blank, or sample were transferred to a 96-well microplate and the absorbance read at 530 nm.

2.3. Ecotoxicity assays after advanced oxidation technologies

Wastewater samples were collected from a WWTP located in Northern Portugal. Triplicate UV, O_3, and UV/O_3 experiments were performed according to Kumar et al. (64), in a reactor (1L) containing 800 mL of WWTP sample spiked with 10 µg L^{-1} of BTL, under magnetic stirring at 450 rpm. All the treatment runs were performed during 10 min. In the ozonation assays, the ozone was produced from pure oxygen and monitored in a BMT 802X ozone generator. An inlet ozone concentration of 50 g Nm^{-3} was set with a constant flow rate of 150 Ncm^3 min^{-1} that was introduced through a ceramic diffuser into the reactor, operating at constant pressure (1 atm) and room temperature. For UV experiments, oxygen was bubbled instead of ozone to keep similar hydrodynamic conditions. A low-pressure Hg-vapour lamp (Heraeus mod. TNN 15/32, λ_{max} =253.7 nm, radiant flux of 3 W) was inserted axially in the reactor and fixed in a quartz immersion tube with refrigeration.

After the experiments, samples were collected and stored at –20 °C for acute immobilization assay with *D. magna* and determination the remove rate.

Acute immobilization assay with *Daphnia magna* in wastewaters

The acute toxicity assay with *D. magna* was performed following the procedures described by MicroBioTests Kit and by international standards guidelines (ISO 6341, and OECD 202). The KIT consists of dormant eggs of the crustacean daphnia magna (ephippia) coated with a chitinous capsule (ephippium), which can be kept for long periods without losing their viability, in the fridge at 4 °C (± 2 °C).

The standard freshwater was prepared using the following chemicals per litter: 67.75 mg of sodium bicarbonate ($NaHCO_3$, ≥99.7%) obtained from Sigma–Aldrich (Missouri, USA); 294 mg of calcium sulphate dihydrate ($CaSO_4.2H_2O$, >99%) and 123.25 mg magnesium sulphate heptahydrate ($MgSO_4.7H_2O$, >99%) purchased from Merck (Darmstadt, Germany); and 5.75 mg of potassium chloride (KCl, >99%) acquired from Panreac (Barcelona, Spain). Before using, the standard freshwater was magnetically stirred and aerated for 30 minutes with an aeration pump. 72h before starting the toxicity assay, the hatching of ephippia was performed by incubating the eggs in a Petri dish with 15 mL standard freshwater at 21 °C under continuous illumination of 6000 lux. To perform a complete test, 140 neonates younger than 24 hours were fed with a suspension of spirulina microalgae 2 h before the beginning of the toxicity assays. Each experiment consisted of a serial dilution of the effluent with the respective organism's standard media at: 100% (undiluted), 75%, 50%, 25%, 12.5% and 6.25% of effluent and a control with standard freshwater. Each dilution series or control was performed with 4 replicates each with 5 neonates corresponding to a total of 20 neonates for each test. A reference test (positive control) was performed with $K_2Cr_2O_7$ to assess the viability, the correct execution, and the sensitivity of the test organisms at five concentrations (0.32, 0.56, 1, 1.8, and 3.2 mg mL^{-1}) to the 24 and 48 hours EC_{50} was calculated.

The toxicity test was performance in multiwell plates placed in bioterium, in the dark at 21 °C. The number of dead or immobilized neonates was recorded in each well after 24 hours and 48 hours of exposure. The test was considered valid when the number of dead plus immobile organisms did not exceed 10% in the controls.

2.4. Determination of BTL

To determine the removal rates of each BTL enantiomer in the effluent samples and for the quantification of BTL racemate and its enantiomers in culture media from the sub-chronic assay, a previously analytical enantioselective gas chromatography-mass spectrometry (GC-MS) method was adapted (36). Briefly, samples of the culture medium (250 mL) or wastewater effluent (250 mL) were filtered and acidified and then pre-concentrated by solid-phase extraction (SPE) with OASIS MCX (150 mg, 6cc) cartridges. The SPE extracts were dried, reconstituted in 200 µL UPW and 200 µL NaOH (1M) and derivatized using the chiral reagent (*R*)-MTPA-Cl, according to the procedure described in our previous work (36). Chromatographic analysis was performed using a Shimadzu-2010 Plus gas chromatography system equipped with an electronically controlled split/splitless Shimadzu AOC-20i autoinjector and QP2010 single quadrupole mass spectrometer and a SH-Rxi-5ms capillary column 30 m x 0.25 mm x 0.25 µm (Crossbond® 5% diphenyl 95% dimethyl polysiloxane from Shimadzu, USA). Injector temperature was set at 280 °C. Column oven temperatures were programmed using several ramps: from140 °C (initial equilibrium time of 50 s) to 215 °C at 11 °C min^{-1} and maintained for 5 min, followed a new ramp at 10 °C min^{-1} up to 285 °C which was maintained for 5 min, with a total run time of 24.32 min. Sample injection (1 µL) was programmed in splitless mode and analysed in selection ion storage (SIS) mode in accordance with the compound's fragments. A matrix-matched calibration curve was constructed from 20 to 320 ng L^{-1} for analyses of culture media samples.

2.5. Statistical analysis

General linear models were used to analyse data from the subchronic assay with the racemate (one-way Anova) and enantiomers (two-way Anova). The normality and homoscedasticity were evaluated and Dunnett contrasts was used to assess significant effects. Reproductive endpoints and mortality were analysed with generalised linear models (negative binomial GLM) followed by Dunnett contrasts. All analyses were performed with jamovi v. 2.3.21 (The jamovi project 2021) with a significance level of 0.05. Plots were built with R v. 4.3.1 (R Core Team 2022), using the graphical interface RStudio v. 2023.06.2+561 (RStudio Team 2022); R packages ggplot2 (Wickham 2016), cowplot (Wilke 2020), and envalysis (Steinmetz 2021). R

studio software was used for determination of the EC_{50} values and evaluation of the acute toxicity assays performed with samples treated by the different AOT.

3. RESULTS AND DISCUSSION

3.1. Semipreparative enantioseparation of BTL

3.1.1. Injection volume optimization

The enantioseparation of (*R,S*)-BTL was performed using a semipreparative 3,5-dimethylphenylcarbamate amylose column based on the methodology published by Spálovská et al. (14). Different proportions of a mixture of 0.1 % of DEA in *n*-Hex and 0.1 % of DEA in EtOH (90:10, 80:20 and 70:30, *v/v*) and different detection wavelength (254 nm and 230 nm) were tested to achieve the best chromatographic conditions (low retention time and good resolution). Enantioseparation was achieved with all conditions, but the increase of EtOH decreased the retention times while enantioresolution was maintained. Therefore, the optimized conditions were established with the mobile phase consisting of 70:30 (*v/v*) of 0.1 % of DEA in *n*-Hex and 0.1 % of DEA in EtOH at a flow rate of 1.5 mL min^{-1} and monitored at 230 nm (**Figure 7**).

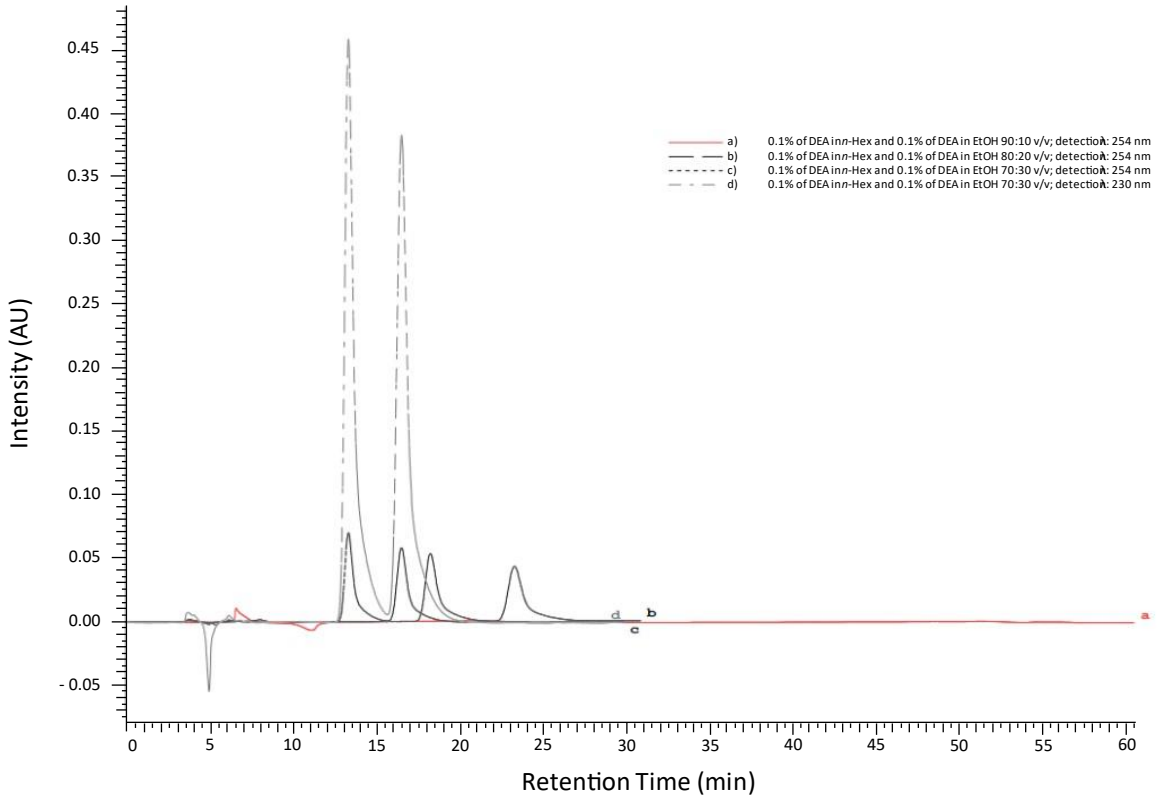

Figure 7| Chromatogram showing the separation of BTL enantiomers in a 1 mg mL^{-1} ethanolic solution by the semipreparative 3,5-dimethylphenylcarbamate amylose column coated with APS-Nucleosil by LC-DAD under normal elution mode. Mobile phase: 0.1 % of DEA in *n*-Hex and 0.1 % of DEA in EtOH; flow rate: 1.5 mL min^{-1}; injection volume: 20 µL. Legend: line a) 0.1 % of DEA in *n*-Hex and 0.1 % of DEA in EtOH, 90:10 (v/v), detection λ: 254 nm; line b) of 0.1 % of DEA in *n*-Hex and 0.1 % of DEA in EtOH, 80:10 (v/v), detection λ: 254 nm; line c) 0.1 % of DEA in *n*-Hex and 0.1% of DEA in EtOH ,70:30 (v/v), detection λ: 254 nm; line d) 0.1% of DEA in *n*-Hex and 0.1% of DEA in EtOH, 70:30 (v/v), detection λ: 230 nm.

The injection volume for the enantiomeric separation of (*R,S*)-BTL was optimized, using a stock solution of 4 mg mL^{-1} BTL in EtOH. Different injection volumes were tested: 20, 50, 70, 90, 100 µL. The strategy consisted of studying different injection volumes considering the column overload capacity, to make the process profitable as possible in terms of purity, yield and number of injections, while maintaining a good resolution. **Figure 8** shows the chromatograms obtained after injection of 20, 50, 70, 90, or 100 µL. The injection volume of 50 µL was selected, considering that higher injection volumes did not increase of the area of the analytes.

