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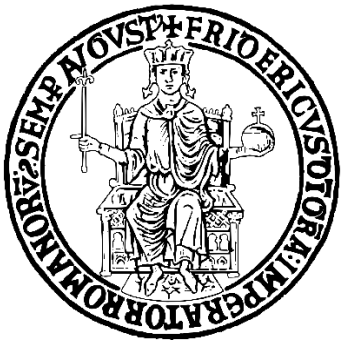

DOCTORATE SCHOOL IN BIOLOGY

XXXV Cycle

"Benzodiazepines effects on *non-target* organisms"

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Abstract

Benzodiazepines, psychotropic drugs for the treatment of insomnia and anxiety, are among the most prescribed remedies worldwide. The massive use and abuse results in the release of active principles and metabolites in the wastewater, where they persist since not eliminated by sewage treatments. They are therefore considered emerging contaminants of great environmental hazard, even at low concentrations, also considering that receptors for benzodiazepines are found in all animals and plants. The fate and consequences of benzodiazepine exposure in aquatic organisms are not fully clear: a large variety of these drugs is available on the market, and a plethora of direct and indirect effects could be exerted on different, accidentally exposed *non-target* species. This Ph.D. project represents the first comparative and multidisciplinary study carried out in parallel on six models, five animals and one plant, with the aim of understanding the extent of the effects of delorazepam, a common-used long-life, and high-potency benzodiazepine. Three concentrations were tested: 1 µg/L, considered environmentally realistic, and 5 and 10 µg/L, considered near-future concentrations based on growing consumption trends worldwide. Behavioural, developmental, and cyto-toxicological effects were considered; standard locomotory and phototactic tests were conducted to determine the effects of delorazepam on nervous and sensory activity. Conventional toxicity tests were performed to clarify the impact on early development and embryo growth, while cytological approaches, by light and electron microscopy, were employed to highlight effects on cell organization and functionality. Finally, molecular and biochemical investigations were carried out to deepen the possible interference with gene expression and protein patterns.

State of art

Pharmaceuticals are one of the cornerstones of human scientific development, known to be essential for the sustainability and maintenance of human health, improving people's quality of life. Hundreds of tons of pharmaceutical compounds are annually dispensed and consumed worldwide. The fate of these drugs, and of their metabolites is becoming a matter of growing interest since they are unproperly discarded in the environment, where accumulate. Households, farms, health facilities, and pharmaceutical industries release pharmaceutical waste into the environment at low concentrations, through routine pharmaceutical use. Pharmaceuticals can also enter the water supply by improper disposal of unused or expired medications. Residues in the environmental compartments are considered "compounds of emerging concern" because they have the potential to cause a considerable impact on human health and ecosystems (Daughton, 2004). They have been found in almost all environmental matrices on several continents, but especially in surface water (lakes, rivers, streams, estuaries), seawater, groundwater, effluents, tributaries of wastewater treatment plants and even drinking water (aus der Beek et al., 2016; Roig and D'Aco, 2016; Fatta-Kassinos et al., 2011; Fekadu et al., 2019). The persistence of pharmaceuticals in the environment has become a focus for the scientific community. Nevertheless, little is known about how pharmaceutical contaminants affect flora and fauna. Advanced analytical techniques have enabled the determination and quantification of almost 3.000 biologically active compounds in the environment (Richardson and Ternes, 2014). Pharmaceutically active compounds are considered pseudo-persistent because of their continuous influx into environmental matrices. This causes the development of a complex "pharmaceutical pool" in many natural matrices, including soils.

Discarded products return to humans via the food chain or drinking water. This happens because wastewater treatment plants (WWTPs) are generally designed to handle easily and moderately degradable organics, therefore were not conceived to treat pharmaceuticals, reason why they are not efficient in their removal. Remediation efficiencies can be less than 10% for some pharmaceutical classes. Various degradation methods, including nanofiltration, oxidation, and photolysis, have been hypothesized to deal with the pharmaceutical accumulation issue, but not all specialized facilities, and more generally, not all governments have the same economic possibilities to implement them. So far, in theory, the most effective technique for removing pharmaceuticals from aqueous solutions is the adsorption method, which exploits the chemical-physical interactions of a substance (liquid or gas) to absorb it onto a solid surface, but it has not yet become a practice (Patel et al., 2019).

Among the drugs of concern, a primary position is deserved by benzodiazepines (BZDs), a class of psychoactive drugs, worldwide one of the most prescribed remedies to treat anxiety, insomnia, and nervous disorders (Argyropoulos and Nutt, 1999; Schmitz, 2016; Nunes et al., 2019). They are classified as central nervous system (CNS) depressants, working by enhancing the effects of the inhibitory neurotransmitter GABA (gamma-aminobutyric acid) on the GABA-A receptors, leading to a regulation of the brain activity and inhibition of excessive neuronal firing, and resulting in sedative, hypnotic (sleep-inducing), anxiolytic (anti-anxiety), anticonvulsant, and muscle relaxant properties (Nunes et al., 2019). They are generally considered to be safe and effective when used as directed by medical prescription, but they can be habit-forming, and their improper use is widely reported. It has been estimated that about 2–7.5% of the population uses BZDs and, among these, 25–75% are long-term users (Vicens et al., 2011; Lugoboni et al., 2018).

Because of their extensive use all over the world, BZDs are ranked among the most found pharmaceuticals in water (Kosjek et al., 2012; Nunes et al., 2019). They have been found ubiquitously in all aquatic systems (wastewater, surface water, drinking water) at concentrations ranging from several nanograms to micrograms per liter (Batt et al., 2016; Wang et al., 2017), a range close to human therapeutic plasma concentrations. Their stability in the environment has been demonstrated, with persistence at unchanged concentrations in lake sediments for several decades (Klaminder et al., 2015). The activity of these drugs is of considerable concern as they are specifically synthesized to elicit responses in humans and animals even at low concentrations (Zuccato et al., 2006), representing therefore a hazard, especially for aquatic species with which they inevitably come into contact (Klaminder et al., 2015). GABA-A receptors, the site of action of BDZs, are evolutionary conserved, from bacteria to invertebrates and vertebrates (Furuhagen et al., 2014) and large-scale effects are foreseeable. The improper activation of the GABA inhibitory system could lead to alteration in behavior with consequent impairment of animal fitness, including, for example, feeding, escape from predators, or changes in reproductive performance. The early expression of GABA-A receptors in embryos (Luján et al., 2005) and, therefore, their early activation, can have direct negative effects on embryo development. The most endangered are aquatic species, often exposed from the *ovo* stage. Last but not least, a possible direct effect on tissues should be considered: BZDs in fact can also exert their action via the peripheral benzodiazepine receptors (TSPO), located on mitochondrial membranes (Papadopoulos et al., 2006) which regulate cell energy metabolism, transmembrane potential, and sensitivity to reactive oxygen species (Casellas et al., 2002). TSPOs are ubiquitously expressed in all tissues and are known to take part in CNS pathological disorders but also to play a key role in neuronal apoptosis, glial cell degeneration, and regeneration (Lang, 2002).

Aim and plan of the research

Pharmaceuticals have long been present in the environment, but their detection and hazardous effects have only emerged in the past 2−3 decades. Despite many publications on this topic, their individual and combined acute and chronic effects on the flora, fauna, and humans are not well understood. The uncertainty regarding pharmaceuticals' effects on *non-target* organisms and the deleterious effects that these compounds may have on ecosystems' functions and structures and human health have been raising concerns among the scientific community. Thus, there is an urgent need for the development of suitable technologies for recovering/remediating environments impacted by the presence of these pollutants, in addition to other reducing or preventive strategies.

The aim of this project was, therefore, to start a multidisciplinary study of the effects of delorazepam (DLZ), a long-life and high-potency benzodiazepine widely marketed in Italy. Its effects have not been extensively tested so far, with respect to the better-known diazepam.

Studies were conducted on a panel of *non-target* organisms, all potentially exposed in nature to contaminated waters, and all widely used in toxicology and ecotoxicology studies (Richards et al., 2006; Carotenuto et al., 2020; 2022; Nunes et al., 2006; Motta et al., 2016; 2019; Curpan et al., 2022; Ribeiro et al., 2015; Herbert et al., 2021). Since the plan was to investigate different aspects, in particular, the effects on behavior, development, and cytotoxicity, different sets of organisms were chosen for each line of research.

To study the effects on **behavior**, three different species were initially chosen: two molluscs, the sessile *Mytilus galloprovincialis* and the vagile freshwater snail *Planorbarius corneus*, and a crustacean, *Artemia salina*. Conventional behavioral tests were carried out: for mussels, the open/closing valve test; for the two vagile species, different types of chambers were set and used to determine the locomotory parameters.

To study the effects of DLZ on **development**, three models were chosen. First, the sea urchin *Paracentrotus lividus*, species shedding gametes directly into the water and, therefore, very convenient for studying the effects of the drug on fertilization and development. Two further models were *Artemia salina* and *Cucumis sativus*, species in which the early embryos are protected by acellular structures, the cyst chorion, and the seed integument respectively. *Cucumis* was chosen also because considered an 'out-group', suitable to carry out a parallel between effects on flora and fauna. GABA signaling in plants is present and modulates growth, development, and stress response (Ramesh et al., 2017). In these models, conventional endpoints such as fertilization, hatching and

germination percentages, growth, and mortality were assessed but parallel morphological, cytological and/or biochemical investigations were also carried out.

The third effect studied, was **cytotoxicity**. DLZ is not reported to cause direct toxicity, however, the presence in tissues of TSPO receptors suggested that local effects might be induced. Model species were *Paracentrotus lividus* eggs and sperms and *Mytilus galloprovincialis* gills. Indirect evidence was also collected from *Cucumis sativus* roots and shoots, and *Artemia salina* nauplii.

Preliminary evidence gathered during **the first year was rather impressive**: profound effects were detected in all the models studied, and in almost all the endpoints examined. At this point a consideration prevailed: what are the effects exerted by DLZ on a more complex vertebrate model? To approach the question, another model species was added to the study, *Xenopus laevis*, whose morphological and genetic similarities with humans make it an excellent model organism for studies on human embryogenesis. Embryonic development was studied in presence of delorazepam and effects on mortality, growth, and teratogenesis were firsts assayed. Results prompted further biochemical, epigenetic, and ultrastructural investigations.

Chapter 1

Benzodiazepines

1.1 Benzodiazepine: history and legislation

Benzodiazepines (BDZs) are one of the world's most widely prescribed pharmacological agents for numerous psycho-physical and neurological conditions including anxiety, insomnia, muscle relaxation, relief of spasticity caused by central nervous system disorders, and epilepsy. They are also used in pre-anesthetic treatment for their amnesic and anxiolytic properties (Cascade and Kalali, 2008).

In 1955, Hoffmann-La Roche chemist Leo Sternbach serendipitously identified the first benzodiazepine, chlordiazepoxide, marketed in 1960 as *Librium.* From this, molecular modifications were pursued in order to obtain enhanced activity: diazepam (*Valium*) was synthesized and commercialized in 1963. In 1975, clonazepam (*Klonopin*) and two years later, lorazepam (*Ativan*) were introduced into the market.