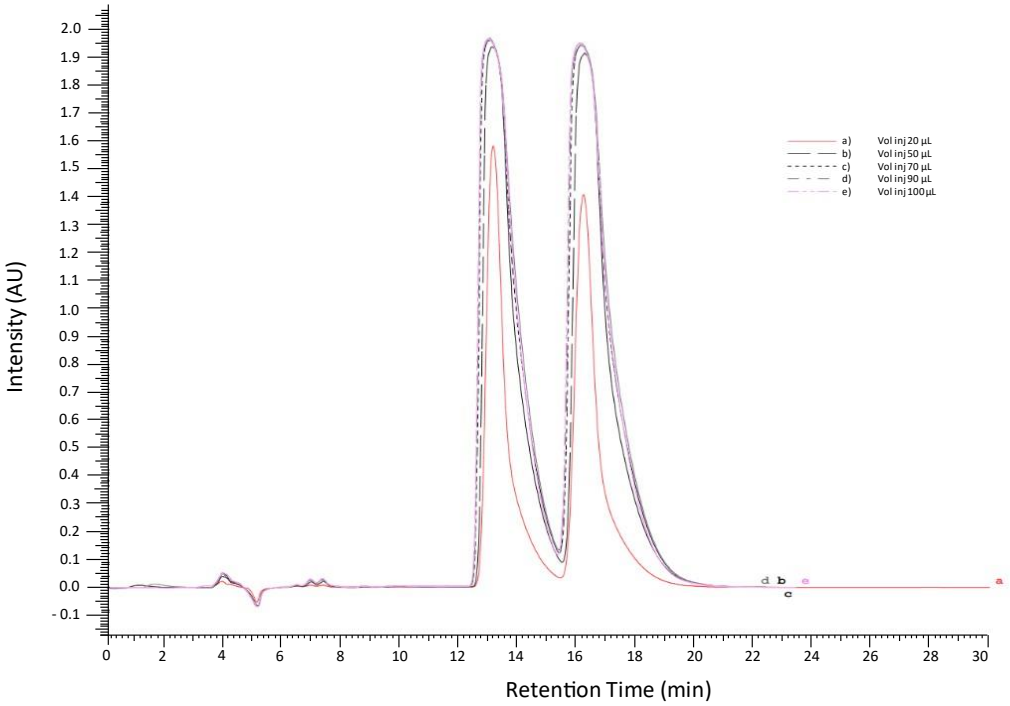

Figure 8| Chromatogram showing the enantioseparation of (*R,S*)-BTL enantiomers of a 4 mg mL^{-1} ethanolic solution in the semipreparative 3,5-dimethylphenylcarbamate amylose column coated with APS-Nucleosil by LC-DAD under normal elution mode. Mobile phase: 0.1 % of DEA in *n*-Hex and 0.1 % of DEA in EtOH, 70:30 v/v; flow rate: 1.5 mL min^{-1}; detection λ: 230 nm. Injection volume: line a) 20 µL; line b) 50 µL; line c) 70 µL; line d) 90 µL; and line e) 100 µL.

3.1.2. Enantioseparation

The elution order of each enantiomer was previously determined under the same conditions by Spálovská et al. (33), which described a longer retention time of the (*R*)-BTL enantiomer over the (*S*)-BTL enantiomer (**Figure 9**). Thus, the (*S*)-BTL enantiomer was the first eluted enantiomer and collected from 12.5 to 14.8 minutes and (*R*)-BTL was collected from 16.2 to 22 minutes. Since baseline separation was not achieved, an intermediate fraction was collected (from 14.8 to 16.2 minutes), to avoid the contamination of previous collected fractions and assure high purity and yield, and then concentrated and reinjected for further enantioseparation.

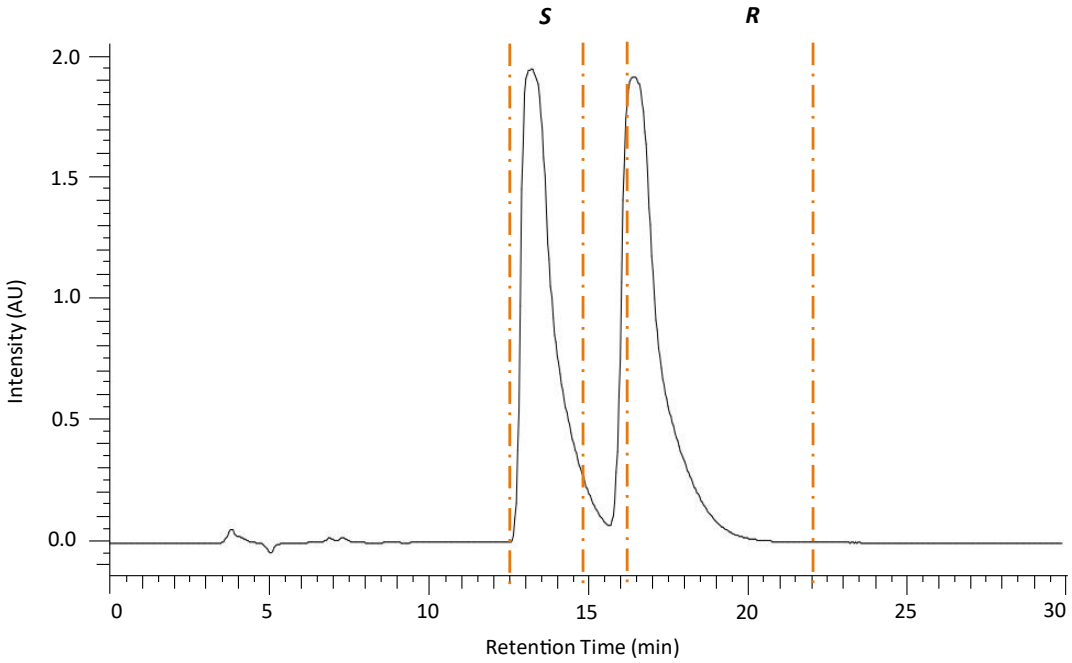

Figure 9| Chromatogram of the enantioseparation of BTL enantiomers of a 4 mg mL^{-1} ethanolic solution in semipreparative 3,5-dimethylphenylcarbamate amylose column coated with APS-Nucleosil by LC-DAD under normal elution mode. Mobile phase: 0.1 % of DEA in *n*-Hex and 0.1% of DEA in EtOH 70:30 v/v; flow rate: 1.5 mL min^{-1}; detection λ: 230 nm; injection volume: 50 µL. The orange dotted line corresponds to the cut-off time, that is, the time when each fraction of enantiomers was collected in the respective flask. The fraction corresponding to (*S*)-BTL was collected from 12.5 min to 14.8 min, the intermediate fraction was collected from 14.8 min to 16.2 min and the fraction corresponding to (*R*)-BTL was collected from 16.2 min to 22 min.

3.1.3. Enantiomeric purity analysis

Enantiomeric purity was evaluated using the semipreparative 3,5-dimethylphenylcarbamate amylose column and the same conditions used for BTL separation. The e.r. of the first fraction corresponding to (*S*)-BTL was higher than 99 % (**Figure 10**). The second fraction corresponding to the (*R*)-BTL was obtained with an e.r. of approximately 97 % (**Figure 10**).

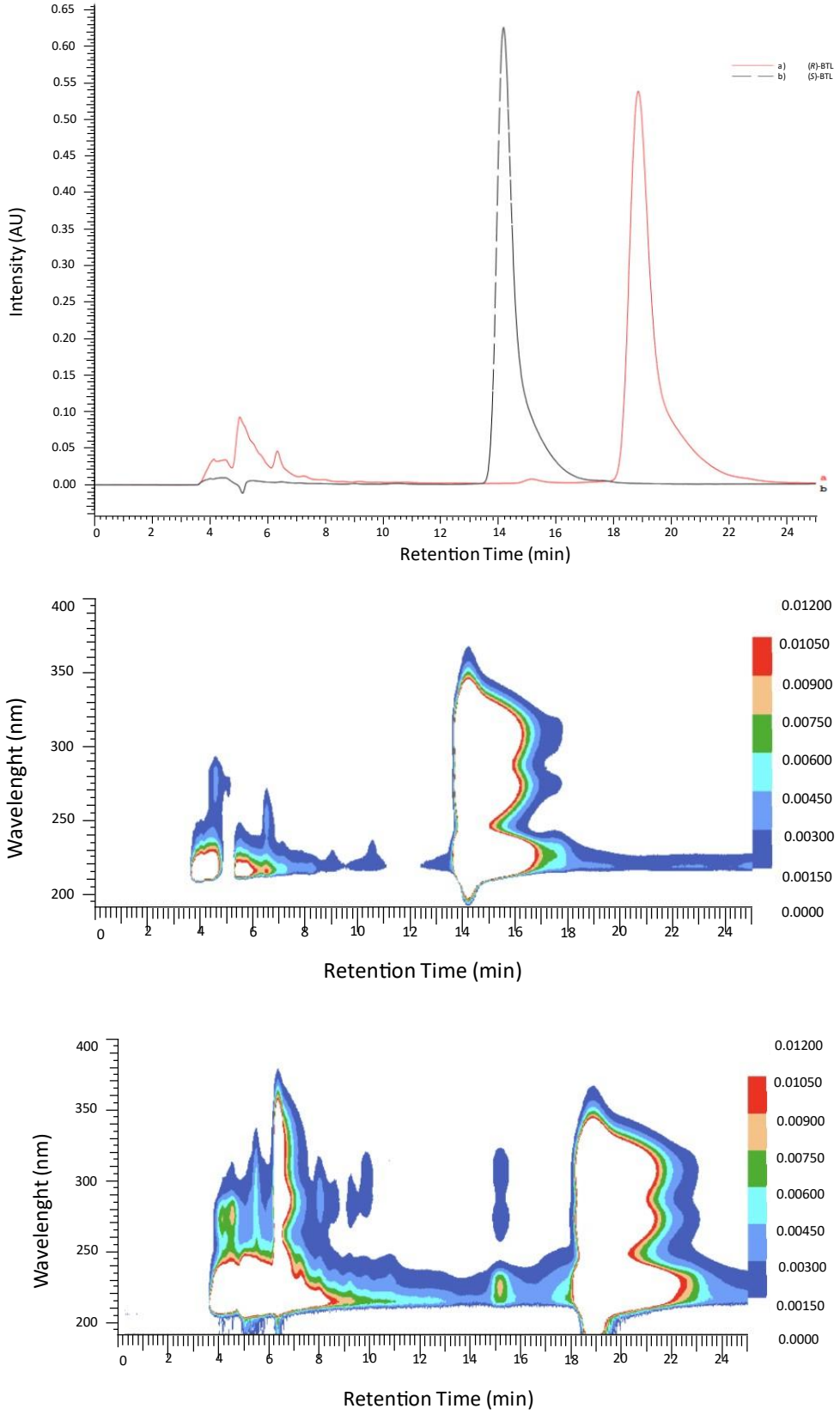

Figure 10| Chromatogram and absorption spectra (absorbance scale: 0.240) showing of the separated fractions of (*S*)-BTL and (*R*)-BTL analysed in the semipreparative 3,5-dimethylphenylcarbamate amylose column coated with APS-Nucleosil by LC-DAD at normal elution mode. Mobile phase: of 0.1 % of DEA in *n*-Hex and 0.1 % of DEA in EtOH, 70:30 v/v; flow rate: 1.5 mL min^{-1}; detection λ: 230 nm; injection volume: 50 µL. Chromatogram representing (*S*)-BTL fraction in dash black line (b) and the corresponding absorption spectra; and (*R*)-BTL fraction in solid red line (a) (absorption spectra below).

3.1.4. Quantification/recovery of enantiomers

For determination of the recovery of the enantiomers from the semi-preparative enantioseparation, an analytical method was performed using a Lux® 3µm i-Amylose-1 (tris-3,5-dimethylphenylcarbamate; 3 µm, 150 x 4.6 mm) column and the mobile phase was tested at different proportions (70:30, 68:32, and 65:35, *v/v*) and different flow rates. The optimized conditions were established with the mobile phase consisting of 0.1 % of DEA in *n*-Hex and 0.1 % of DEA in EtOH, with a ratio of 65:35 (*v/v*), a flow rate of 0.7 mL min^{-1}, a detection λ of 230 nm, and an injection volume of 10 µL.