Initially, benzodiazepines appeared to be less toxic and less likely to cause dependence than other similar drugs. They worked on analogous neurotransmitters as barbiturates and exploited similar effects: depression of the central nervous system and production of a sense of calm, but with a specific improvement: lack of respiratory depression, a safety concern related to the use of barbiturates. Medical professionals greeted benzodiazepines enthusiastically at first, so that already in the mid-to-late 1970s, benzodiazepines topped all “most frequently prescribed” lists.

Normal-dose physical dependence was first suspected in the early 1970s but it was not until the early 1980s that scientific evidence was adduced to establish its reality and frequency: the spectre of abuse and dependence was already a reality (Lader, 1991; Wick, 2013). As a result, individual benzodiazepines and the entire class began to appear on guidelines and in legislation, with attempts to limit them to short-term use.

Benzodiazepines are produced by licensed pharmaceutical companies and authorized and marketed according to national legislation. They are prescription-only medicines and are subject to additional restrictions on their supply, use, and possession under drug control laws. They are recommended for short-term use at the lowest possible dose to reduce the risks of tolerance, dependence, and withdrawal symptoms. In most countries, benzodiazepines are controlled under drug control laws and are dispensed only after prescription only, in agreement with the 1971 United Nations Convention on Psychotropic Substances, which currently controls 38 benzodiazepines. These are alprazolam, bromazepam, brotizolam, camazepam, chlordiazepoxide, clobazam, clonazepam, clorazepate, clotiazepam, cloxazolam, delorazepam, diazepam, estazolam, ethyl loflazepate, etizolam (since 2020), flualprazolam (since 2020), fludiazepam, flurazepam, flunitrazepam, halazepam, haloxazolam, ketazolam, loprazolam, lorazepam,

lormetazepam, medazepam, midazolam, nimetazepam, nitrazepam, nordazepam, oxazepam, oxazolam, phenazepam (since 2016), pinazepam, prazepam, temazepam, tetrazepam and triazolam (EMCDDA, 2021)

In 2021, the International Narcotic Control Board (INCB) listed the most marketed benzodiazepines at 21. Italy is the main manufacturer, covering more than 50% of world production, followed by India, Switzerland, China, and the USA. (INCB, 2021).

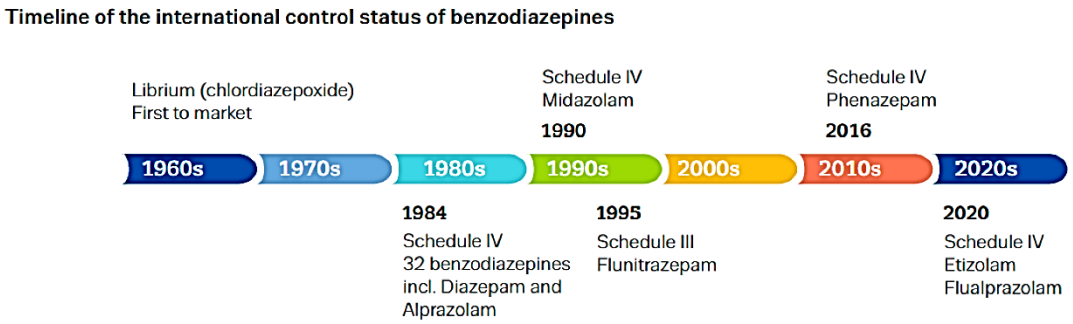

Figure 1. Timeline of the international control status of benzodiazepine (EMCDDA, 2021)

1.2 Structure and classification of benzodiazepines

The term *benzodiazepine* is the chemical name for the heterocyclic ring system, which is a fusion between the benzene and diazepine ring systems. Their core chemical structure is formed by the fusion of a benzene ring and a diazepine ring (Figure 2). The "benzo" prefix indicates the benzene ring fused onto the diazepine ring (Moss, 1998).

Figure 2. Benzodiazepine structure

Different compounds have different side groups attached to this central structure in positions 1, 2, 5, or 7. The different side groups affect the binding of the molecule to the GABA-A receptor, therefore the BDZs' action can vary slightly depending on their nature (Langer et al., 2020) and selectivity with respect to the receptors. This can modulate the pharmacological properties, the potency of the effect, and the pharmacokinetic conditions (duration of the effect, distribution, etc.).

These differences are mainly manifested in the hypnotic/anxiolytic sedative effect, in the time of life, in the elimination velocity, in the hepatic metabolism, in the drug liposolubility, in the administration mechanism, in the potency, in the action onset (fast <1h, medium 1–2h, slow >2h) (Lechuga and Indart, 1996; Ashton, 2002; Danza et al., 2009).

1.2.1 Classification according to the chemical structure

- ***1.4 Benzodiazepines***

  In this category, the diazepine ring has two nitrogen atoms in positions 1 and 4. Depending on the different bonded radicals we can find the keto benzodiazepines, the main class of BZDs such as Diazepam and Delorazepam, the hydroxy benzodiazepines, characterised by the presence of a hydroxyl group in position 3 such as Lorazepam and Oxazepam, and the nitro benzodiazepines, characterised by the presence of a nitro group in position 7 such as Clonazepam and Nitrazepam.

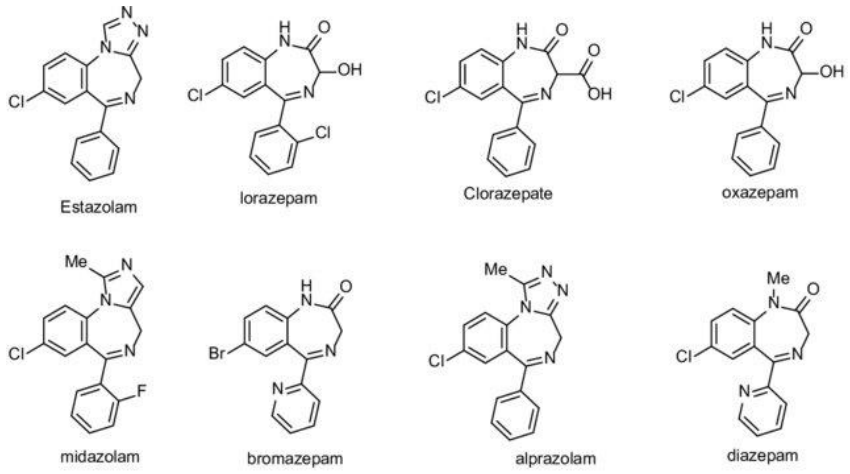

Figure 3. 1,4 benzodiazepines

- ***1.5 Benzodiazepines***

They differ from the previous ones for the position of the carbon and nitrogen atoms in positions 4 and 5 of the diazepine ring, such as Clobazam.

- ***Triazolobenzodiazepines***

These are characterized by the presence of a condensed triazole ring at position 1, such as Triazolam.

- ***Imidazolobenzodiazepines***

Distinguished by the presence of a condensed imidazole ring in position 1, such as midazolam.

1.2.2 Classification according to half-life

Benzodiazepines differ significantly in potency, lipophilicity, elimination half-lives, and onset and duration of action. These differences bring up the difference in their clinical utility and give rise to 3 categories of benzodiazepines: short-acting, intermediate-acting, and long-acting agents (Fig. 4).

	Onset (hours)	Action Duration	Half-Life (hours)	Potency	Equivalent Doses (mg)
flurazepam	1	Long	* 40–250	low	15–30
chlordiazepoxide	1.5	Long	* 36–200	low	10–25
diazepam	1	Long	* 36–200	low	5–10
clorazepate	1	Long	* 36–200	low	7.5–15
clonazepam	1	Long	18–50	high	0.25–0.5
temazepam	0.5	Intermediate	8–22	low	30
lorazepam	2	Intermediate	10–20	high	1
oxazepam	3	Short	4–15	low	15–20
alprazolam	1	Short	6–12	high	0.5
triazolam	0.5	Short	2–5	high	0.25–0.5

* active metabolites.

Figure 4. Pharmacokinetic differences between benzodiazepines (Guina and Merrill, 2018)

- **Short-acting** benzodiazepines have an average elimination half-life of 1-12 hours. This category includes Alprazolam (Xanax), Bromazepam (Lexotan), and Lorazepam (Tavor).
- **Intermediate-acting** benzodiazepines have an average elimination half-life of 24-48 hours. Representative examples of this category include Flunitrazepam (Roipnol), and Nitrazepam (Mogadon).
- **Long-acting** benzodiazepines have an average elimination half-life of more than 48 hours (1-4 days). To this category belong, among others, Delorazepam (EN), Diazepam (Valium), Prazepam (Prazene), and Flurazepam (Dalmadorm). Drugs in this category have long-acting pharmacologically active metabolites (often desmethyldiazepam) and accumulate extensively during multiple dosages (Griffin et al., 2013).

1.3 Benzodiazepine pharmacology

1.3.1 Interaction with GABA-A receptors

BZDs act as positive allosteric modulators on the gamma amino butyric acid (GABA)-A receptor, a ligand-gated chloride-selective ion channel, enhancing the activity of the chief inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Its role is in reducing neuronal excitability and, in humans, it is also responsible for the regulation of muscle tone. This results in the sedative, hypnotic (sleep-inducing), anxiolytic (anti-anxiety), anticonvulsant, and muscle relaxant properties for which the drugs are prescribed. Three GABA receptors exist, A, B, and C. The GABA-A receptor is the only one with which BZDs interact.

The GABA-A receptor comprises five glycoprotein subunits, each with multiple isoforms, arranged like a rosette around a central pore, crossing the cell membrane, which is permeable to chloride and other anions. GABA-A receptors contain 2 α subunits, 2 β subunits, and 1 γ subunit. Each receptor complex has 2 GABA-binding sites but only 1 BZD-binding site. The benzodiazepine binding site is in a specific pocket at the pairing (intersection) of the α and γ subunits. When BZDs bind to the pocket, they induce a conformational change in the GABA-A receptor, allowing GABA to bind. Benzodiazepine site ligands, therefore, do not act directly to open the channel, but rather modulate the capacity of GABA to do so, resulting in augmentation or diminution of its inhibitory effects. When GABA binds with the GABA-A—benzodiazepine receptor complex, it acts as an agonist: inducing conformational changes, which increase the permeability of the central pore to chloride ions. The resulting chloride flux hyperpolarizes the neuron, reducing its excitability and producing a general inhibitory effect on neuronal activity. Classical benzodiazepines in clinical use act to enhance the effectiveness of GABA uniquely by lowering the concentration of GABA required for opening the channel (Nutt and Malizia, 2001). Pharmacological effects of BZs are mediated via positive modulation of four different subtypes of GABA-A receptors, namely those containing the α1, α2, α3, or α5-subunit, in addition to the γ2 subunit (Sieghart, 2006). Sedative effects of BZs are principally attributed to the α1-GABA-A receptor subtype, anxiolytic actions to α2-/α3- containing receptors, anterograde amnesic effects to α1/α5 subtypes and anticonvulsant activity partially to α1-GABA-A receptors (Milić et al., 2012). In addition to GABA and benzodiazepines, other psychoactive compounds, such as barbiturates and anaesthetic steroids, can also bind to the receptor and open the chloride channel.