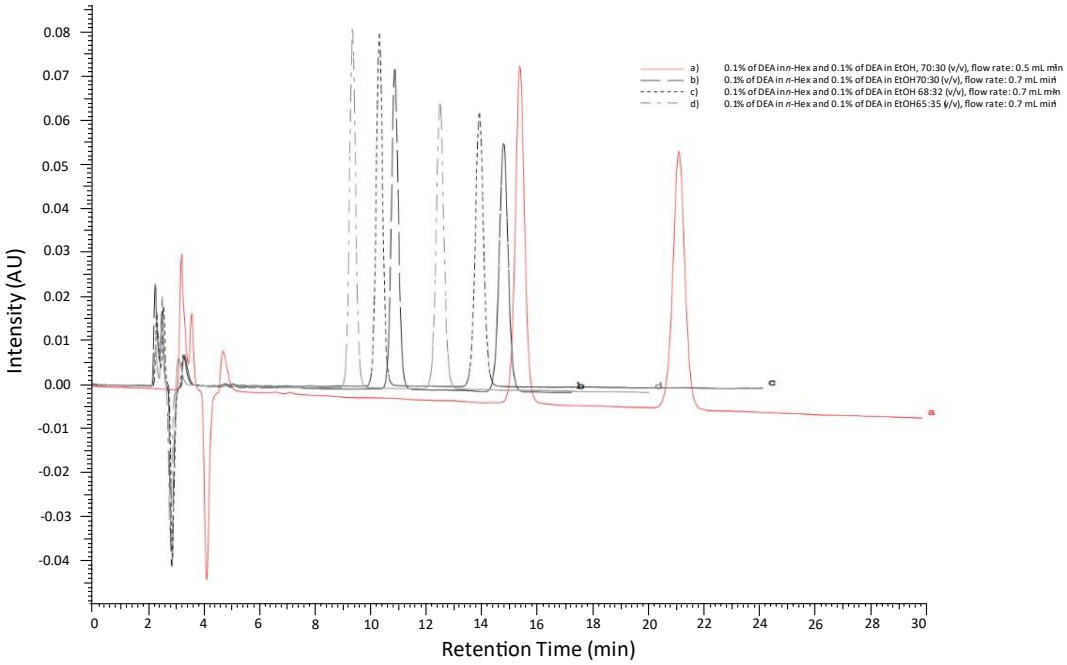

Figure 11| Chromatogram showing the separation of BTL enantiomers in a 100 µg mL^{-1} ethanolic solution by the analytical column (Lux® 3µ i-Amylose-1 column) under normal elution mode. Mobile phase: 0.1 % of DEA in *n*-Hex and 0.1 % of DEA in EtOH; detection λ: 230 nm; injection volume: 10 µL. Legend: line a) 0.1% of DEA in *n*-Hex and 0.1% of DEA in EtOH, 70:30 (*v/v*), flow rate: 0.5 mL min^{-1}; line b) of 0.1 % of DEA in *n*-Hex and 0.1 % of DEA in EtOH ,70:30 (*v/v*), flow rate: 0.7 mL min^{-1}; line c) of 0.1 % of DEA in *n*-Hex and 0.1 % of DEA in EtOH ,68:32 (*v/v*), flow rate: 0.7 mL min^{-1}; line d) 0.1 % of DEA in *n*-Hex and 0.1 % of DEA in EtOH, 65:35 (*v/v*), flow rate: 0.7 mL min^{-1}.

The analytical method was validated to further quantify the enantiomers and to determine the percentage of recovery obtained in the semipreparative procedure. The calibration curves were found to be linear of 2.5 to 75 µg mL^{-1} with R^2 greater than 0.9992.

Table 2| Results of BTL enantiomers recovery obtained by semipreparative chromatography.

Enantiomer	Range (μg mL^{-1})	Liner equation	Correlation level (R^2)	Recovery concentration (μg mL^{-1})	Recovery (%)
(*S*)-BTL	2.5 to 75	y = 13884x - 10319	0.9992	308.9	8.8
(*R*)-BTL	2.5 to 75	y = 13854x - 9943	0.9993	338.8	9.7

If a 100% recovery was obtained, it would be expected to obtain 3.5 mg of each enantiomer. Calculating and multiplying by the dilution factor, an (*S*)-enantiomer concentration of 308.9 μg mL^{-1} and 338.8 μg mL^{-1} of (*R*)-enantiomer was obtained. The recovery percentage was 8.8 % of (*S*)-BTL and 9.7 % (*R*)-BTL. During the evaporation process, racemization occurs that may justify the loss of a large amount of the enantiomers and the low recovery.

3.2. Subchronic assay

The assay was carried out during 9 days, starting with daphnia with less than 24 h. Concentrations were selected in order to include environmental reported levels, 0.1 μg L^{-1} and two higher sublethal concentrations, 1 μg L^{-1} and 10 μg L^{-1} for (*R,S*)-BTL and 0.1 and 1.0 μg L^{-1} for each enantiomer. Different parameters namely morphophysiological, reproductive and biochemical, were evaluated considering different daphnia life (juveniles and adults) stages of development.

Furthermore, an enantioselective GC-MS method was used for the quantification of BTL in test culture media. Results showed that concentrations of both racemate and enantiomers in the culture media were within nominal expected concentrations used in the sub-chronic assay (0.1; 1 and 10 μg L^{-1}).

3.2.1. Morphophysiological parameters

Morphological changes were observed in both juveniles and adults exposed to the racemate or each of the isolated enantiomers. A significant increase in body size was observed at the highest concentration 10 μg L^{-1} in juveniles (day 3) exposed to the racemate but no changes were found after 9 days of exposure in adults (**Figure 12, Table 3**). Regarding enantiomers, an enantioselective effect was observed with an increase in body size in the organisms exposed to

the (*R*)-enantiomer at both concentrations, whereas no changes were found in those exposed to (*S*)-enantiomer. No significantly changes were found in adults, as occurred for the racemate (**Figure 12**, **Table 3**).

A significant increase in heart size was noted in the organisms exposed to all concentrations of the racemate in juveniles, without any changes in their heart area. On the contrary, a significant decrease in the heart size and area was found in the juveniles exposed to the (*S*)-enantiomer at 1 µg L^{-1}. Interestingly, there was no impact on both heart size and area of those exposed to (*R*)-BTL. The same lack of effects was found in both heart parameters in the adults (day 9) exposed to either the racemate or each pure enantiomer. In turn, a significant increase in the heart rate were registered after exposure to the racemate in juveniles (0.1 µg L^{-1}) and adults (1 and 10 µg L^{-1}). No changes were found in juveniles exposed to the enantiomers whereas an increase was observed in the adults exposed to (*R*)- and (*S*)-enantiomer at the highest concentration.

In general, these morphophysiological changes agree with our previous study performed with the racemate, which reports changes in the body size, heart size and area, and heart rate of daphnia exposed to BTL at 10 µg L^{-1}. Nevertheless, lower concentrations and enantioselective effects were not investigated in that study (16). In the present study, the enantioselective effects observed for body size, heart size and area demonstrate that both (*R*) and (*S*)- enantiomers can negatively interfere with critical life stages of daphnia. Juveniles seems to be more susceptible than adults, but it is worth to mention that most changes for both racemate and enantiomers were found at concentrations higher than those usually reported in wastewaters. Other studies conducted by our group with MDMA and AMP showed that both substances can interfere with morphophysiological parameters of daphnia, with differences found for some effects between racemate and enantiomers. For instance, a significant increase in body size was observed in adult daphnia exposed to AMP racemate, whereas a significant decrease was found in those exposed to (*S*)-AMP (17). In another study, the exposure to MDMA racemate caused a significant decrease in body size, heart area and size in juveniles exposed to a concentration of 1 µg L^{-1}, and a significant increase in body size of adults was found at a concentration of 10 µgL^{-1}, whereas (*S*)-MDMA caused a significant decrease of body size at a concentration of 1 µg L^{-1} (5). The cellular and molecular mechanisms underlying the changes induced were not explored and were beyond the scope of the referred study; however, changes in food intake and metabolism have been shown to interfere with body growth and reproduction (65–67).

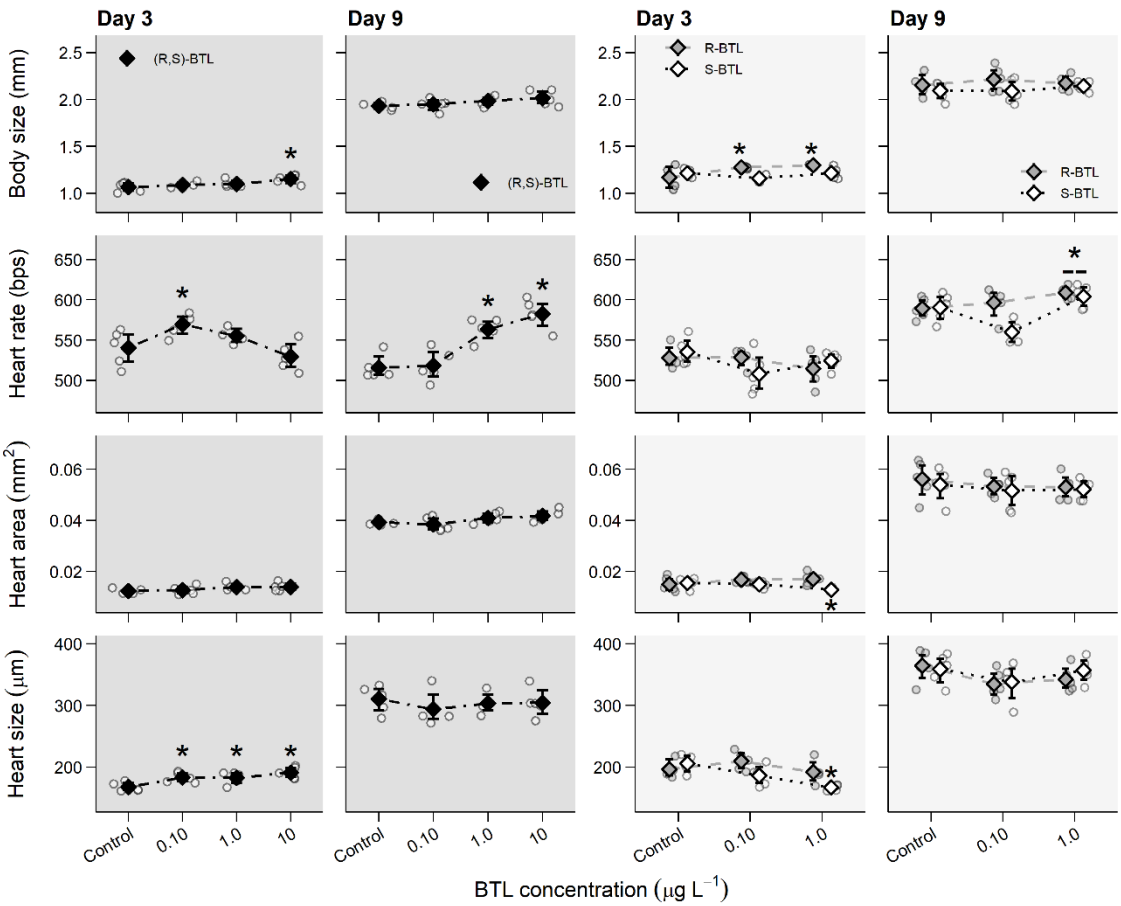

Figure 12| Morphophysiological effects of (*R,S*)-BTL (in the left panel) and the pure enantiomers (in the right panel) determined at day 3 and day 9 in *D. magna*. Note: Asterisks (*) represent significant differences relatively to the control.

Table 3| Statistical analysis of morphophysiological effects of BTL racemate and pure enantiomers on *D. magna*, determined at day 3 and 9. Significant effects ($p < 0.05$) in bold.

Variable	Source of variation	Day 3			Day 9		
		df	F	*p*	df	F	*p*
Body size (µm)	(*R*,*S*)	3, 16	3.41	**0.043**	3, 15	1.66	0.218
	Enantiomer	1, 22	5.32	**0.031**	1, 23	3.47	0.075
	Concentration	2, 22	2.90	**0.076**	2, 23	0.275	0.762
	Interaction	2, 22	4.54	**0.022**	2, 23	0.544	0.588
Heart size (µm)	(*R*,*S*)	3, 16	6.06	**0.006**	3, 16	0.467	0.709
	Enantiomer	1, 22	5.29	**0.031**	1, 24	0.218	0.645
	Concentration	2, 22	5.96	**0.009**	2, 24	2.84	0.078
	Interaction	2, 22	3.67	**0.042**	2, 24	0.444	0.646
Heart area (μm^2)	(*R*,*S*)	3, 16	1.68	0.211	3, 16	2.44	0.102
	Enantiomer	1, 24	7.04	**0.014**	1, 24	0.524	0.476
	Concentration	2, 24	0.727	**0.494**	2, 24	0.634	0.539
	Interaction	2, 24	3.90	**0.034**	2, 24	0.0290	0.971
Heart rate (bpm)	(*R*,*S*)	3, 15	5.32	**0.011**	3, 16	19.8	**< 0.001**
	Enantiomer	1, 24	0.0257	0.874	1, 24	1.41	**0.025**
	Concentration	2, 24	1.8618	0.177	2, 24	5.10	**0.002**
	Interaction	2, 24	2.3672	0.115	2, 24	2.01	**0.025**

d.f. = degrees of freedom; F: value of statistical test; *p*: probability (statistical differences ≤ 0.05).

3.2.2. Reproduction parameters

No significant changes were observed in the number of ovigerous daphnia and number of eggs per daphnia for both racemate and each enantiomer (**Figure 13, Table 4**). No neonates were observed after 9 days of exposure of the organisms to the racemate. Similar results were obtained in a previous study (16). However, (*R*)-BTL caused a significant increase in the number of neonates at the concentration of 0.1 µg L^{-1}. No impact in the number of neonates was observed in the organisms exposed to (*S*)-BTL. Although only first reproductive events were considered which may hide possible effects on reproductive parameters, these findings are of high importance as BTL is expected to occur at varying enantiomeric fractions. Nevertheless, the 21-day reproduction assay should be performed to clarify these results and to investigate possible enantioselective effects. In fact, various studies have been shown negative effects of PAS in daphnia reproduction. For instance, a 21-day exposure to METH showed a significant increase in

the total number of neonates (24), whereas exposure to cocaine caused a significant decrease (68). In an enantioselective study, daphnia exposed to AMP racemate caused an increase in the number of neonates at concentration of 10 µg L^{-1}, whereas (*S*)-AMP caused a significant decrease the number of eggs per daphnia (17).

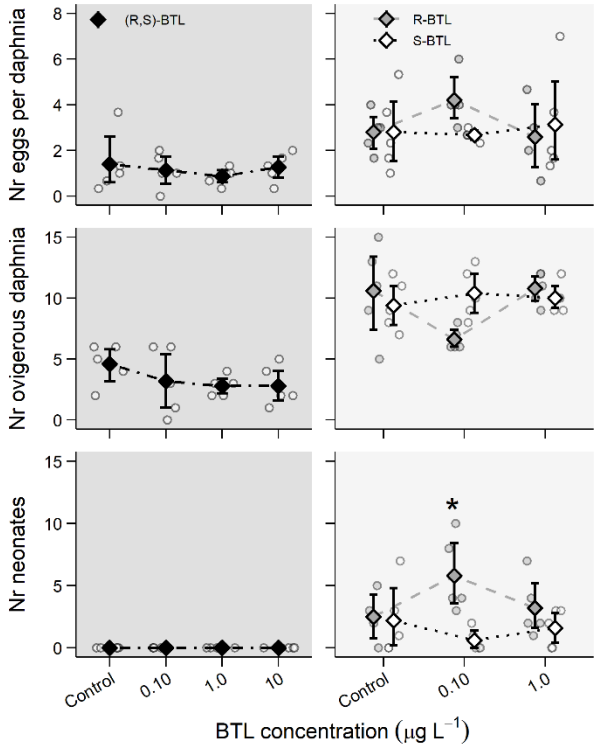

Figure 13| Effects of BTL racemate (in the left panel) and enantiomers (in the right panel) in *D.magna* reproduction. Note: Asterisks (*) represent significant differences relatively to the control.

Table 4| Statistical analysis of effects of BTL racemate and enantiomers on *D. magna* reproduction Significant effects ($p < 0.05$) in bold.

Variable	Source of variation	df	X^2	*p*
Nº of ovigerous	(*R,S*)	3, 16	3.08	0.380
	Enantiomer	1, 24	0.519	0.471
	Concentration	2, 24	2.628	0.269
	Interaction	2, 24	4.517	0.105
Nº of eggs per daphnia	(*R,S*)	3, 16	0.698	0.876
	Enantiomer	1, 24	1.58	0.451
	Concentration	2, 24	1.59	0.209
	Interaction	2, 24	1.20	0.548
Nº of neonates	(*R,S*)			
	Enantiomer	1, 23	6.94	**0.001**
	Concentration	2, 23	1.30	0.854
	Interaction	2, 23	5.19	**0.028**

d.f. = degrees of freedom; X^2: value of statistical test; *p*: probability (statistical differences ≤ 0.05).

3.2.3. Mortality

A significant increase in mortality was found in organisms exposed to the racemate at the highest concentration, 10 µg L^{-1} (**Figure 14, Table 5**).

No significant mortality was observed for enantiomers, which is similar to previous studies performed with other PAS such as MDMA (5)and AMP (17).

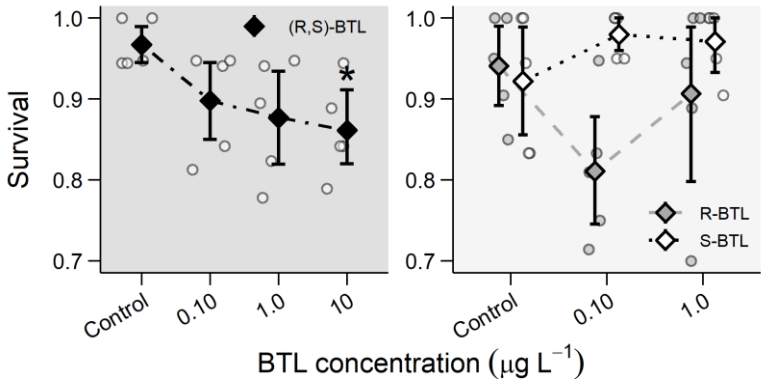

Figure 14| Effects of BTL racemate (in the left panel) and enantiomers (in the right panel) on *D. magna* mortality. Note: Asterisks (*) represent significant differences relatively to the control.

Table 5| Statistical analysis of effects of BTL racemate and enantiomers on *D. magna* mortality. Significant effects ($p < 0.05$) in bold. No *p* value for enantiomer and interaction due to statistical artefact.

Variable	Source of variation	df	X^2	*p*
Mortality	(*R*,*S*)	3	8.06	**0.0448**
	Enantiomer	1	10.5	-
	Concentration	1	2.53	0.28
	Interaction	2	9.57	-

d.f. = degrees of freedom; X2: value of statistical test; *p*: probability (statistical differences ≤ 0.05).

3.2.4. Biochemical parameters

Exposure to BTL racemate caused a significant decrease of AChE activity at all tested concentrations (0.1, 1 and 10 µg L^{-1}). On the contrary, a significant increase in AChE activity was observed in the organisms exposed to (*R*)-BTL at 0.1 µg L^{-1} but no changes were found for (*S*)-BTL (**Figure 15, Table 6**). Regarding CAT, a significant decrease was observed when the racemate was spiked at 0.1 µg L^{-1} but no changes were found at superior concentrations nor for ROS. TBARS levels were not impacted by the racemate at the two lower concentrations tested, but a significant increase of TBARS was found, in the exposure to 10 µg L^{-1}. Regarding the exposure to the isolated enantiomers, no changes were found in the CAT activity nor TBARS

levels for both enantiomers. However, a significant decrease in ROS was found for the organisms exposed to (*S*)-BTL at 1 μg L^{-1}.

Some studies showed that exposure to PAS can induce oxidative stress and affect the activity of several enzymes in non-target organisms. Parolini et al. (46) showed that benzoylecgonine induced oxidative stress and lead to the inhibition of AChE activity on *D. magna* after 48 h of exposure at environmental concentrations (0.5 and 1 μg L^{-1}). A similar increase in ROS and oxidative stress was observed following exposure to citalopram and mirtazapine (69). AChE is an enzyme that plays a crucial role in the regulation of the central nervous system and is an important biomarker in the central nervous system function (46,69). A reduction in the activity of AChE in aquatic organisms exposed to environmental pollutants has been attributed to oxidative stress (69). Nevertheless, in this study, no changes in ROS levels were observed despite the significant decrease of AChE activity resulting from the exposure to the racemate. Only the exposure to 10 μg L^{-1} led to an increased level of TBARS. An increased AChE was observed for (*R*)-BTL at 0.1 μg L^{-1} but no changes were found for ROS or TBARS at this exposure level. Nevertheless, an increase in mortality was found in the organisms exposed to (*R*)-BTL that can be related to the increased AChE activity. Other studies performed with PAS have also been shown a decreased AChE activity. For instance, reduced AChE activity was found for exposure of organisms to MDMA at concentration 10 μg L^{-1} (5), and for exposure to three concentrations (0.1, 1 and 10 μg L^{-1}) of AMP (17).

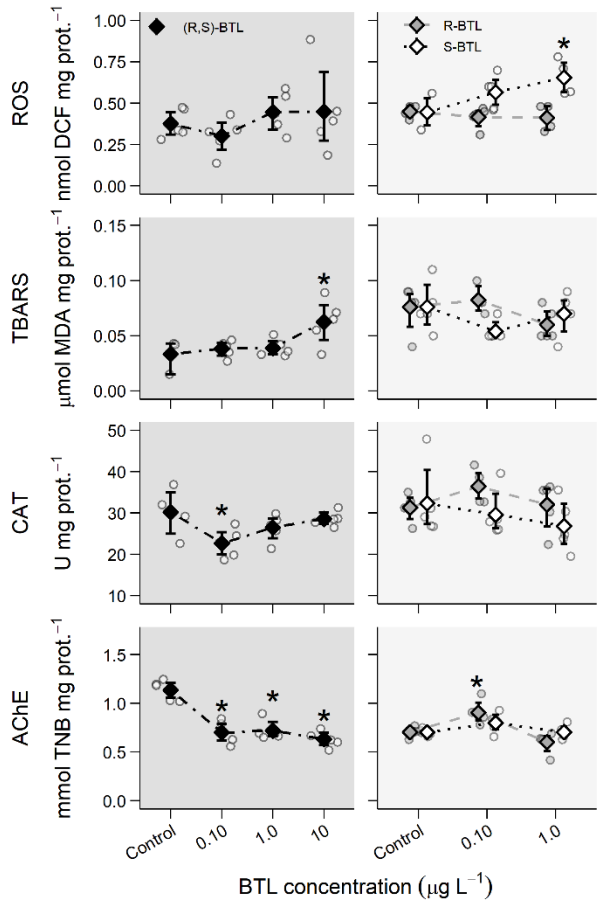

Figure 15| Effects of BTL racemate (in the left panel) and enantiomers (in the right panel), on biochemical parameters. Note: Asterisks (*) represent significant differences relatively to the control.

Table 6| Statistical analysis of biochemical effects of BTL racemate and enantiomers on *D. magna*. Significant effects ($p < 0.05$) in bold.

Variable	Source of variation	df	F	*p*
CAT (U CAT mg^{-1} protein)	(*R*,*S*)	3, 15	3.53	**0.041**
	Enantiomer	1, 24	2.915	0.101
	Concentration	2, 24	0.964	0.3340.396
	Interaction	2, 24	1.234	0.306
AChE (mmol TNB mg $^{-1}$ protein)	(*R*,*S*)	3, 16	25.7	**< 0.001**
	Enantiomer	1, 24	7.67e^{-4}	0.978
	Concentration	2, 24	14.64	**< 0.001**
	Interaction	2, 24	3.92	**0.034**
TBARS (µM MDA mg^{-1} protein)	(*R*,*S*)	3, 14	4.02	**0.030**
	Enantiomer	1, 23	0.840	0.369
	Concentration	2, 23	0.343	0.281
	Interaction	2, 23	2.861	0.078
ROS (nmol DCF mg^{-1} protein)	(*R*,*S*)	3, 16	0.939	0.445
	Enantiomer	1, 24	11.84	**0.002**
	Concentration	2, 24	0.003	0.997
	Interaction	2, 24	3.532	**0.045**

d.f. = degrees of freedom; F: value of statistical test; *p*: probability (statistical differences ≤ 0.05).