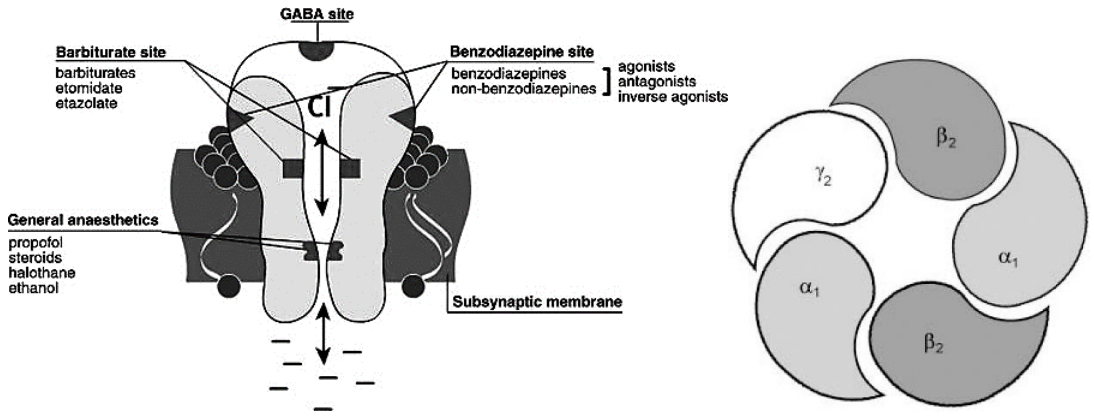

Figure 5. GABA receptor with target sites. The composition of the receptor sub-units, particularly α and γ sub-units, seems to determine the benzodiazepine pharmacology of the receptor, with different subtypes having different sensitivities to benzodiazepine receptor ligands.

Specific receptors for GABA are not restricted to the CNS, indeed GABA-A receptor has been found in a wide range of peripheral tissues, including parts of the peripheral nervous system, endocrines, and non-neural tissues such as smooth muscle and the female reproductive system (Watanabe et al., 2002).

1.3.2 Interaction with Translocator protein (TSPO)

First described as a high-affinity binding site for diazepam in the rat kidney in 1977, this receptor was therefore named as a "peripheral" benzodiazepine receptor (PBR), reflecting its expression in peripheral tissues, in contrast with the "central" benzodiazepine receptor, which is expressed mainly in the central nervous system (CNS). Lately, to represent more accurately its subcellular role and putative tissue-specific functions, a second label was instituted, Translocator Protein (TSPO).

TSPO is an 18 kDa mitochondrial transmembrane protein consisting of 169 aminoacids, which is mainly located in the outer membrane. The three-dimensional structure of TSPO, which is highly hydrophobic and rich in tryptophan, is characterized by five α-helices spanning one phospholipid layer of the mitochondrial membrane (Anholt et al., 1986; Antkiewicz-Michaluk et al., 1988; Bernassau et al., 1993). The TSPO is believed to form a complex with several proteins of the outer and inner mitochondrial membrane collectively known as the mitochondrial permeability transition pore (MPTP), an important regulator of apoptotic and necrotic cell death during injury. Therefore, it mainly regulates cell energy metabolism, transmembrane potential, and sensitivity to reactive oxygen species. TSPO is expressed in glial cells and ependymal cells in the brain, and

also in peripheral tissues, particularly abundant in peripheral endocrine tissues, such as the adrenal glands, testis, and ovary. TSPO density can be modulated under a variety of physiological or pathological conditions (Papadopoulos et al., 2006; Casellas et al., 2002).

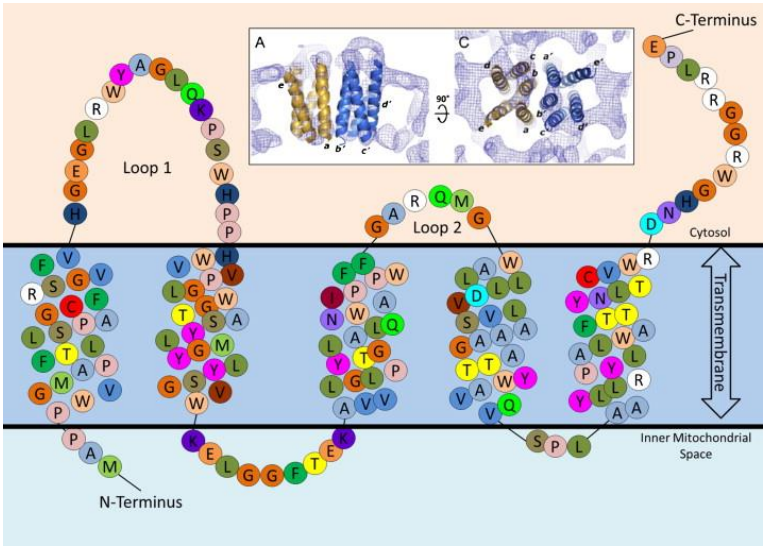

Figure 6. Schematic draw of TSPO. Cytosolic loop 1 and loop 2 are the binding sites for benzodiazepines (Raffa and Pergolizzi, 2019)

Among the functional roles attributed to the TSPO, steroidogenesis is perhaps the best characterized. The TSPO is responsible for the translocation of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. This action is the rate-limiting step of steroidogenesis. Once inside the mitochondria, the cholesterol side-chain cleavage enzyme (CYP11A1 – a member of the cytochrome P450 family) converts cholesterol to pregnenolone. Pregnenolone is recruited to the endoplasmic reticulum to form other important steroid products (Papadopulos et al., 1997).

Besides that, studies of TSPO function have yielded a diverse list of activities, including the regulation of cell death, mitochondrial protein import and cellular proliferation, Ca^{2+} ion regulation, mitochondrial respiration and oxidation, transport of porphyrins and heme biosynthesis and immunomodulation (Austin et al., 2013).

TSPOs also expressed in CNS take part in pathological processes linked to several nervous system disorders, such as epilepsy, cerebral ischemia, nerve injury, and neurodegeneration, by playing important roles in neuronal apoptosis, degeneration and regeneration of glial cells, with similar results also in animal experiments (Lang et al, 2002).

The evidence that TSPO is involved in the secretion of neurosteroids, whose levels have been reported to be changed in several diseases, has suggested that TSPO may be implicated in the pathogenic mechanisms of anxiety. Indeed, the important involvement of TSPO in anxiety in humans has been extensively demonstrated by numerous studies in which TSPO expression levels have been reported to be changed. Specifically, TSPO density is up-regulated in acute stress conditions and down-regulated in chronic or repeated stress (Taliani et al., 2009).

1.4 Benzodiazepines Metabolism

Most compounds undergo extensive phase I metabolic transformations, mainly mediated by the cytochrome P450 (CYP) enzyme family. The compounds are predominantly metabolized by CYP3A4 enzymes. To a lesser extent, CYP3A5, CYP2B6, CYP2C18 and CYP2C19 enzymes are also involved in the metabolism of benzodiazepines (Fukasawa et al., 2007; Gafni et al., 2003; Mizuno et al., 2009). Glucuronidation and acetylation by uridine 5′-diphosphoglucuronosyltransferase (UGT) and N-acetyltransferase 2 (NAT2), respectively, are the major enzymes involved in phase II metabolism of benzodiazepines.

Some metabolites are also marketed as pharmaceutical products, such as temazepam, nordazepam and oxazepam, the main metabolites of diazepam. Some metabolites of triazolobenzodiazepines, for example, the α-OH metabolite in the case of alprazolam and triazolam, show high binding affinities towards GABA-A and are considered to be at least as active as the parent compound (Hester and Von Voigtlander, 1979). On the contrary, 4-OH metabolites generally show reduced biological activity (Baselt, 2011). Significant binding affinity at the GABA-A receptor was also reported for α-OH-midazolam glucuronide, a phase II metabolite of midazolam (Rudge et al., 2006)

1.5 Misuse and Abuse

Despite the worsening public health indicators associated with BZDs use, and although BZDs have been ranked at high risk of dependence among licit and illicit drugs (Nutt et al., 2010), the abuse and misuse of these psychotropics remain overlooked by policymakers and the scientific community (Lembke et al., 2018), probably because the perceived risk associated with benzodiazepines is still underestimated.

The matter of fact is that despite clinical recommendations for use of BZDs (should be considered as a treatment for specific clinical situations and short-term use, no more than 2–4 weeks), long-term

BZD users range from 6 to 76% of total users, a phenomenon affecting almost 2% to 7.5% of the population in developed countries (Fang et al.,2009; Zandonai et al., 2022). Benzodiazepine-related overdose deaths increased by more than 400% from 1996 to 2013 and emergency department visits for benzodiazepines increased by more than 300% from 2004 to 2011. Contextually, the number of benzodiazepine prescriptions not only increased by 67% from the mid-1990s to 2013, but the quantity (i.e., dose equivalents) increased more than 3-fold during this period (Bachhuber et al., 2016; Votaw et al., 2019).

It is estimated that, in the U.S. alone, there are now 94 million prescriptions for various benzodiazepines, nearly one prescription for every three citizens. A total of 30.6 million adults (12.6%) reported benzodiazepine use in the past year—25.3 million (10.4%) as prescribed and 5.3 million (2.2%) misuse. BZDs are also the third most misused illicit or prescription substance among adults and adolescents in the U.S. (Votaw et al., 2019).

A 2008–2009 general population survey of over 20,000 individuals ages 15–64 in Sweden found that 2.2% of participants misused benzodiazepines and other sedatives in the previous year (Abrahamsson and Hakansson, 2015). The same rate of current benzodiazepine misuse was found in a 2008–2009 household survey of 2,280 individuals ages 15 and older residing in Thailand (Puangkot et al., 2010). Similar rates of past-year misuse have been reported in general population samples in Brazil (Galduróz et al., 2005) and Australia (Hall et al., 1999). Although few studies outside of the U.S. have examined trends in misuse, a nationwide study of 179,114 school-age respondents (14–18 years old) in Spain found that the prevalence of tranquillizer, sedative, and sleeping pill misuse increased from 2.4% in 2004 to 3.0% in 2014 (Carrasco-Garrido et al., 2018).

According to a recent report, benzodiazepine misuse is most common in young adults. The highest past-year rate of combined sedative/tranquillizer misuse was observed among 18 to 25-year-olds (5.8%), followed by 26 to 34-year-olds (4%). In the U.S., the typical age of onset of benzodiazepine misuse is during early adulthood (18–25 years) (Boyd et al., 2018). It seems that the transition to college may be a particularly risky time; a study of college sophomores found a 102.9% increase in the prevalence of misuse from pre-college – one of the greatest rates of increase among all substances during this period (Arria et al., 2008; Votaw et al., 2019).

Little is known about benzodiazepine misuse in older adults, despite high rates of prescribing in this group. Rates of tranquillizer and sedative misuse are lower in adults over the age of 50, as compared to younger age groups, anyway, the proportion of individuals with past-year tranquillizer misuse who are over the age of 50 doubled from 2005 to 2006 to 2013–2014 (from 7.9% to 16.5%; Palamar et al., 2019).