PAS are pervasive environmental contaminants posing risks to wildlife and humans. Information about BTL toxicity is scarce and there is no information about possible enantioselective effects on the development of daphniids. A summary of the results of the ecotoxicity study can be found in **Figure 16**. Our study confirms the ecotoxicity of BTL and the enantioselectivity of some effects (body size, heart size and area, number of neonates and enzymatic activity). Either the enantiomeric mixture and the enantiopure molecules may affect all parameters (morphophysiological, reproductive and biochemical) in *D. magna*, although the racemate and the (*R*)-enantiomer seems to be more toxic than (*S*)-BTL and early development stages (juveniles) are more susceptible than adults in the case of morphophysiological endpoints. The results also showed the increase of mortality in the organisms exposed to racemate.. However, most changes were observed at levels higher than those usually reported in the aquatic ecosystems (1 and 10 µg L^{-1}). Nevertheless, it is important to stress that changes in morphophysiological, biochemical and reproductive parameters may reduce the fitness of this species in aquatic ecosystems when exposed to multi-stressors. According to some studies, changes in morphophysiological responses such as growth and heart rate can be related with

changes in food intake, respiration, or metabolic rate(65–67). Future studies should be performed to understand the potential cellular and molecular mechanisms underlying these changes.

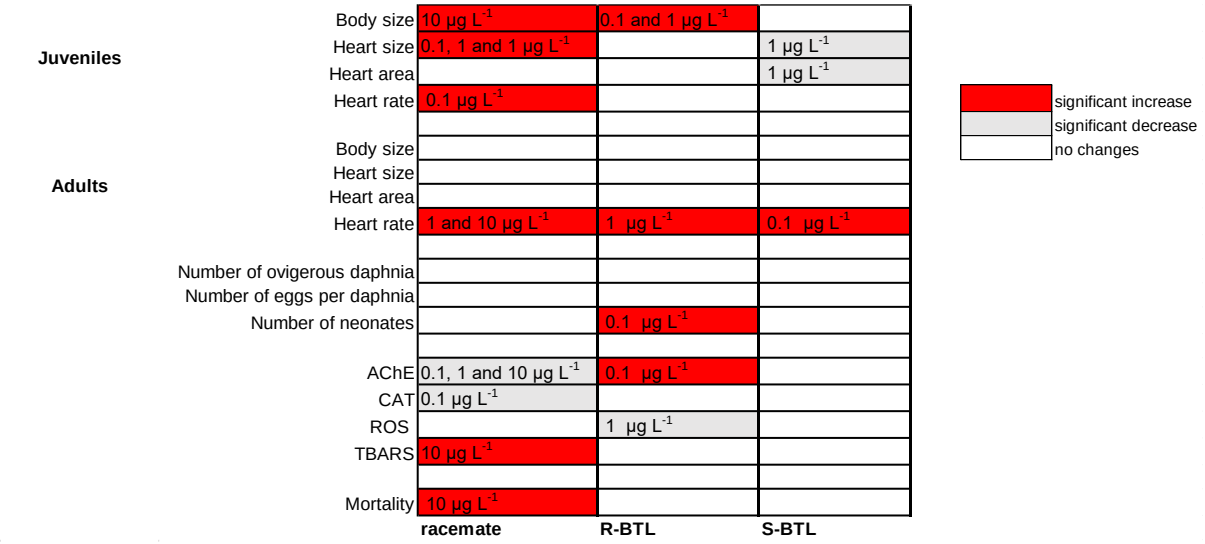

Figure 16| Summary of BTL ecotoxicity assay with the racemate and isolated enantiomers.

3.3. Degradation and acute toxicity

Over the past few years, the detection of PAS in wastewater of different countries has been growing (19–23,25,28) and recent studies have suggested the upgrading of WWTPs with AOTs as a good approach to enhance MPs removals, in comparison to those achieved by the current conventional treatments. However, some AOTs may generate toxic by-products and thus, when evaluating the removal efficiency of a given treatment option, it is also important to assess the toxicity of the final effluents. For instance, ecotoxicity and genotoxicity of lorsatan was assessed before and after treatment by UVC/photolysis and UV/H_2O_2 (70). Results showed high degradation rate, but genotoxicity was observed after 480 minutes of treatment with UV/H_2O_2.

In this work, wastewater samples were spiked with BTL at 10 µg L^{-1} and different technologies as O_3, UV and combination of both O_3/UV were applied. The removal rate of BTL was determined and the acute immobilisation test was performed to investigate toxicity before and after application of the AOTs.

The toxicity was assessed before application of any treatment and the results of the acute immobilization assay with *D. magna* are shown in **Figure 16 and Table 7**. Unspiked effluent and effluent spiked with BTL showed similar EC_{50} values, 84.1, after 24 h-exposure, but after 48

h of exposure, the EC_{50} of BTL-spiked effluent was lower than that of the unspiked effluent (*i.e.*, EC_{50} of 74.1 for unspiked effluent and EC_{50} of 62.5 for BTL spiked effluent), evidencing a higher toxicity of the spiked effluent and suggesting an impact of BTL on the effluent toxicity (**Figure 16**). However, no significant differences were observed among both samples (**Table 7**).

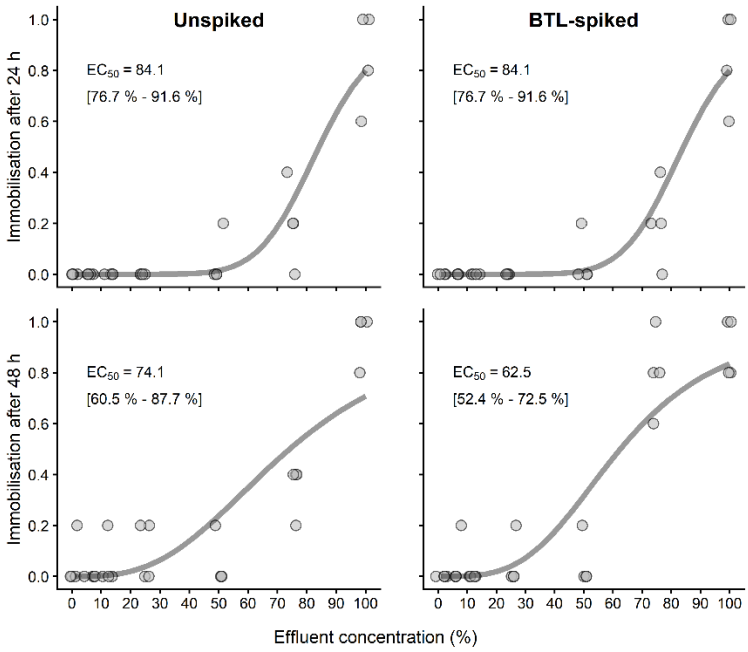

Figure 16| Survival of *D. magna* exposed to different concentrations of unspiked effluent and effluent spiked with BTL, after 24 h and 48 h.

Table 7| Statistical analysis of EC_{50} of unspiked effluent and effluent spiked after 24 h and 48 h of exposure. Significant effects ($p < 0.05$) are in bold.

Exposure time	*p*-value
24 h	0.9994
48 h	0.1782

Regarding application of AOTs, a reduction of toxicity after 24 h of exposure was found in the samples treated by all AOTs. All treated samples had an $EC_{50/24h}$ value greater than the $EC_{50/24h}$ value of the untreated spiked effluent showing lower toxicity of treated effluents (**Figure 17 and Table 8**). A significant increase of the $EC_{50/24h}$ values was observed comparing untreated spiked effluent with treated spiked effluent with UV or O_3, which suggest the positive impact of these two treatments in the removal of BTL and other substances occurring in the effluent. However, no significant differences in $EC_{50/24h}$ values were observed between these two treatments. Nonetheless, $EC_{50/24h}$ of the effluent treated with UV was higher than the effluent treated with O_3, demonstrating lower toxicity of the UV treatment. The combination of UV/O_3 led to an $EC_{50/24h}$

lower than both single treatments, and close to the value of the untreated effluent, suggesting that this combined treatment can lead to the formation of by-products that may be more toxic than the parent compound.

After 48 h of exposure, a significant decrease in toxicity was observed for all treated samples, but no significant differences were found among treatments. Nevertheless, the treatment with O_3 showed the greatest $EC_{50/48h}$ value and thus, it is suggested less toxicity to *D. magna* comparatively to UV and UV/O_3. These results are surprising, considering that O_3 is known to generate by-products that may be more toxic than the original compounds. Again, UV/O_3 showed the lowest $EC_{50/48h}$ demonstrating that the combined treatment can originate toxic by-products.

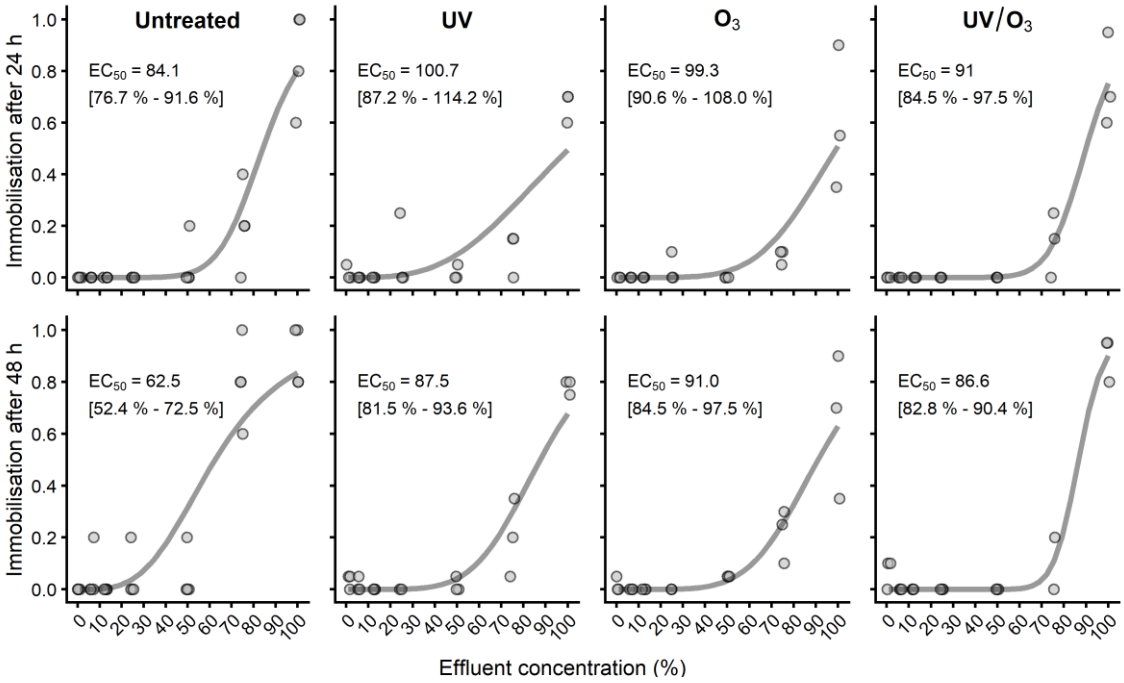

Figure 17| Proportion of survival of *D. magna* exposed to different concentrations of effluent spiked with BTL (untreated) and different treatment (O_3, UV, and O_3/UV) after exposure (24 h and 48 h).

Table 8| Statistical analysis of EC_{50} in untreated effluent spiked with BTL and in the same effluent after different treatments (O_3, UV, and O_3/UV), after exposure (24 h and 48 h). Significant effects ($p < 0.05$) are in bold.

Exposure time	Treatment	*p*-value
24 h	Untreated vs UV	**0.031948**
	Untreated vs O_3	**0.009677**
	Untreated vs UV/O_3	0.175480
	UV vs O_3	0.882811
	UV vs UV/O_3	0.128010
	O_3 vs UV/O_3	0.056530
48 h	Untreated vs UV	**0.0002448**
	Untreated vs O_3	**0.0016053**
	Untreated vs UV/O_3	**0.0002003**
	UV vs O_3	0.2905205
	UV vs UV/O_3	0.1664870
	O_3 vs UV/O_3	0.0703863

The removal rate of BTL was calculated by using a GC-MS method published elsewhere (36). **Table 9 and Figure 18** show the removal rate obtained by applying the 3 different treatments (UV, O_3, and O_3/UV), which were similar (23.26% – 28.89%), being higher for the second eluted diastereomers, corresponding to the (*R*)-enantiomer. Nevertheless, UV demonstrated a slight lower removal rate than the O_3-based treatments, whereas single O_3 sled to the highest removal.

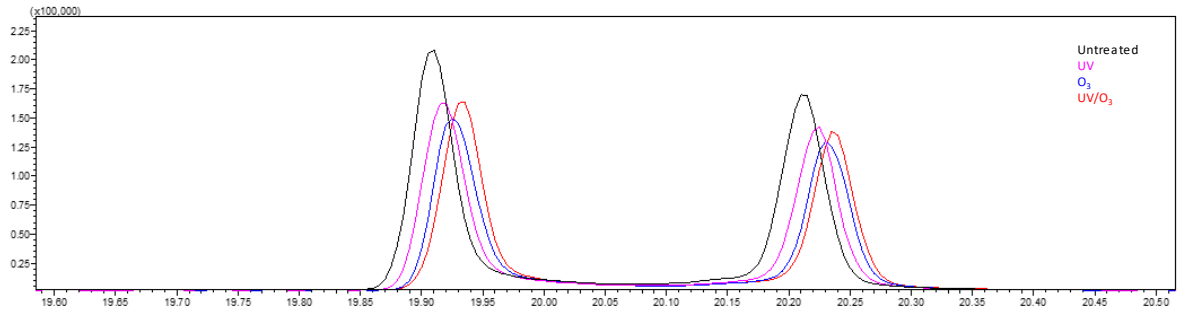

Figure 18| Chromatograms of the fragment ion 288 m/z obtained for showing the different treatments. Effluent untreated (black), UV-treated effluent (pink), O_3-treated effluent (blue), and UV/O_3-treated effluent (red).

Table 9| Determination of removal rate (%) of BTL in effluent treatment with different treatments (O_3, UV, and O_3/UV).

Treatment	Removal rate (%)		Standard deviation	
	S	*R*	*S*	*R*
UV	23.26	26.19	8.26	9.83
O_3	28.73	28.89	6.15	5.69
UV/O_3	25.52	27.51	1.92	1.79

Interestingly, both UV-based treatments had a slight difference between the removals of both enantiomers. This may be related to the indirect photolysis that can occur when UV is applied in natural samples with a heterogeneous composition of organic matter that may act as photosensitizers.

Differences of removals between treatments corroborates EC_{50} values from acute immobilisation. Indeed, O_3 treatment showed lower toxicity to *D. magna* after 48h of exposure, and also the higher removal rate (29%) for both enantiomers. It is interesting to note that the treatment that showed the highest toxicity to *D. magna,* was not the treatment leading to the lowest removal rate of BTL. This result emphasis that toxicity may not be originated only form the BTL itself, but also from its transformation products. It is also interesting to stress that all AOTs did not completely removed BTL, in fact, removal rates are low. Therefore, it is expected the occurrence of BTL in effluent samples and receiving waters with the potential of contaminate ecosystems.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

Many PAS are chiral and available as racemates. Considering that enantiomers may behave differently in biological systems, the enantioselectivity in ecotoxicity should be considered for an accurate risk assessment of chiral PAS in the environment.

To study the possible toxicity of BTL and its enantiomers to aquatic organisms, *D. magna* was used as model organism and enantiomers were isolated by an optimized chiral semipreparative LC method. The recovery percentage was as low as 8.8% of (*S*)-BTL and 9.7% of (*R*)-BTL, which can be explained by the instability and racemization that occurs during the separation procedure, causing loss of a large amount of enantiomers and consequently low recovery. However, both enantiomers of BTL were isolated with a high enantiomeric purity (>97 %).

The results obtained in this work allow to understand that exposure to BTL racemate and enantiomers at reported concentrations, can induce significant morphophysiological responses,and modulation of the AChE and CAT activity and lipid peroxidation in *D. magna*. Heart size, area and rate showed enantioselective effects over time, demonstrating the relevance of these studies for an accurate environmental risk assessment. While (*S*)-BTL induce effects in heart development, (*R*)-BTL induces AChE activity.

AOTs demonstrate successful reduction in the immobilization of *D. magna*, UV showing lower toxicity after 24 h of exposure while surprisingly O_3 showed the lowest toxicity after 48 h of exposure and can be explain with the higher removal rate (29%) comparatively to the other treatments.

Further studies should be carried out to determine the impact of BTL racemate and enantiomers on daphnia, as well as other non-target organisms. Furthermore, knowing that the environmental contaminants do not exist alone, but rather as a mixture with a number of other concomitant substances including natural organic matter, more studies should consider these complex mixtures and the potential harm that these combinations of contaminants may have on aquatic organisms.

It is expected that in the future BTL will be detected more frequently and al higher levels in the aquatic environment, with potential consequences for aquatic organisms and thus, more studies should be carried out. AOTs and other advanced treatments (e.g., using membrane technologies) to remove not only the parent compound but also the by-products formed should

be investigated aiming the mitigation this substance (and others) in the environment, thus reducing their impact on animals and humans.

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Appendix

A. Preparation of the media for microalgae maintenance

The micronutrient stock solution was prepared by adding the following nutrients to 250 mL with distilled water: 46.4 mg of boric acid (H_3BO_3, ≥99.8%) obtained from Merk (Darmstadt, Germany); 104 mg of manganese (II) chloride hexahydrate ($MnCl_2.6H_2O$, ≥98%) and 0.36 mg of cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$, >99%) purchased from Panreac (Barcelona, Spain); 0.88 mg of zinc chloride ($ZnCl_2$, >97%), 1.8 mg of sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$, ≥98%) and 75 mg of disodium ethylenediamine tetraacetic acid (EDTA) dihydrate ($Na_2EDTA.2H_2O$, ≥98.5%) acquired from Sigma-Aldrich (Missouri, USA); and 39.94 mg of iron (III) chloride hexahydrate ($FeCl_3.6H_2O$,≥ 97%) purchased from Panreac (Barcelona, Spain).

The individual macronutrient stock solutions were prepared in 100 mL of distilled water. The solutions contained 2.6 g of sodium nitrate ($NaNO_3$, >99%) and 1.5 g of sodium bicarbonate ($NaHCO_3$, ≥99.7%) acquired from Sigma-Aldrich (Missouri, USA); 1.5 g magnesium sulphate heptahydrate ($MgSO_4.7H_2O$, >99%) purchased from Merck (Darmstadt, Germany); 0.10 g of potassium hydrogen phosphate (K_2HPO_4, >99%) acquired from Panreac AppliChem ITW Reagents (Darmstadt, Germany); and 1.22 g of magnesium chloride hexahydrhate ($MgCl_2.6H_2O$, >98%) and 0.44 g of calcium chloride dihydrate ($CaCl_2.2H_2O$, ≥ 99%) acquired from Riedel-de-Haën (North Caroline, USA).

B. Preparation of standards and samples for biochemical assays

Table 1A| Chemicals used in the determination of biochemical parameters.

Chemicals	Company	Chemicals	Company
Sodium hydroxide (NaOH)	Sigma-Aldrich (St. Louis, EUA or Steincheim, Germany)	2,7-dichlorofluorescein diacetate (H_2DCFDA, ≥97%)	Sigma-Aldrich (St. Louis, EUA or Steincheim, Germany)
Acetylthiocholine iodide (ATCI)		Malondialdehyde (MDA, ≥96%)	
Coomassie Plus (The Better Bradford Assay™ Reagent)		Potassium dihydrogen phosphate (KH_2PO_4, ≥99%)	
Bovine serum albumin (BSA, ≥96%)		Disodium hydrogen phosphate (Na_2HPO4)	Roic Farma (Barcelona, Spain)
5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, ≥98%)		Dimethyl sulfoxide (DMSO, ≥99.9%)	Fisher (Geel, Belgium)
2,7-dichlorofluorescein (DCF, 90%)		Potassium chloride (KCl)	PanReac AppliChem ITW Reagents (Darmstadt, Germany)
Ammonium molydate tetragydrate (AMT)		Catalase (CAT; ≥4.0 units/mg protein; ref. C3515-10MG) at 69629 U/mL	Aspergillus niger
Hydrogen peroxide (H_2O_2)		Trichloroacetic acid (TCA)	VWR (Leuven, Belgium)
Thiobarbituric acid (TBA, ≥98%);		Tris base	Merk (Darmstadt, Germany)
Sodium dodecyl sulphate (SDS, ≥98.5%);		Sodium chloride (NaCl)	
Butylated hydroxytoluene (BHT, ≥99%)		Hydrochloric acid (HCl) 37%	Honeywell Fluka (Seelze, Germany)

Table 2A| Preparation of standards for BSA calibration curve.

BSA Standards	C_fBSA (mg/mL)	V_i BSA (µL)	PBS (pH 7.4) (µL)	Bradford reagent (µL)
B	0.0000	0	100	100
1	0.0015	3	97	
2	0.0030	6	94	
3	0.0045	9	91	
4	0.0060	12	88	
5	0.0075	15	85	
6	0.0100	20	80	
7	0.0125	25	75	
8	0.0175	35	65	
9	0.0250	50	50	

Table 3A| Preparation of standards for DCF calibration curve

DCF Standards	C_f DCF (mg/mL)
B	0
1	0.0293
2	0.0586
3	0.0781
4	0.1562
5	0.3125
6	0.625

Table 4A| Preparation of standards and samples for CAT activity.

CAT Standards	C_f CAT (U/mL)	V_i CAT (C=13.9258 U/mL) (µL)	PBS (pH 7.4) (µL)	60 mM SPB/0.065 M H_2O_2 (µL)	32 mM AMT (µL)
B	0	0	100	100	250
1	0.16	1	99		
2	0.31	2	98		
3	0.63	5	95		
4	1.0	7	93		
5	1.25	9	91		
6	2.0	14	86		
7	2.5	18	82		
8	3.0	21	79		
9	4.0	28	72		
10	5.0	35	65		
11	6.0	42	58		
12	7.0	50	50		

Table 5A| Preparation of standards for MDA calibration curve.

MDA Standards	C_f MDA (mg/mL)	V_i MDA stock solution (C= 5 mM) (µL)	UPW (µL)
B	0	0	1000
1	2.5	0.5	999.5
2	5	1	999
3	10	2	998
4	20	4	996
5	30	6	994
6	50	10	990
7	80	16	984
8	100	20	980

C. Abstract and Poster communication presented in Internacional congress "CHIRALITY 2023"

PC-127

Enantioseparation and ecotoxicity assessment of butylone on the microcrustaean *Daphnia magna*

Ana Rita Carvalho,[1,2] Ana Mafalda Morão,[1] Virgínia M. F. Gonçalves,[1,3] Maria Elizabeth Tiritan,[1,4,5] Ana Rita Lado Ribeiro,[6,7] João Soares Carrola,[8,9] Maria Manuela Amorim,[2] Cláudia Ribeiro[1]

[1]*TOXRUN – Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, 4585-116 Gandra, Portugal*
[2]*CISA - ESS, Polytechnic Institute of Porto, 4200-072 Porto, Portugal*
[3]*UNIPRO – Oral Pathology and Rehabilitation Research Unit, University Institute of Health Sciences (IUCS-CESPU), 4585-116 Gandra, Portugal*
[4]*CIIMAR, University of Porto,4450-208 Matosinhos, Portugal*
[5]*Laboratory of Organic and Pharmaceutical Chemistry, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal*
[6]*LSRE-LCM, Faculty of Engineering, University of Porto, 4200-465 Porto, Portugal*
[7]*ALiCE, Faculty of Engineering, University of Porto, 4200-465 Porto, Portugal*
[8]*Department of Biology and Environment, University of Trás-os-Montes and Alto Douro, CITAB, Vila Real, Portugal*
[9]*Inov4Agro – Institute for Innovation, Capacity Building and Sustainability of Agri-food Production*
Email: beth@ff.up.pt

The aquatic environment is the reservoir of the majority of contaminants including new psychoactive substances (NPS). Belonging to the group of NPS, butylone (BTL) is a chiral synthetic cathinone consumed in the form of racemate[1]. However, enantiomers may exhibit different biological activities including toxicity in non-target aquatic organisms[2]. This study aimed to separate the two enantiomers of BTL and to assess the sub-chronic effects on *Daphnia magna*, focusing on reproductive and biochemical parameters. The enantiomers were separated by liquid chromatography using a semipreparative chiral column (APS-Nucleosil coated with a 3,5-dimethylphenylcarbamate of amylose). Daphnids (<24 h) were exposed for 9 days to concentrations of 0.1, 1, or 10 μg/L of *rac*-BTL and 0.1 or 1 μg/L of each BTL enantiomer (5 replicates per concentration and a control). Enantiomers were obtained with high enantiomeric purity, i.e., ~99.9% for (*R*)-BTL and ~97% for (*S*)-BTL. A significant decrease in acetylcholinesterase (AChE) and catalase (CAT) activity was noted in the organisms exposed to *rac*-BTL whereas (*R*)-BTL led to a significant increase of AChE and CAT, at 0.1 μg/L. No significant adverse effects were observed on the reproductive parameters for the racemate and enantiomers. Thus, BTL may cause enantioselective toxicity effects to daphnia related to enzymatic activity. Ongoing studies are expected to provide new knowledge on BTL adverse effects related to other specific endpoints.