Regarding the reasons leading to the abuse, over 75% of NSDUH (National Survey on Drug Use and Health) respondents with past-year tranquilizer misuse reported that they misused prescription to help with conditions for which benzodiazepines are indicated, such as sleep, tension, or emotions. They are often used to self-manage psychiatric disorders, anxiety, and insomnia and as a way of coping with traumatic experiences, pain and loss, boredom, and isolation. They are also misused out of curiosity or for their pleasurable effects and their ability to both alter perceptions of time and enhance the effects of opioids and other substances (Chen et al., 2011; Votaw et al., 2019)

1.6 Consumption in Italy

In Italy, the cost of benzodiazepines is not reimbursed by the National Health Service, so data on their use are difficult to gather and can be inferred only from specific questionnaires and information about sales. A study dated 1996 reported that over half of the people who took benzodiazepines (9% of the population, in 1996) were chronic consumers (daily use, for more than 6 months) and, of these, 15%–44% were addicted (Magrini et al., 1996)

According to AIFA, Italy is not among the European countries with the highest consumption of BZD, although there was a more than double increase in consumption between 2000 and 2015. From a more recent study on the consumption of psychoactive drugs in Italy between 2015 and 2017, what emerges was that the trend in the use of these medicines was almost stable. An increase in the utilization of BZDs was recorded in 2017, when consumption raised to about 50 DDD/1000 * inhabitants per day, with an increase of about 8% compared to the previous year (AIFA, 2018). *(* the mean number of defined daily doses (DDD) per 1000 inhabitants*).

According to the National Report on the Use of Medicines in Italy of 2021, benzodiazepines (together with contraceptives, drugs for erectile dysfunction, NSAIDs and antipyretics) were class C drugs most purchased by Italians at least for the last 5 years (class C drugs, classified by AIFA, are those medicines that are fully paid for by the patient and, therefore, not reimbursed by the NHS). In the 2021 report at the top of the ranking, benzodiazepine derivatives (anxiolytics) recorded an expenditure of 400.9 million euros, equal to 11 .6% of the total expenditure.

ATC I	Categoria terapeutica	DDD/1000 ab *die*	Δ % 21-20	Spesa *(milioni)*	%*	Δ % 21-20
N	Derivati benzodiazepinici (ansiolitici)	27,3	-2,8	400,9	11,6	0,0
N	Anilidi	7,5	4,2	319,3	9,2	8,5
G	Farmaci usati nella disfunzione erettile	2,1	10,5	237,7	6,9	11,6
G	Associazioni fisse estro-progestiniche	19,9	-1,0	214,0	6,2	2,4
N	Derivati benzodiazepinici (ipnotici e sedativi)	21,2	-0,9	143,7	4,1	2,1
D	Corticosteroidi attivi, associazioni con antibiotici	4,9	6,5	94,1	2,7	6,2
A	Lassativi ad azione osmotica	2,2	10,0	76,0	2,2	17,5
J	Vaccini influenzali	0,1	-	74,9	2,2	228,5
N	Analoghi delle benzodiazepine	5,8	3,6	73,3	2,1	5,8
S	Corticosteroidi antimicrobici in associazione	3,1	6,9	71,9	2,1	9,4
M	Altri miorilassanti ad azione centrale	1,2	9,1	69,2	2,0	15,1
R	Corticosteroidi	4,8	2,1	68,8	2,0	5,2
N	Altri psicostimolanti e nootropi	1,3	8,3	63,8	1,8	15,2
M	Altri miorilassanti ad azione periferica	0,0	-	58,3	1,7	35,6
B	Eparinici	2,4	9,1	56,1	1,6	13,3
N	Preparati antivertigine	2,9	3,6	53,0	1,5	5,6
R	Mucolitici	4,2	-12,5	47,4	1,4	-8,7
M	Bifosfonati	0,0	-	45,2	1,3	5,9
G	Preparati sequenziali estro-progestinici	3,5	0,0	44,7	1,3	6,4
N	Benzamidi	0,3	0,0	39,7	1,1	2,6
	Totale prime 20	**114,8**	**0,3**	**2252,1**	**65,0**	**8,9**
	Totale	**212,8**	**7,9**	**3465,4**	**100,0**	**6,0**

* calcolata sul totale della spesa

Figure 7. Top twenty class C therapeutic categories with the most expensive prescription in 2021: comparison 2020-2021

The territorial BZDs consumption went from 40 DDD/1000 inhabitant for die in 2015 to 54,3 DDD/1000 in 2021, with an average annual variation of +5.2%. Alprazolam and lorazepam, in 2021, remained the highest spending substances and account for 41% of the total benzodiazepines.

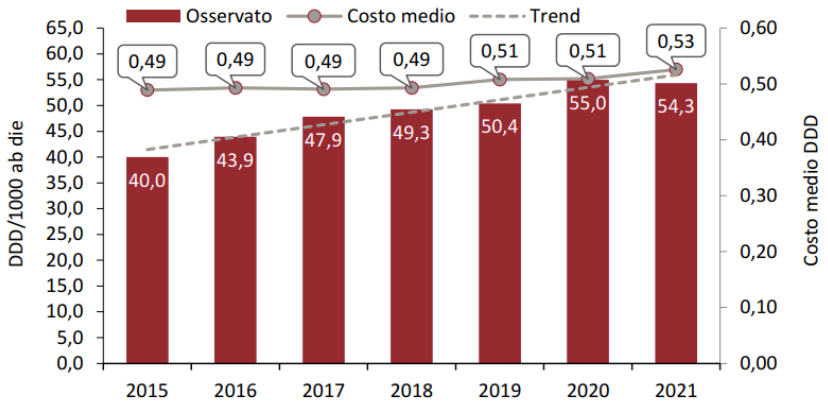

Figure 8. BZDs: time trend 2015-2021 of territorial consumption and average cost

Sottogruppi e sostanze	Spesa *pro capite*	Δ % 21-20	CAGR % 15-21	DDD/1000 ab *die*	Δ % 21-20	CAGR % 15-21	Costo medio DDD	Δ % 21-20
Ansiolitici	6,77	0,7	5,7	27,3	-2,3	4,3	0,68	3,3
Ipnotici	2,43	2,9	6,6	21,2	-1,0	5,2	0,31	4,2
Sedativi	1,24	6,5	12,1	5,8	4,3	11,0	0,58	2,4
Benzodiazepine	**10,43**	**1,8**	**6,5**	**54,3**	**-1,1**	**5,2**	**0,53**	**3,3**
alprazolam	2,28	2,4	8,6	10,4	-0,5	6,9	0,60	3,2
lorazepam	2,00	-0,9	3,4	10,4	-4,0	2,1	0,53	3,5
zolpidem	1,19	6,7	12,5	5,6	4,5	11,3	0,58	2,4
lormetazepam	1,11	3,7	6,6	15,2	-0,6	5,3	0,20	4,5
bromazepam	0,85	-0,9	4,7	1,4	-4,7	3,1	1,65	4,3
delorazepam	0,82	3,2	6,4	2,6	0,7	5,5	0,88	2,8
triazolam	0,77	3,0	7,2	3,7	-1,6	5,4	0,57	5,0
diazepam	0,38	-2,2	5,4	1,3	-2,9	4,9	0,77	1,0
brotizolam	0,33	2,0	6,3	1,4	-4,0	4,4	0,64	6,6
flurazepam	0,15	1,5	6,5	0,7	0,2	5,1	0,62	1,5

Figure 9. BZDs: *pro capite* expenditure and consumption (DDD/1000 inhabitants per day) comparison 2015-2021

All the Italian regions, the report observes, with the exception of Valle d'Aosta, recorded an increase in the consumption of prescription drugs, and consequently, an increase in spending volumes. The moderate variability of consumption and expenditure for self-medication and class C prescription drugs is mainly explained by differences in income between the Regions but also by a different attitude of doctors and patients in the use of these drugs.

For example, while for class C drugs with prescription, the *pro capite* expenditure of Campania is almost double that of the Autonomous Province of Bolzano (81.60 euros vs 41.40), with regard to self-medication drugs the expenditure *pro capite* in Liguria is 72% higher than in Basilicata (48.20 euros vs 28.10) and more than 21% more is spent in the North than in the South. The greatest increases in the consumption of class C drugs were recorded in the Marches (+35.6%), in Umbria (+28.0%).

Regione	Classe C con ricetta			
	Spesa *pro capite*	Δ % 21-20	DDD/1000 ab *die*	Δ % 20-19
Piemonte	61,20	6,8	227,8	3,6
Valle d'Aosta	51,60	-4,4	236,2	-2,6
Lombardia	62,00	6,3	222,0	6,3
PA Bolzano	41,40	9,8	145,5	2,5
PA Trento	44,70	4,0	188,7	10,8
Veneto	51,50	3,0	206,3	6,3
Friuli VG	47,50	5,1	191,6	8,0
Liguria	70,00	3,7	272,8	0,3
Emilia R.	55,90	5,5	220,3	9,7
Toscana	57,70	4,3	244,3	7,2
Umbria	54,30	21,5	196,0	28,0
Marche	55,10	23,5	222,6	35,6
Lazio	60,50	4,1	208,1	6,4
Abruzzo	48,90	5,8	161,5	4,9
Molise	47,70	16,6	157,3	2,3
Campania	81,60	12,7	226,5	8,8
Puglia	49,80	5,5	177,5	7,1
Basilicata	42,40	5,5	178,4	20,6
Calabria	54,10	12,5	218,7	11,6
Sicilia	50,80	2,6	183,0	11,5
Sardegna	55,00	5,8	219,2	2,8
Italia	**58,50**	**6,8**	**212,8**	**7,9**
Nord	58,10	5,4	219,6	5,9
Centro	58,40	7,4	220,7	11,1
Sud ed Isole	59,10	8,0	198,0	8,7

* sono inclusi i farmaci classificati in C-Non Negoziata

Figure 10. Territorial pharmaceutical prescription 2021 for class C prescription drugs: comparison 2020-2021

1.7 Benzodiazepine use after COVID-19 pandemic

The COVID-19 outbreak has led people to realize a drastic changes in lifestyle, with social distancing, periods of social isolation and loneliness, which have resulted in negative consequences on mental well-being. It was found that people were three times more likely to have anxiety or depressive disorders in 2020 compared to the previous year, and more than one in three individuals presented one or both disorders (Twenge & Joiner, 2020). Significant was also the psychological impact of COVID-19 among individuals who tested positive, and experienced anxiety symptoms, fear, and a lack of hope regarding the uncertainties in treatment and health outcomes. According to a survey conducted by the American Psychiatric Association in March 2020, 48% of the general population reported anxiety, 36% reported that the pandemic was severely affecting their mental health, and 19% reported insomnia. For this reason, some authors have typified COVID-19 as a "psychiatric epidemic". In this scenario of anxiety, panic attack and insomnia, depressive symptoms, anger, and fear, benzodiazepine prescriptions have risen, with also anti-anxiety medications becoming easier to access through telemedicine (EMCDDA, 2021; Turna et al., 2021).

In 2021, a survey to gather information about the use of benzodiazepines during the COVID-19 pandemic in the general population was conducted. Of a total number of 240 participants, 65% consumed benzodiazepines due to anxiety, 41.7% for tension, 45.8% due to insomnia, 36.7% due to fear, and 42.5% due to restlessness. One-fourth of the benzodiazepines were prescribed by a family doctor, and one-fifth of the respondents assumed the drug on their own initiative without a doctor's advice and 4% without a prescription. Overall, the use of benzodiazepines shows an increase rate of 20.9% (Isjanovski et al., 2021).

The web interest and, therefore, the probable consumption of benzodiazepines has significantly increased also in Italy after the COVID-19 pandemic. An infodemiological analysis, conducted to gather more information on the increase in consumption of BZDs, reported an increase of the 18% for Alprazolam, 13% for Bromazepam, 14% for Clonazepam, and 8% for Lorazepam (Mattiuzzi et al., 2022).

1.8 Delorazepam

Delorazepam (Chlordesmethyldiazepam) is a long-acting benzodiazepine, derived from diazepam. Chemically it differs from diazepam by demethylation in position 1 and the introduction of another chlorine atom in position 2. It has found a large application in treating insomnia and anxiety due to its high elimination half-life (80–115 h) and greater potency compared to diazepam (1 mg delorazepam = 10 mg diazepam) (Altamura et al., 2013); it produces a major active metabolite known as lorazepam, commercially available, that represents about 15 – 34% of the parent drug (Bareggi et al., 1988).

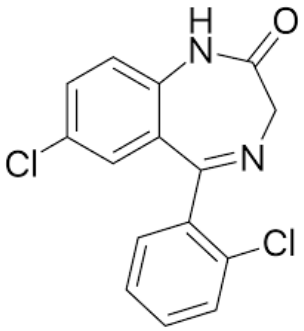

Figure 11. Delorazepam

Like all benzodiazepines, delorazepam acts at limbic, thalamic and hypothalamic levels and can produce depression of the central nervous system: sedation, hypnosis, decreased anxiety, relaxation of skeletal muscles and reduced aggression (in laboratory animals). It is indicated in the treatment of anxiety, psychosis with an anxiety component, insomnia and epilepsy (Bareggi et al., 1986; Moosmann and Auwärter, 2018). It is therefore widely marketed, especially in Italy, where it is among the most consumed and abused benzodiazepines (Lombardi et al., 2021).

A study carried out in in Italy, in the 2020, aimed at studying frequency and characteristics of BZDs causing adverse events requiring emergency intervention, or even hospitalization. Delorazepam

ranked first causing adverse effects in 11.6% of cases, together with lorazepam (21.4%), alprazolam (18.2%), and bromazepam (8.9%). It required an emergency intervention in 52.5% of patients.

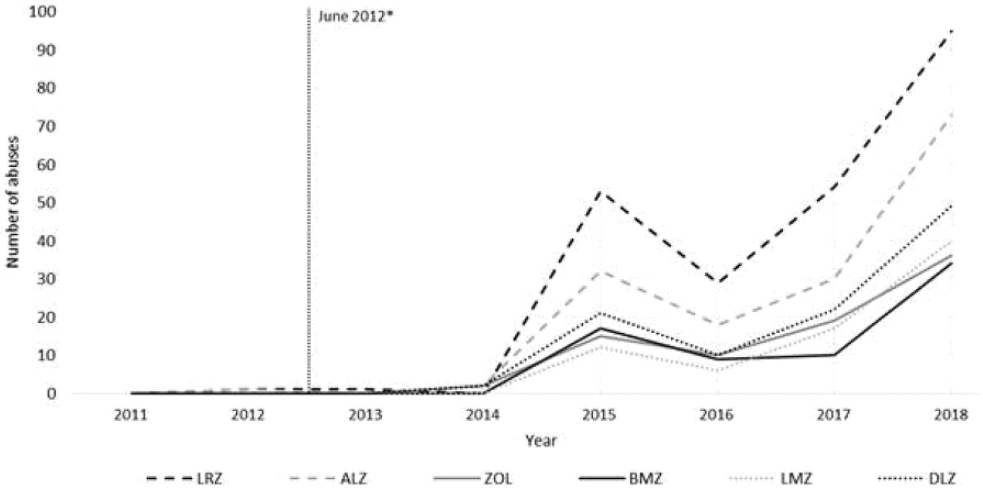

Figure 12. Trend of "abuse/misuse" and "overdose" reporting (2012–2018). ALZ alprazolam, BMZ bromazepam, DLZ delorazepam, LMZ lormetazepam, LRZ lorazepam, ZOL zolpidem (Lombardi et al., 2020).

1.9 Environmental contamination by delorazepam

Environmental pollution by benzodiazepines is increasing generating a major threat to aquatic ecosystems worldwide. Several biochemically active BZDs have been found in aquatic systems globally, due to the vast and constant release in wastewater (Bade et al., 2020).

A recent review analyzed 219 scientific papers from which 1642 data/entries were obtained, each corresponding to one target compound in one aqueous matrix. Results demonstrate a strict correlation between the country's average income and the concentration of drugs in surface water (Cunha et al., 2017). Among the drugs investigated, citalopram was the most cited followed by oxazepam, and lorazepam.

Abbreviations

D-WWI	Domestic wastewater influent
D-WWE	Domestic wastewater effluent
DI-WWI	Domestic and industrial wastewater influent
DI-WWE	Domestic and industrial wastewater effluent
DH-WWI	Domestic and hospital wastewater influent
DH-WWE	Domestic and hospital wastewater effluent
DHI-WWI	Domestic, hospital, and industrial wastewater influent
DHI-WWE	Domestic, hospital, and industrial wastewater effluent
S-WWI	Slaughterhouse wastewater influent
S-WWE	Slaughterhouse wastewater effluent
LDI-WWI	Leachate, domestic and industrial wastewater influent
LDI-WWE	Leachate, domestic and industrial wastewater effluent
PD-WWI	Predominantly domestic wastewater influent
PD-WWE	Predominantly domestic wastewater effluent
H-WWI	Hospital wastewater influent
H-WWE	Hospital wastewater effluent
DW	Drinking water
SW	Surface water
GW	Groundwater
SeaW	Seawater
EW	Estuary water
LE	Leachate from landfills

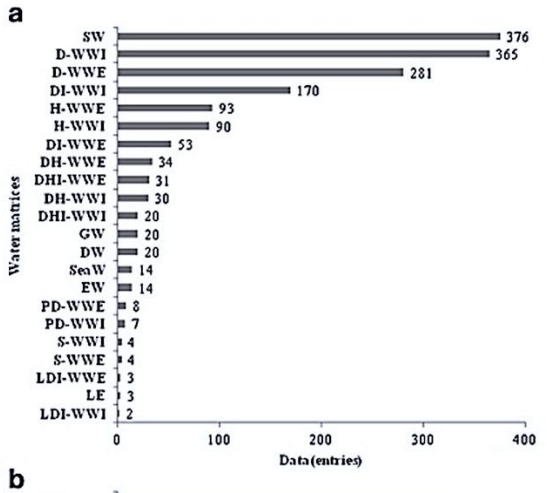

Fig. 1 **a** Amount of data/entries per aqueous matrix (22 in total) contaminated with seven psychoactive drugs included in this review; **b** amount of data per psychoactive drug found in 219 scientific papers

Figure 13. a) Amount of data/entries per aqueous matrix (22 in total) contaminated with seven psychoactive drugs; b) amount of data per psychoactive drug found in 219 scientific papers. Adapted from Cunha et al., 2017.

Lorazepam and its degradation product, including delorazepam, are present at concentrations ranging from nanograms to micrograms, the higher concentrations being registered in hospital wastewater. As far as Italy is concerned, there is no information on de/lorazepam concentration in the rivers. In contrast, data can be found for European basins. In Galicia (Spain), for example, lorazepam is detectable at an average concentration of 562 ng/L with a maximum on influent wastewater of over 10 µg/L (Esteban et al., 2012).

Table 2
Percentage (%) of samples and average concentrations (ng/L) with detectable concentrations in the influent (I) and effluent (E) wastewater of sewage treatment plants, downstream rivers (DSSTP) and tap water in the watersheds of Galicia in 2008-2009.

	N	I (%)	Average (ng/L)	N	E (%)	Average (ng/L)	N	DSSTP (%)	Average (ng/L)	N	Tap water (%)	Average (ng/L)
Antidepressants												
Amitriptiline	15	20	24	15	27	22	6	-	-	75	-	-
Citalopram	15	33	114	15	73	149	6	33	10	75	-	-
Clomipramine	15	-	-	15	7	4	6	-	-	75	1	27
Fluoxetine	15	7	16	15	60	28	6	-	-	75	-	-
Nortriptiline	15	-	-	15	13	11	6	-	-	75	-	-
Sertraline	15	13	113	15	27	33	6	-	-	75	-	-
Venlafaxine	15	47	401	15	67	317	6	50	67	75	1	44
Antiepileptics												
Carbamazepine	15	30	73	15	40	181	6	33	63	75	-	-
Anxiolytics												
α-Alprazolam	15	13	20	15	-	-	6	-	-	75	-	-
Alprazolam	15	20	27	15	20	17	6	17	17	75	1	11
Lorazepam	15	87	10598	15	67	689	6	50	167	75	3	562
Nordiazepam	15	13	16	15	40	17	6	-	-	75	-	-
Oxazepam	15	47	83	15	67	84	6	-	-	75	-	-
Tetrazepam	15	40	92	15	53	64	6	-	-	75	-	-

I: percentage of samples with detectable concentrations in influent wastewater; E: percentage of samples with detectable concentrations in effluent wastewater; DSSTP: percentage of samples with detectable concentrations 50 meters downstream of sewage treatment plants.

Figure 14. Detection of lorazepam in watersheds of Galicia. (Esteban et al., 2012)

Table 1 Minimum and maximum concentrations (ng L^{-1}) of each target psychoactive drug in each investigated aqueous matrix (n = number of observations by category)