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(2) Langa, I.; Tiritan, M. E.; Silva, D.; Ribeiro, C. *Chemosensors* **2021**, *9*, 224.

Enantioseparation and ecotoxicity assessment of butylone on the microcrustacean *Daphnia magna*

Ana Rita Carvalho[1,2], Ana Mafalda Morão[1], Virgínia M. F. Gonçalves[1,3], Maria Elizabeth Tiritan[1,4,5], Ana Rita Lado Ribeiro[6,7], João Soares Carrola[8,9], Maria Manuela Amorim[2], Cláudia Ribeiro[1]

[1] 1H-TOXRUN – One Health Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, 4585-116 Gandra, Portugal; [2]CISA - ESS, Polytechnic Institute of Porto, 4200-072 Porto, Portugal; [3]UNIPRO – Oral Pathology and Rehabilitation Research Unit, University Institute of Health Sciences (IUCS-CESPU), 4585-116 Gandra, Portugal; [4]CIIMAR, University of Porto, 4450-208 Matosinhos, Portugal; [5]Laboratory of Organic and Pharmaceutical Chemistry, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal; [6]LSRE-LCM, Faculty of Engineering, University of Porto, 4200-465 Porto, Portugal; [7]ALiCE, Faculty of Engineering, University of Porto, 4200-465 Porto, Portugal; [8]Department of Biology and Environment, University of Trás-os-Montes and Alto Douro, CITAB, Vila Real, Portugal; [9]Inov4Agro – Institute for Innovation, Capacity Building and Sustainability of Agri-food Production.

E-mail: beth@ff.up.pt

INTRODUCTION

The aquatic environment is the reservoir of the majority of contaminants including new psychoactive substances (NPS). Butylone (BTL) is a NPS chiral synthetic cathinone, synthesized as a racemate[1]. However, due to enantioselective metabolism and biodegradation, BTL may occur in different enantiomeric fractions (EF). Enantiomers may exhibit different biological activities including toxicity in non-target aquatic organisms[2] (Figure 1). The aim of this study is to investigate the possible enantioselective effects of BTL.

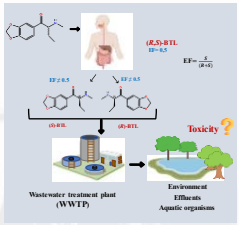

Figure 1 - Enantioselective metabolism and biodegradation of BTL and environmental sources of BTL.

Methods

Enantioseparation of BTL

A liquid chromatograph with a diode array detector was used for the enantioseparation of BTL (semipreparative chromatography) and for evaluation of enantiomeric purity (analytical chromatography).

Table 1. Chromatographic conditions for semipreparative and analytical assays.

	Semipreparative	Analytical
Chiral stationary phase	Homemade Semipreparative chiral column: APS-Nucleosil coated with a *tris*-3,5-dimethylphenylcarbamate of amylose (500 Å, 7 µm, 20%, w/w;Macherey-Nagel), packed into a stainless-steel 200× 70 mm i.d. column.	Lux® Amylose *tris* -3,5-dimethylphenylcarbamate (3 µm, 150 x 4.6 mm).
Mobile phase	0.1% DEA n-Hex/EtOH (70:30, *v/v*)	0.1% DEA n-Hex/EtOH (65:35, *v/v*)
Flow rate	1.5 mL/min	0.7 mL/min
Detection	230 nm	230 nm

Ecotoxicity assays

- Daphnids (< 24 h) were exposed for 9 days to concentrations of 0.1, 1, or 10 µg/L of *rac*-BTL or 0.1 or 1 µg/L of each BTL enantiomer (5 replicates per concentration and a control).
- On the 9th day, it was determined the following reproductive parameters: number of daphnia with eggs, number of eggs per daphnia and number of neonates. Acetylcholinesterase and catalase activity were also determined.

Results

Enantioseparation of BTL

BTL enantioseparation was performed under normal phase elution mode. The (*S*)-BTL enantiomer fraction was first eluted and collected from 12.5 to 14.8 min and the (*R*)-BTL fraction was collected from 16.2 to 22 min (Figure 2).

An intermediate fraction was collected (from 14.8 to 16.2 min).

The enantiomeric purity of each fraction was evaluated using the same equipment. (Figure 3).

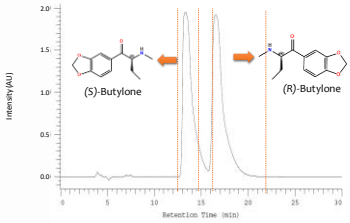

Figure 2 - Enantioseparation of (*R,S*) BTL. Column: APS-Nucleosil coated with a 3,5-dimethylphenylcarbamate of amylose. Mobile phase: n-Hex (0.1%DEA) and /EtOH (0.1%DEA), 70:30, *v/v*; flow rate: 1.5 mL/min; volume of injection: 50 µL at 4 mg/mL in n-Hex/EtOH (70:30, *v/v*); detection: 230 nm at room temperature.

Enantiomers were obtained with high enantiomeric purity, *i.e.*, ~99.9% for (*R*)-BTL and ~97% for (*S*)-BTL (Figure 3).

The analytical method was developed to quantify the enantiomers and determine the percentage of recovery of the semipreparative procedure. The calibration curves were found to be linear in the range of 2.5 to 75 µg/mL with a R^2 greater than 0.9992.

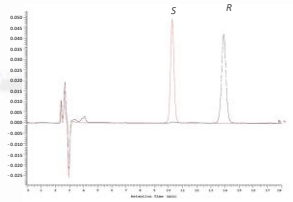

Figure 3 - Chromatogram of BTL fractions. Column: amylose tris (3,5-dimethylphenylcarbamate); Mobile phase: n-Hex (0.1%DEA) and /EtOH (0.1%DEA), 65:35, v/v; flow rate: 0.7 mL/min; volume injection: 10 µL; detection: 230 nm a) (red line) corresponds to the first fraction - (*S*)-BTL and b) (black line) corresponds to the second fraction- (*R*)-BTL.

Ecotoxicity assays

No significant effects were observed on reproductive parameters of both racemate and enantiomers regarding the number of daphnids with eggs, number of eggs per daphnia, and number of neonates for the enantiomers (Figure 4).

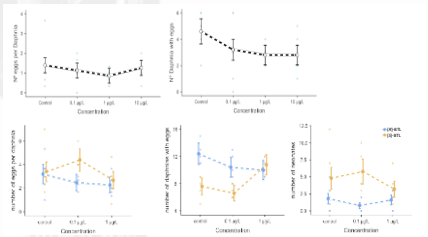

Figure 4 - Reproductive parameters (number of daphnia with eggs, number of eggs per daphnia, and number of neonates) at 9th day of exposure to three different concentrations of *rac*-BTL (0.1, 1 or 10 µg/L) and two different concentrations of (*R,S*)-BTL (0.1 or 1 µg/L). Asterisks (*) represent significant differences compared to the control.

Significant decrease were observed in catalase activity at the lower concentration (0.1 µg/L) for the *rac*-BTL. No effects were noted for the enantiomers (Figure 5). Also, a significant decrease of acetylcholinesterase activity were observed at all *rac*-BTL concentrations whereas (*R*)-BTL showed an increase showed at the lower concentration.

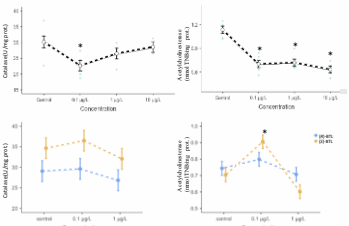

Figure 5 - Evaluation of enzymatic activity (catalase (U CAT/mg protein) and acetylcholinesterase (mmol TNB/mg protein) after exposure to three different concentrations of *rac*-BTL (0.1, 1 or 10 µg/L) and two different concentrations of (*R,S*)-BTL (0.1 or 1 µg/L). Asterisks (*) represent significant differences compared to the control).

CONCLUSION

- The enantiomers of BTL were **successfully separated** with high enantiomeric purity (higher than 97%).
- The present study demonstrates that **exposure to BTL may cause enantioselective toxicity** effects to daphnia related to **enzymatic activity**.
- Ongoing studies are expected to provide new knowledge on BTL adverse effects related to other specific endpoints.

ACKNOWLEDGEMENTS

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D. Abstract and Poster communication presented in nacional congress TOXRUN 2023

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Poster 39

Butylone enantioseparation and ecotoxicity evaluation on *Daphnia magna*

A. R. Carvalho [1,2,*], A. M. Morão [1], V. M. F. Gonçalves [1,3], M. E. Tiritan [1,4,5], A. R. Lado [6,7], J. S. Carrola [8,9], M. M. Amorim [2] and C. Ribeiro [1,*]

[1] TOXRUN-Toxicology Research Unit, University Institute of Health Sciences, IUCS-CESPU, CRL, 4585-116 Gandra, Portugal
[2] CISA, ESS, Polytechnic Institute of Porto, Rua Dr. António Bernardino de Almeida, 400, 4200-072 Porto, Portugal
[3] UNIPRO-Oral Pathology and Rehabilitation Research Unit, University Institute of Health Sciences (IUCS), CESPU, 4585-116 Gandra, Portugal
[4] Interdisciplinary Center of Marine and Environmental Research (CIIMAR), University of Porto,4450-208 Matosinhos, Portugal
[5] Laboratory of Organic and Pharmaceutical Chemistry, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal
[6] LSRE-LCM - Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal
[7] ALiCE - Associate Laboratory in Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal
[8] Department of Biology and Environment, University of Trás-os-Montes and Alto Douro, CITAB, Vila Real, Portugal
[9] Inov4Agro – Institute for Innovation, Capacity Building and Sustainability of Agri-food Production

* Correspondence: anartcarvalho@gmail.com; claudia.ribeiro@iucs.cespu.pt

Abstract

Background: The synthetic drug butylone (2-methylamino-1-(3,4-methylenedioxyphenyl)butan-1-one, BTL) is a chiral cathinone consumed in the form of racemate [1]. After consumption, BTL is metabolized and excreted along with its metabolites, and its residues are carried by sewerage systems to wastewater treatment plants (WWTPs) [2]. Both human metabolism and biodegradation in WWTPs may be enantioselective causing a change in its enantiomeric fraction (EF). However, enantiomers may exhibit different biological activities including toxicity on non-target aquatic organisms [3]. **Objective:** This study aimed to separate both enantiomers and assess the sub-chronic effects on *Daphnia magna* focusing on morpho-physiological and reproductive parameters. **Methods:** The enantiomers were separated by liquid chromatography using a homemade semipreparative chiral column (APS-Nucleosil coated with a 3,5-dimethylphenylcarbamate of amylose). Daphnia (with less than 24 h) were exposed for 9 days to concentrations of 0.1, 1, or 10 µg/L, with a total of 5 replicates per concentration and a control. **Results:** Morpho-physiological alterations were observed, except in the heart area. A tendency to the increase of body size, heart size and mortality were observed for the higher concentrations (1 and 10 µ/L). The daphniids with eggs tended to decrease. Analysis of other endpoints (ongoing) are required to draw accurate conclusions. **Conclusions:** The present study demonstrates that exposure to BTL may cause effects on mortality and morphology of *D. magna*. The ongoing studies will bring new knowledge on BTL adverse effects and the possible enantioselective toxicity effects on this non-target aquatic organism.