Aqueous matrix	Alprazolam		Bromazepam		Citalopram		Clonazepam		Diazepam		Lorazepam		Oxazepam	
	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
D-WWI	1.74 (n = 34)	2580	1.3 (n = 7)	3662	0.4 (n = 131)	650	– (n = 0)	–	1.7 (n = 33)	196	2.1 (n = 32)	10,598	5.6 (n = 128)	3300
D-WWE	0.81 (n = 37)	176	0.5 (n = 9)	15,542	1.5 (n = 78)	2100	– (n = 0)	–	0.642 (n = 47)	720	1.2 (n = 55)	920	6.3 (n = 55)	3817
DI-WWI	8.77 (n = 2)	12.6	68.5 (n = 2)	94.2	5.9 (n = 78)	359	– (n = 0)	–	3.2 (n = 7)	69.3	33 (n = 12)	443.8	9.3 (n = 69)	882
DI-WWE	6.2 (n = 3)	10	25.8 (n = 2)	37.8	7.7 (n = 8)	840,000	– (n = 0)	–	3.45 (n = 6)	63.2	22.8 (n = 17)	399.8	10.8 (n = 17)	908
DH-WWI	19.1 (n = 2)	49.1	– (n = 0)	–	12.7 (n = 10)	719	– (n = 0)	–	2 (n = 8)	434	41 (n = 6)	446	54 (n = 4)	1160
DH-WWE	11.3 (n = 2)	33.5	– (n = 0)	–	9.2 (n = 14)	240	– (n = 0)	–	6.53 (n = 7)	22	80 (n = 5)	347	28 (n = 6)	1130
DHI-WWI	– (n = 0)	–	– (n = 0)	–	35.7 (n = 12)	2040	– (n = 0)	–	590 (n = 2)	1180	109.3 (n = 3)	330.2	77 (n = 3)	600
DHI-WWE	– (n = 0)	–	– (n = 0)	–	12.2 (n = 4)	7525	– (n = 0)	–	45.5 (n = 3)	4000	31 (n = 11)	246.8	50 (n = 3)	400
S-WWI	– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	9 (n = 4)	16	– (n = 0)	–	– (n = 0)	–
S-WWE	– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	2 (n = 4)	8	– (n = 0)	–	– (n = 0)	–
LDI-WWI	10 (n = 1)		– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	30 (n = 1)		– (n = 0)	–
LDI-WWE	10 (n = 1)		– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	10 (n = 1)		30 (n = 1)		– (n = 0)	–
PD-WWI	69 (n = 1)		– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	62 (n = 2)	85	31 (n = 2)	95	50 (n = 2)	210
PD-WWE	25 (n = 2)	57	– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	28 (n = 2)	100	22 (n = 2)	47	50 (n = 2)	210
H-WWI	4.58 (n = 16)	168	40 (n = 2)	158	9.43 (n = 23)	888	– (n = 0)	–	2 (n = 17)	244	20 (n = 22)	1325	28 (n = 10)	2200
H-WWE	12.2 (n = 2)	29	125 (n = 7)	205	19 (n = 19)	162	49 (n = 14)	145	2 (n = 32)	660	38 (n = 15)	205	17 (n = 8)	7434
DW	2.3 (n = 6)	11	– (n = 0)	–	1.5 (n = 2)	3.4	– (n = 0)	–	0.47 (n = 4)	23.5	4 (n = 2)	562	2.5 (n = 6)	91
SW	0.3 (n = 24)	5900	0.73 (n = 0)	19	0.33 (n = 107)	76,000	– (n = 0)	–	0.14 (n = 91)	625	1.6 (n = 85)	705.5	1.3 (n = 62)	1400
GW	6.4 (n = 1)		– (n = 0)	–	13.1 (n = 4)	1400	– (n = 0)	–	3.88 (n = 6)	35.1	1.2 (n = 5)	54	10 (n = n = 4)	210
SeaW	– (n = 0)	–	– (n = 0)	–	0.9 (n = 13)	27	– (n = 0)	–	– (n = 0)	–	41.8 (n = 1)		– (n = 0)	–
EW	– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	2.68 (n = 3)	8.6	1.2 (n = 5)	5.9	12 (n = 6)	30
LE	– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	– (n = 0)	–	89,900 (n = 1)		20 (n = 2)	3760

Minimum concentrations refer to minimum quantifiable concentrations

D-WWI domestic wastewater influent; *D-WWE* domestic wastewater effluent; *DI-WWI* domestic and industrial wastewater influent; *DI-WWE* domestic and industrial wastewater effluent; *DH-WWI* domestic and hospital wastewater influent; *DH-WWE* domestic and hospital wastewater effluent; *DHI-WWI* domestic, hospital, and industrial wastewater influent; *DHI-WWE* domestic, hospital, and industrial wastewater effluent; *S-WWI* slaughterhouse wastewater influent; *S-WWE* slaughterhouse wastewater effluent; *LDI-WWI* leachate, domestic, and industrial wastewater influent; *LDI-WWE* leachate, domestic, and industrial wastewater effluent; *PD-WWI* predominantly domestic wastewater influent; *PD-WWE* predominantly domestic wastewater effluent; *H-WWI* hospital wastewater influent; *H-WWE* hospital wastewater effluent; *DW* drinking water; *SW* surface water; *GW* groundwater; *SeaW* seawater; *EW* estuary water; *LE* leachate from landfills

Environ Sci Pollut Res (2017) 24:24076–24091

Figure 15. Minimum and maximum concentrations (ng L−1) of each target psychoactive drug in each investigated aqueous matrix (n = number of observations by category, focus on lorazepam. (Cunha et al., 2017)

Although diazepam and other benzodiazepines has been proven to be toxic for several microorganisms (Cerveny et al., 2020, Silva et al., 2020, Oggier et al., 2010; Lebreton et al., 2021b, Ogueji et al., 2017) for de/lorazepam no ecotoxicological assay is reported in scientific literature. Delorazepam is detected in human remains, for forensic scopes (Bonete et al., 2018; Gerace et al., 2015) but no information can be found on toxicity in conventional toxicological models such as the crustacean *Daphnia magna* or the bacteria *Vibrio fischeri* (ISPRA, 2011). No information is also available for models such as *Artemia salina*, or other copepods, molluscs or other invertebrates that play a key role in the ecosystems.

Chapter 2

Xenopus laevis

2.1 Environmental concentrations of a delorazepam-based drug impact on embryonic development of non-target *Xenopus laevis*

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2.2 Water contamination by delorazepam induces epigenetic defects and genomic instability in the embryos of the clawed frog *Xenopus laevis*

Submitted

2.3 Structural and functional damage to retina, intestine and skeletal muscle in *Xenopus laevis* embryos exposed to the commonly used psychotropic benzodiazepine delorazepam

Submitted

2.1

Environmental concentrations of a delorazepam-based drug impact on embryonic development of non-target *Xenopus laevis*

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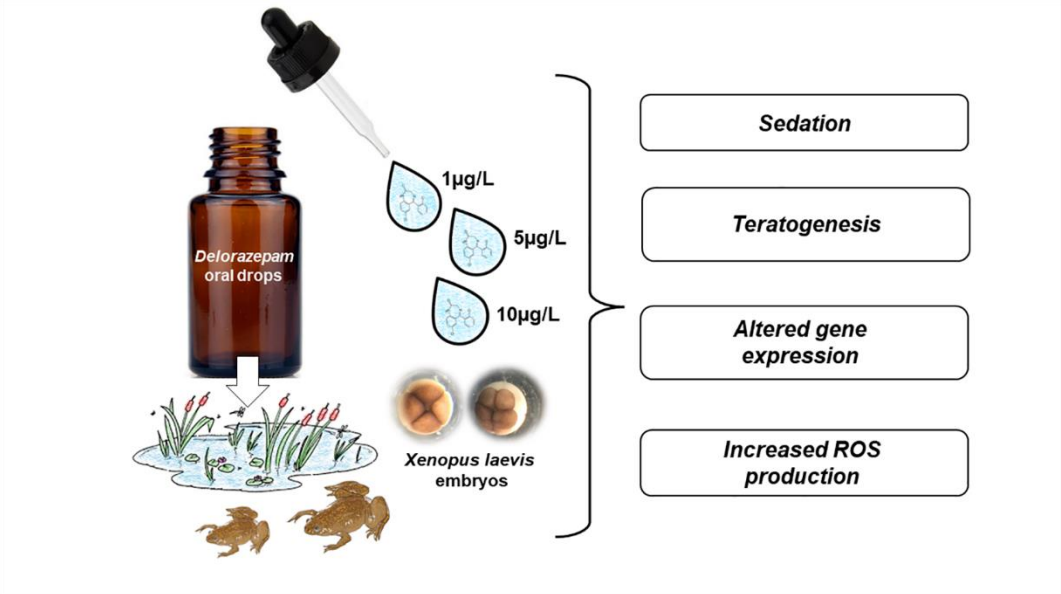


Highlights

- Benzodiazepines are an emerging class of water pollutants
- The effects of a delorazepam-based drug were evaluated on *Xenopus laevis* embryos, amphibian model
- Results demonstrate that delorazepam sedates the embryos and is teratogenic
- Delorazepam increased ROS production, lipid hydroperoxides levels, GPX and GR activity
- Delorazepam alters the expression of developmental and pro-inflammatory cytokines genes

Abstract

Benzodiazepines, psychotropic drugs used for treating sleep disorders, anxiety and epilepsy, represent a major class of emerging water pollutants. As occurs for other pharmaceutical residues, they are not efficiently degraded during sewage treatment and persist in effluent waters. Bioaccumulation is already reported in fish and small crustaceans, but the impact and consequences on other "non-target" aquatic species are still unclear and nowadays of great interest. In this study, we investigated the effects of a pharmaceutical preparation containing the benzodiazepine delorazepam on the embryogenesis of *Xenopus laevis*, amphibian model species, taxa at high risk of exposure to water contaminants. Environmental (1 µg/L) and two higher (5 and 10 µg/L) concentrations were tested on tadpoles up to stage 45/46. Results demonstrate that delorazepam interferes with embryo development and that the effects are prevalently dose-dependent. Delorazepam reduces vitality by decreasing heart rate and motility, induces marked cephalic and abdominal edema, as well as intestinal and retinal defects. At the molecular level, delorazepam increases ROS production, modifies the expression of some master developmental genes and pro-inflammatory cytokines. The resulting stress condition significantly affects embryos' development and threatens their survival. Similar effects should be expected as well in embryos belonging to other aquatic species that have not been yet considered targets for these pharmaceutical residues.

Keywords: environmental toxicity; FETAX test; teratogenicity, gene expression; oxidative stress.

Introduction

Benzodiazepines (BZDs), psychotropic drugs used for treating insomnia and anxiety (Argyropoulos et al., 1999), are worldwide one of the most prescribed remedies (Schmitz, 2016; Nunes et al., 2019). Massive use and abuse (Votaw et al., 2019) result in a vast and constant release of these drugs and/or their active metabolites in the wastewater (Bade et al., 2020). Since they are not efficiently degraded during sewage treatment (Patel et al., 2019), BZDs accumulate in effluent waters and sediments (Klaminder et al., 2015; Lei et al., 2021), reaching concentrations ranging from µg/L to ng/L (Calisto & Estevens, 2009). As a consequence, BZDs represent nowadays an important class of emerging pollutants (Nunes et al., 2019) and therefore a potential environmental hazard, even at low concentrations, especially for aquatic species with which they inevitably come into contact (Klaminder et al., 2015). GABA receptors, targets for BZDs, are evolutionary very conserved, from

bacteria (Guthrie et al., 2000) to animals (Furuhagen et al., 2014) and therefore large-scale effects are foreseeable. BZDs bioaccumulate in invertebrates (Lebreton et al., 2021a) and vertebrates, inducing relevant behavioral (Cerveny et al., 2020) and physiological alterations (Silva et al., 2020), including interferences with gene expression, enzymes activities (Oggier et al., 2010; Lebreton et al., 2021b) and oxidative stress (Ogueji et al., 2017).

The behavior and fate of BZDs in the aquatic environment are still not fully clear and so are the effects exerted on non-target species which may come accidentally into contact with these drugs. In particular, not very much is known about the effects on amphibians even if they are a class of vertebrates at high risk of exposure being bound to the aquatic environment both during embryonic development and adult life. In this study, therefore, we investigated the effects of a benzodiazepine delorazepam-based drug (DLZ) on the embryo development of a model species, *Xenopus laevis,* widely used in toxicology and environmental studies (Carotenuto et al., 2020; Carotenuto et al., 2022).

Delorazepam, a derivative of diazepam, is one of the benzodiazepines with the highest elimination half-life (80-115 hours) ant produces a major active metabolite known as lorazepam that represents about 15 – 34 percent of the parent drug (Bareggi et al., 1988). Like all benzodiazepines, it has anxiolytic, skeletal muscle relaxant, and hypnotic properties (Bareggi et al., 1986; Moosmann et al., 2018).

Xenopus embryos were exposed to a largely consumed pharmaceutical product (oral drops) containing delorazepam at a concentration of 1 mg/ml. Preparation was used as it is, assuming that trace components are not relevant functionally or toxicologically. Preparation was diluted to a final concentration of DLZ of 1 µg/L, calculated considering the average concentration of different benzodiazepines in European waste and coastal waters (Fick et al., 2017; Calisto & Estevens, 2009). Two higher concentrations were also tested, 5 and 10 µg/L, for comparison and to mimic the simultaneous exposure to multiple BZDs occurring in nature.

The effects of the drug were determined by a modified version of the FETAX test and its conventional endpoints (Bernardini et al, 1994; Carotenuto et al., 2021): mortality, length, and occurrence of malformation, *in toto* and at the retinal level, proven target of embryo's toxicity (Hauptman et al.,1993; Simoniello et al., 2014). Following the occurrence of malformations, a preliminary gene expression analysis was also carried out to assess the possible influences of DLZ on the expression of early development genes, on the cytokine-mediated immunological response, and on the detoxification processes (see Table S1 and Carotenuto et al., 2021). In addition, in consideration of the sedative activity of DLZ, the impacts on embryonic swimming performance and heartbeat rate

were determined. Changes in redox state were evaluated by ROS content analysis and by determining lipids oxidative damage, antioxidant enzyme activity (glutathione peroxidase and reductase), and i*n vitro* susceptibility to oxidants.

2. Materials and methods

2.1 Animals

Adult *Xenopus laevis*, obtained from Nasco (Fort Atkinson, Wisconsin, USA), were kept and used at the Department of Biology of the University of Naples, Federico II, according to the guidelines and policies dictated by the University Animal Welfare Office in agreement with international rules and strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health of the Italian Ministry of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Naples Federico II (Permit Number: 2014/0017970). All procedures were performed according to Italian ministerial authorization (DL 116/92) and European regulations on the protection of animals employed for experimental and other scientific purposes. All surgical procedures were performed under tricaine (MS222, Sigma) and organized to minimize suffering. To obtain eggs, *X. laevis* females were injected in the dorsal lymphatic sac with 500 units of Gonase (AMSA) in amphibian Ringer solution (111 mM NaCl, 1.3 mM $CaCl_2$, 2 mM KCl, 0.8 mM $MgSO_4$, in 25 mM Hepes, pH 7.8). Fertilized eggs and embryos were obtained by standard insemination methods (Bernardini et al, 1994) and staged according to Nieuwkoop and Faber (1956).

2.2 Embryos' treatment

Three *in vitro* fertilizations were performed. For each fertilization, triplicate Petri dishes were set for controls (3 dishes containing 10 embryos for a total of 30 embryos) and delorazepam treatments (3 dishes containing 10 embryos for each concentration, for a total of 90 embryos). The experiment in triplicate produced a total of 360 embryos, 270 of which were exposed to the drug. The conventional FETAX assay was modified by anticipating the contact of the embryos with the drug at stage 4/8 cell, to emulate the environmental situation of contact with the drug and to study the effects on early development. 10 embryos at stage 4/8 for each treatment were selected for testing and placed in a 10 cm diam glass Petri dish containing 50 mL of FETAX solution (Frog Embryo Teratogenesis Assay-Xenopus pH 7.4; 106 mM NaCl, 11 mM $NaHCO_3$, 4 mM KCl, 1 mM $CaCl_2$, 4 mM $CaSO_4$, 3 mM $MgSO_4$), (Mouche et al., 2017).

For the treatment, a largely consumed pharmaceutical product was used. In form of oral drops, it contains the active principle delorazepam at a 1 mg/ml concentration and excipients in unspecified quantities (purified water, ethanol, glycasol N, glycerol, propylene glycol, sodium saccharin).

Solutions were prepared by dissolving the drug in FETAX solution with different dilutions to obtain 1 µg/L, 5 µg/L, and 10 µg/L. Sibling embryos grown in FETAX solution were used as controls. All embryos were exposed up to stage 45/46 in a static condition, i.e., solutions were not renewed, so to determine the potentially embryotoxic effects of the delorazepam-based drug and/or its active metabolites. All the experiments were carried out at 21 °C, under a 12 h light: 12 h dark photoperiod. The pH (7.4) of the solutions in the Petri dishes containing the embryos was checked daily. Embryo's survival and phenotypes were checked daily, and dead embryos were recorded and immediately removed.

2.3 Determination of embryo's phenotype, length, heart rate, and motility

For phenotype analysis, the survived embryos at stage 45/46 were anesthetized in FETAX containing 100 mg/L MS-222 (SIGMA) and placed under an MZ16F UV stereomicroscope equipped with a Leica DFC 300Fx camera. A photo of each class of most common malformation was taken in ventral, lateral, and dorsal positions. For length, heartbeat, and motility determination, thirty embryos from each treatment were randomly collected. A stereomicroscope equipped with an eyepiece micrometer was used to determine the length of the embryo. Heart rate was determined by counting the number of beats in a series of 30 seconds examinations, carried out in triplicate at a distance of 1 min (Carotenuto et al., 2016). For motility evaluation, the selected embryos were transferred into separate glass Petri dishes (diameter: 10 cm) containing 50 mL of FETAX solution, and let acclimatize for 5 minutes, protected by a black curtain from any possible disturbance exerted by the researcher. Single embryos were filmed for 60 seconds, and videos were analyzed by the software Tracker Video Analysis and Modeling Tool (Open-Source Physics). Speed and swimming activity data were normalized using the respective controls. The average velocity was determined as the distance traveled per second (cm/s), in the 60 seconds trials; time inactive (freeze) was quantified as average time (in seconds) spent resting, in the 60 seconds trials.

2.4 Histological analysis

Ten randomly selected embryos from each treatment were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M PBS pH 7.4 for 24h at 4 °C, and post-fixed in 1% osmium tetroxide for 1 hour at 4 °C (Avallone et al., 2015). After washing in 0.1 M PBS pH 7.4 at 4 °C, samples were dehydrated in ascending ethanol, and propylene oxide and embedded in Epon 812 (60 °C, 48 h). Semi-thin sections (1.5 µm) of the eyes were cut and stained with 1% toluidine blue solution prepared in 1% sodium tetraborate buffer. For each embryo, 30 serial sections were examined with a Zeiss Axiocam camera applied to a Zeiss Axioskop microscope (Zeiss, Jena, Germany). Measurements of retina layers thickness and cell diameter were performed with the AxioVision software.

2.5 RNA and Real-Time PCR

For each treatment group, total RNA was extracted from a pool of 6 embryos with the Direct-zol RNA Mini Prep kit (ZymoResearch, Irvine, CA, USA) following the manufacturer's instruction and used for cDNA synthesis using the SuperScript Vilo cDNA synthesis kits (Life Technologies Massachusetts, USA). Primers were designed using the software Primer 3 Plus (Table S1). Real-time PCR was performed using Power SYBR Green Master Mix kits (Life Technologies) using the 96-well optical reaction plate in 20 µL total reaction volume. Reactions were conducted on an AriaMx Real-time PCR System. The magnitude of change in gene expression relative to control was determined by the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001).

2.6 Redox state analysis

2.6.1 Preparations of homogenates

The analysis of redox state was performed on six samples for each experimental group. The embryos were finely minced and homogenized in a cold homogenization medium (HM, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.1% fatty acid-free albumin, 10 mM Tris, pH 7.4) using a glass Potter-Elvehjem homogenizer set at 500 rpm for 1 min. Total protein content was measured by the biuret method and the homogenates were used for the following measures.

2.6.2 ROS content determination

The ROS content was measured according to Napolitano et al., 2022. In brief, 25 µg of homogenate proteins diluted in 200 µL of monobasic phosphate buffer were incubated for 20 min with 10 µM DCFH-DA at room temperature. Then, $FeCl_3$ was added to a final concentration of 100 µM, and the mixture was incubated for 30 minutes. The conversion of DCFH-DA to the fluorescent product DCF was measured using a multimode microplate reader (Synergy™ HTX Multimode Microplate Reader, BioTek) with excitation and emission wavelengths of 485 and 530 nm. Background fluorescence (conversion of DCFH to DCF in the absence of homogenate and mitochondria) was corrected with parallel blanks. ROS production was expressed as Relative Fluorescence Units per µg protein.

2.6.3 Oxidative damage to lipids

The level of lipid hydroperoxides (HPs) was used to measure the extent of the lipid peroxidative processes in the homogenates of the embryos. The measure was spectrophotometrically performed by using a system of two coupled enzymatic reactions catalyzed by glutathione peroxidase and glutathione reductase, respectively, in the presence of GSH and H_2O_2. The HPs levels were calculated

by the rate of NADPH oxidation at 340nm and expressed as nmol NADPH oxidized/minutes per mg of proteins.

2.6.4 Activities of the antioxidant enzymes GPX and GR

GPX activity in 0.02 mg proteins of the homogenates was assayed at 25 °C by using H_2O_2 as substrate according to Flohé and Günzler (1984). The reaction was spectrophotometrically followed at 340 nm by the oxidation of NADPH in the presence of GSH and GR. GR activity of 0.02 mg proteins of the homogenates was assayed at 25 °C by measuring the rate of NADPH oxidation after the addition of GSSG. Each procedure was performed by using a multi-mode microplate reader (Synergy™ HTX Multi-Mode Microplate Reader, BioTek), and both enzymes' activities were expressed as nmol NADPH oxidized/minutes per mg of proteins.

2.6.5 *In vitro* susceptibility to oxidants

The *in vitro* susceptibility of homogenates to oxidants was evaluated by the change in hydroperoxide levels induced by treatment of 1 mg of homogenate proteins/mL with Fe and ascorbate (Fe/As), at concentrations of 100/1000 µM, for 10 min at room temperature (Venditti et al., 2016). The reaction was stopped by the addition of 0.2% 2.6-di-t-butyl-p-cresol (BHT) and the hydroperoxide levels were evaluated as previously described.

2.6.6 Cytochrome oxidase activity (COX)

COX activity of 0.1 mg proteins of the homogenate was polarographically determined at 25 °C by using a respirometer Hansatech (Hansatech Instruments Ltd, United Kingdom). The measure was performed in 1.0 mL of buffer solution (30µM Citc 3131, 10 mM Sodium Malonate, 75 mM Hepes, 4 µM Rotenone, 0.5 mM 2.4-dinitrophenol, pH 7.4) after membranes solubilization with 1% Lubrol and in presence of a mixture of TMPD plus Ascorbate (30 mM plus 400mM). COX activity was expressed as µmol O/min per mg of proteins.