Keywords: ecotoxicity; psychoactive drugs; *Daphnia magna*; enantioseparation

Acknowledgments

This work is financially supported by national funds through the FCT/MCTES (PIDDAC), under the project PTDC/CTA-AMB/6686/2020. ARLR acknowledges the support from LA/P/0045/2020 (ALiCE), UIDB/04033/2020 (CITAB/Inov4Agro), UIDB/50020/2020 and UIDP/50020/2020 (LSRE-LCM), funded by national funds through FCT/MCTES (PIDDAC), and FCT funding under the Scientific Employment Stimulus - Individual Call (2022.00184.CEECIND).

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Butylone enantioseparation and ecotoxicity evaluation on *Daphnia magna*

Ana Rita Carvalho [1,9*], Ana Mafalda Morão [1], Virgínia M. F. Gonçalves [1,2], Maria Elizabeth Tiritan [1,3,4], Ana Rita Lado Ribeiro [5,6], João Soares Carrola [7,8], Maria Manuela Amorim [9], Cláudia Ribeiro [1]

[1]TOXRUN-Toxicology Research Unit, University Institute of Health Sciences, IUCS-CESPU, CRL, 4585-116 Gandra, Portugal
[2]UNIPRO-Oral Pathology and Rehabilitation Research Unit, University Institute of Health Sciences (IUCS), CESPU, 4585-116 Gandra, Portugal
[3]Interdisciplinary Center of Marine and Environmental Research (CIIMAR), University of Porto, 4450-208 Matosinhos, Portugal
[4]Laboratory of Organic and Pharmaceutical Chemistry, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal
[5]LSRE-LCM - Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200 -465 Porto, Portugal
[6]ALiCE - Associate Laboratory in Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200 -465 Porto, Portugal
[7]Department of Biology and Environment, University of Trás-os-Montes and Alto Douro, CITAB, Vila Real, Portugal;
[8]Inov4Agro – Institute for Innovation, Capacity Building and Sustainability of Agri-food Production;
[9]CISA, ESS, Polytechnic Institute of Porto, Rua Dr. António Bernardino de Almeida, 400, 4200 -072 Porto, Portugal
*E-mail: anaritacarvalho@gmail.com ;

INTRODUCTION

New psychoactive substances (**NPS**) belong to a heterogenous group of substances, comprising analogues of existing controlled drugs or pharmaceutical psychoactive substances [1].
According to the European Monitoring Centre for Drugs and Drug Addiction 2022 annual report, **synthetic cathinones** (SC) are the second largest group of NPS [2].
Cathinone is a psychostimulant substance, with effects similar to that of amphetamine, which can be of natural origin, or produced synthetically. SC are compounds derived from cathinone that are also known as 'bath salts'[3].

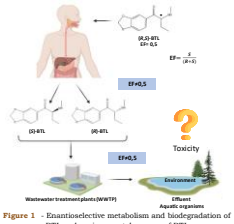


Figure 1 - Enantioselective metabolism and biodegradation of BTL and environmental sources of BTL.

Butylone (BTL) is a chiral SC and is produced in the form of racemate (**Figure 1**). It emerged on the European market in 2008 [1].
After consumption, BTL is metabolized and excreted together with its metabolites, and the residues are carried by sewerage systems to wastewater treatment plants (WWTP) [6] and later reach streams and rivers.
Both human metabolism and biodegradation in WWTPs may be **enantioselective** and its enantiomeric fraction (EF) may change. However, enantiomers may exhibit different biological activities including toxicity on non-target aquatic organisms [7].

MATERIALS AND METHODS

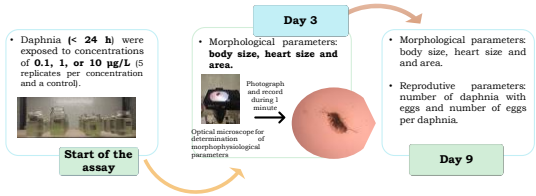
Enantioseparation of butylone

Semi-preparative chromatography
A stock solution of (*R,S*) BTL was prepared in *n*-Hex/EtOH (70:30, *v/v*) at a concentration of 4 mg/mL.
A liquid chromatograph with a diode array detector was used for semipreparative chromatography and evaluation of enantiomeric purity.

Semi-preparative chromatography
Chiral stationary phase: homemade semipreparative chiral column (APS-Nucleosil coated with a 3,5-dimethylphenylcarbamate of amylose).
Mobile phase: 0.1% DEA *n*-Hex/EtOH (70:30, *v/v*) and flow rate 1.5 mL/min.
Detector: λ_{max} = 230nm.

Analytical chromatography
Chiral stationary phase: Amylose tris (3,5-dimethylphenylcarbamate)
Mobile phase: 0.1% DEA *n*-Hex/EtOH (65:35, *v/v*)

Ecotoxicity evaluation on *Daphnia magna*



RESULTS AND DISCUSSION

Enantioseparation

BTL enantiomers were separated according to the methodology adapted by Spálovská et al. (2018). The analysis was performed under normal elution mode (Figure 2). (*S*)-BTL enantiomer was first eluted and collected from 12.5 to 14.8 min and (*R*)-BTL was collected from 16.2 to 22 min (Figure 2). An intermediate fraction was collected (from 14.8 to 16.2 min) to assure high enantiomeric purity. The enantiomeric purity of each fractions was evaluated by analytical chromatography using the same equipment and chromatographic conditions (Figure 3).

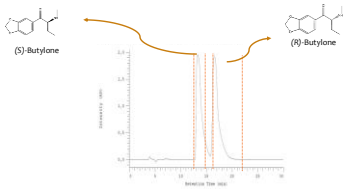


Figure 2 - Chromatogram showing the enantioseparation of BTL in the semipreparative chiral column (APS-Nucleosil coated with a 3,5-dimethylphenylcarbamate of amylose).

Enantiomers were obtained with high enantiomeric purity, *i.e.*, ~99.9% for (*R*)-BTL and ~97% for (*S*)-BTL (Figure 3).
The analytical method was developed to quantify the enantiomers and determine the percentage of recovery of the semipreparative procedure. The calibration curves were found to be linear in the range of 2.5 to 75 µg/mL with a R^2 greater than 0.9992. The recovery percentage was 8% for each enantiomer.

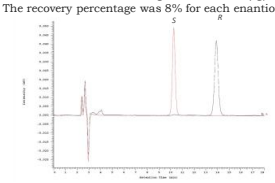


Figure 3 - Chromatogram showing the analysis of BTL fractions a) (red line) corresponds to the first fration, i.e., (S)-BTL and b) (black line) corresponds to the second fration, i.e., to (R)-BTL.

Ecotoxicity evaluation on *Daphnia magna*

BTL effects on morphological parameters are shown in Figure 4. At day 3, a **significant increase** in **body size and heart size** were observed at 10 µg/L and at 1 and 10 µg/L , respetively. (Figure 4), however at **day 9** no significant effects in both body size and heart size. No significant effects were observed at both day 3 and 9 in heart area.

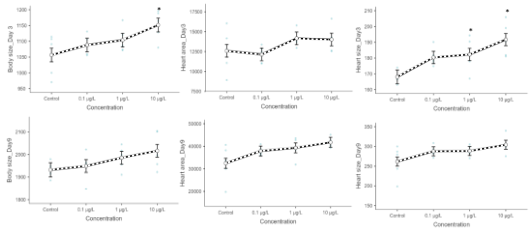


Figure 4- Morphological parameters (body size, heart area and size) at days 3 and 9 after exposure to the three different concentrations of BTL (0.1, 1 and 10 µg/L). Asterisks (*) represent significant differences compared to the control).

No significant effects were observed for the reproductive parameters (number of daphnids with eggs and number of eggs per daphnia) significant effects (Figure 5).

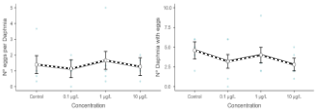


Figure 5- Reproductive parameters (number of daphnia with eggs and number of eggs per daphnia) at day 9 of exposure to three different concentrations of BTL (0.1, 1 and 10 µg/L).

CONCLUSIONS

- The enantiomers of BTL were successfully separated with a high enantiomeric purity (higher than 97%).
- The present study demonstrates that exposure to BTL may cause effects on morphology in the juveniles (body size and heart size at day 3) of *D. magna*.
- The ongoing studies will bring new knowledge on BTL adverse effects and the possible enantioselective toxicity effects on this non-target aquatic organism.

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E. Abstract communication will present in XXVII Encontro Luso-Galego de Química (November 22-24,2023)

Assessment of Butylone degradation by advanced oxidation technologies and ecotoxicity of treated wastewater.

Ana R. Carvalho[1,2,*], Ana M. Morão[1], Virgínia M.F. Gonçalves[1,3], Maria E. Tiritan[1,4,5], Ivan Langa[1], Ana M. Gorito[8,9], M. Fernando R. Pereira[8,9], Adrián M.T. Silva[8,9], João S. Carrola[6], Bruno B. Castro[7,8], Maria M. Morim[2], Ana R. L. Ribeiro[9,10], Cláudia Ribeiro[1]

[1] 1H-TOXRUN – One Health Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, Gandra, Portugal;
[2]CISA - ESS, Polytechnic Institute of Porto, 4200-072 Porto, Portugal;
[3]UNIPRO – Oral Pathology and Rehabilitation Research Unit, University Institute of Health Sciences, Gandra, Portugal;
[4]CIIMAR, University of Porto,4450-208 Matosinhos, Portugal
[5]Laboratory of Organic and Pharmaceutical Chemistry, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal
[6]Department of Biology and Environment, University of Trás-os-Montes and Alto Douro, CITAB/Inov4Agro, Vila Real, Portugal
[7]CBMA - Centre of Molecular and Environmental Biology / ARNET - Aquatic Research Network, University of Minho, Portugal
[8]IB-S - Institute of Science and Innovation for Bio-Sustainability, University of Minho, Portugal
[9]LSRE-LCM, Faculty of Engineering, University of Porto, 4200-465 Porto, Portugal
[10]ALiCE, Faculty of Engineering, University of Porto, 4200-465 Porto, Portugal
* anartcarvalho@gmail.com

Psychoactive substances (PAS) include pharmaceuticals and illicit drugs that are frequently detected in environmental samples due to their widespread use and recalcitrance in wastewater treatment plants (WWTP), posing risks to wildlife and human health [1]. Butylone is a synthetic cathinone (2-methylamino-1-(3,4-methylenedioxyphenyl) butan-1-one, BTL) and emerged in the European drug market, as a new designer drug, in 2008 [2]. Advanced oxidation technologies (AOT), including UV- and ozone-based treatments, have been demonstrating to be effective in removing a wide range of micropollutants [3]. This study aims to evaluate the degradation of BTL in secondary-treated wastewater by AOTs and the ecotoxicity of treated effluents. For that, wastewater samples were spiked with BTL at 10 µg L^{-1} and different AOTs (UV, ozonation (O_3), and UV/O_3) were applied. The removal rate of BTL was determined using a gas chromatography–mass spectrometry (GC-MS) method and the Acute Immobilisation Test was performed using *Daphnia magna* to investigate the toxicity before and after application of the AOT. Before treatment, no significant effects were observed due to exposure to unspiked or BTL-spiked effluents but EC_{50} at 48 hours evidenced a higher toxicity of the spiked effluent, suggesting an impact of BTL on the effluent toxicity. The treatment with O_3 showed the greatest $EC_{50/48h}$ value and thus, suggesting to be less toxic to *D. magna,* comparatively to UV and UV/O_3. The removal rates obtained by applying the 3 different treatments (UV, O_3, and UV/O_3) were similar (ranged between 23.3% and 28.9%). UV demonstrated a slightly lower removal rate than the UV/O_3 treatments, whereas O_3 led to the highest removal. AOTs demonstrated a successful reduction in the immobilization of *D. magna*, with UV showing lower toxicity after 24 hours whereas O_3 showed the lowest toxicity after 48 hours, which may be related to the higher removal rate (29%). To reduce the adverse impacts of this substance (and others) on animals and humans, AOT and other advanced treatments (*e.g.*, membrane technologies) should be investigated, aiming to remove not only the parent compound but also its by-products.

Acknowledgements

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F. Article

Carvalho AR, Morão AM, Gonçalves VMF, Tiritan ME, Gorito AM, Pereira MFR, Silva AMT, Carrola JS, Castro BB, Amorim MM, Ribeiro ARL, Ribeiro C. Toxicity of butylone and its enantiomers to *Daphnia magna* and its degradation by advanced oxidation technologies (in preparation).