2.7 Statistical analysis

Data were processed with GraphPad-Prism 8 software (GraphPad Software, Inc., San Diego, CA, USA). The survival distributions in control and experimental groups were assessed in terms of significance using the Mantel-Cox test. To evaluate differences in heartbeat, length, motility, and oxidative stress among groups, the data were checked for compliance with parametric tests, then One-Way ANOVA followed by Tukey's pairwise comparison tests were performed. For Real-Time PCR, statistical significance was determined using Two-Way ANOVA with the Bonferroni test. Data were

expressed as mean ± SD; probability was considered statistically significant at $p< 0.05$ (*), very significant at $p< 0.01$ (**) and at $p<0.001$ (***), and extremely significant at $p<0.0001$(****).

3. Results

3.1 Embryo survival and body malformations

The embryos grown in presence of DLZ showed a significant dose-dependent increase in mortality ($p<0.0001$; Table S2, Figure 1A): the percent of death reached 32.2% in 1 µg/L DLZ, 44.4% in 5 µg/L and 53.3% 10 µg/L DLZ. In controls, mortality remained at 11.1%. Control embryos showed an average length of 0.97 mm. No significant variations were registered in embryos exposed to DLZ at 1 and 5 µg/L while, in 10 µg/L treatment, a moderate but significant decrease in length was observed if compared to control and 1 µg/L (0.87 mm; $p<0.05$; Fig. 1B).

The incidence of malformations also follows a dose-dependent trend (Table S3). Control embryos showed a low rate of malformation, 3.7%, and anomalies consisted of moderate and diffused swelling. In presence of DLZ, the percentages of malformations raised to 21.3% after 1 µg/L exposition and to 62.0% and 69.0% in embryos exposed to 5 or 10 µg/L.

Different types of malformations were common to the three dosages used, albeit with different frequencies (Table S3). Diffuse edema was the most common (61.5 to 74.2%), a condition making it impossible to distinguish the cephalic from the abdominal region (Fig. 2E-F). The head edema was the second most represented abnormal condition (16.1 to 38.5%) where, although the presence of abdominal swelling (Fig. 2C-D), the cephalic area was distinguishable (Fig. 2C-D). A bent tail condition was also occasionally observed (9.7%) but only in 5 µg/L embryos (Fig. 2H).

In control embryos the intestine appeared well organized, properly convoluted (Fig. 2B); in contrast, in DLZ-treated embryos it appeared immature (Table S3), misfolded, apparently elongated, and/or dilated (Fig. 2D-F), often with clearly anomalous loops (Fig. 2F). The edema was frequently so consistent to cause an outbreak of the abdominal wall, with consequent extrusion of part of the intestine (Fig. 2G). In embryos with the bent tail condition, the immaturity of the intestine folding was a constant condition (Fig. 2H).

Body alterations were often accompanied by an alteration in the presence and/or distribution of the dorsal pigment (Fig. 2C, E) if compared to the control (Fig. 2A).

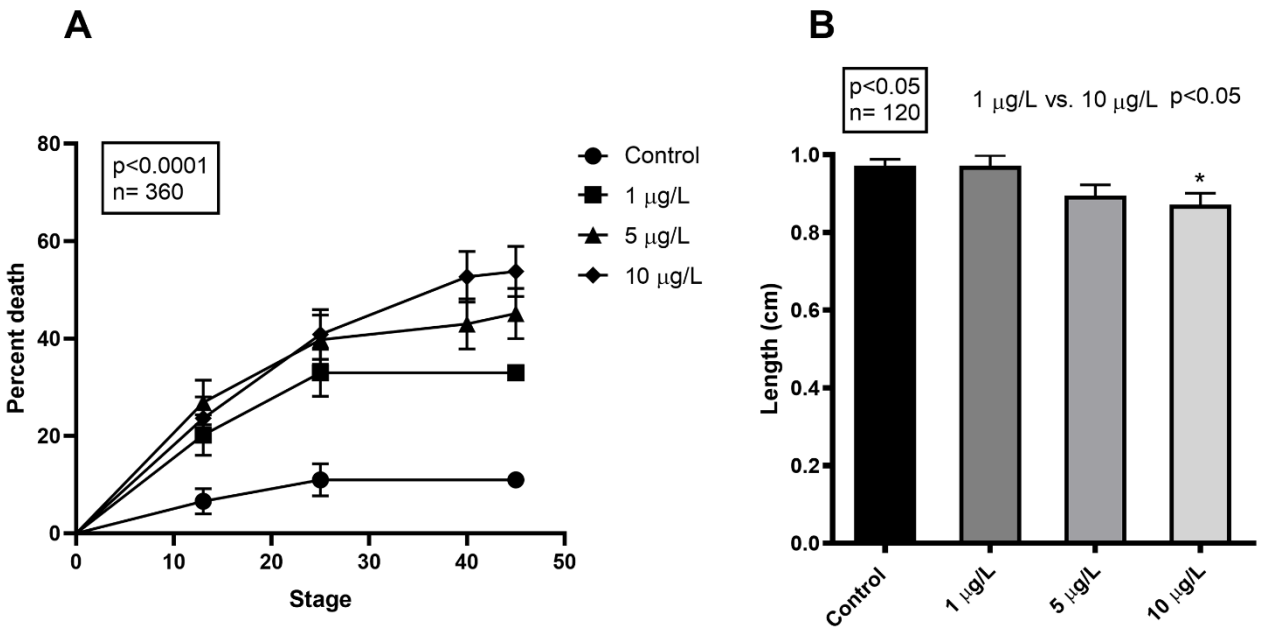


Figure 1. Mortality and length in *Xenopus laevis* embryos exposed to delorazepam. (A) Mortality percentage significantly increases at all the concentrations and in all stages examined. Chi-square test for trend p<0.0001. (B) Significant growth retardation in embryos exposed to 10 μg/L if compared to control and 1 μg/L mean length. Chi-square test for trend p<0.05. Data are means ± SD; total number of embryos examined (n); Statistic Unit = 12. * p<0.05; **p<0.01; ***p<0.001.

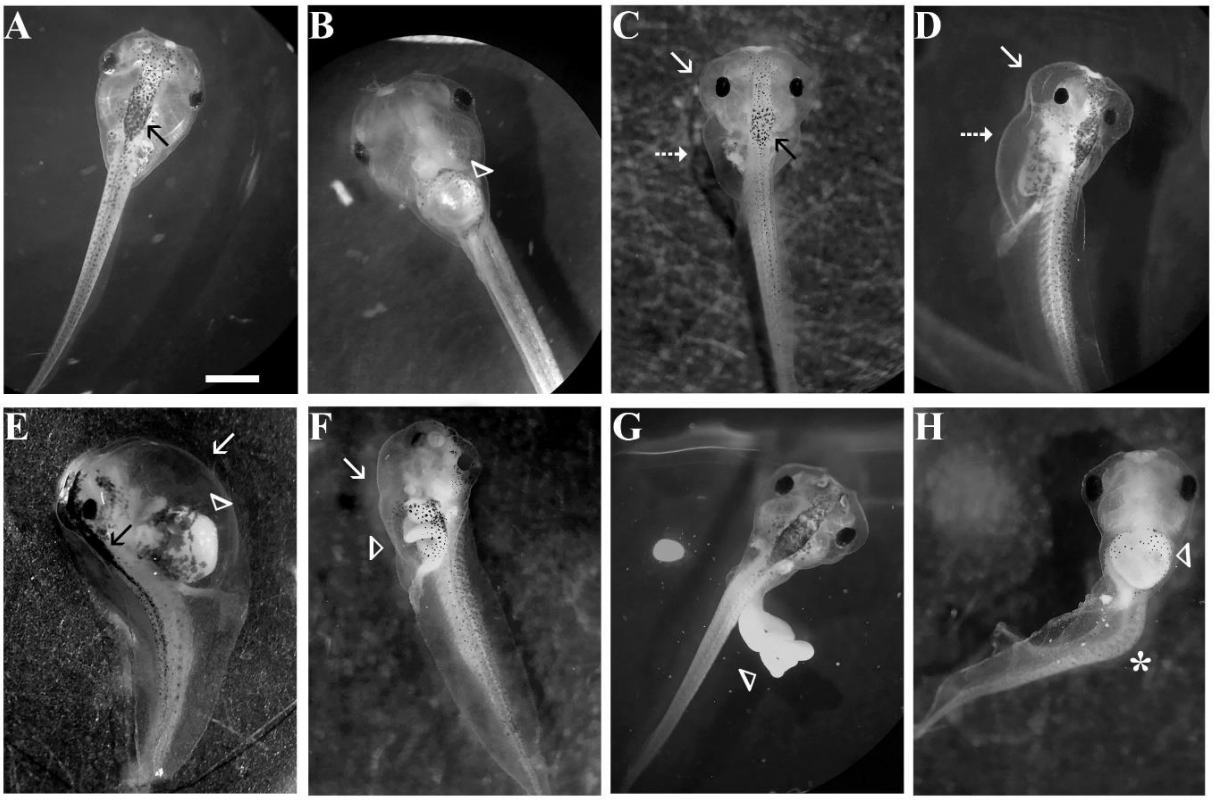


Figure 2. Altered phenotypes in *Xenopus laevis* embryos exposed to delorazepam. (A) Dorsal and (B) ventral view of control embryo displaying the typical gross morphology with spiralized intestine (arrowhead). Notice the correct distribution of the dorsal pigment (black arrow); (C) Dorsal and (D) lateral view of embryos after exposition to delorazepam; head (arrow) and abdominal (dot arrow) edema, immature intestine (arrowhead), and reduced dorsal pigment (black arrow). (E-F) Lateral view of diffuse edema (arrow) with the presence of an immature intestine (arrowhead) and increased dorsal pigmentation (black arrow); (G) Leaking of the intestine (arrowhead) following the outbreak of abdominal edema; (H) Bent tail condition (asterisk) with immature intestine (arrowhead). Bar = 1.25mm.

3.2 Retinal defects

The microscopic analyses of the control eyes (Fig. 3A) showed a typical differentiating retina. Ganglion cells (GCL) were organized in a monolayer, the inner plexiform layer (IPL) appeared a thick and dense reticulum of fibrils**.** The inner nuclear layer (INL) was multilayered, with tightly packed cells among which Muller cells were recognizable by the denser cytoplasm. The outer plexiform layer (OPL) was barely visible below a thick outer nuclear layer (ONL). It was characterized by the presence of large oil droplets, in the inner segment of cones photoreceptors, and by the contact with a regular pigmented epithelium. After exposure to DLZ, the thickness of the different layers (fig. 3E) and cell size (fig. 3F) significantly increased. At the environmental

concentration of 1 µg/L, GCL and INL are thicker, ganglion cells and inner nuclear layer cells are larger and disarranged (Fig 3B) if compared to controls. Increasing the dose to 5 µg/L (Fig. 3C) or 10 µg/L (Fig. 3D) caused a further dose-dependent increase in cells diameters (fig. 3F) and layers thickness (fig. 3E). This latter alteration was particularly evident in GCL, becoming multilayered, and in OPL, becoming distinguishable between INL and ONL. Significant changes are also observed in cell organization. In the INL, cells appeared loosely arranged and Muller cells increased in number, especially at 10 µg/L treatment. As a consequence, the retina appeared disorganized (Fig. 3C-D), thicker and the volume of the vitreous chamber markedly reduced (Fig. 3B-D). Moreover, oil drops were more numerous, bigger, and dispersed in the entire retina (Fig. 3B-D). No significant morphological changes were detected in the inner plexiform layer (IPL).

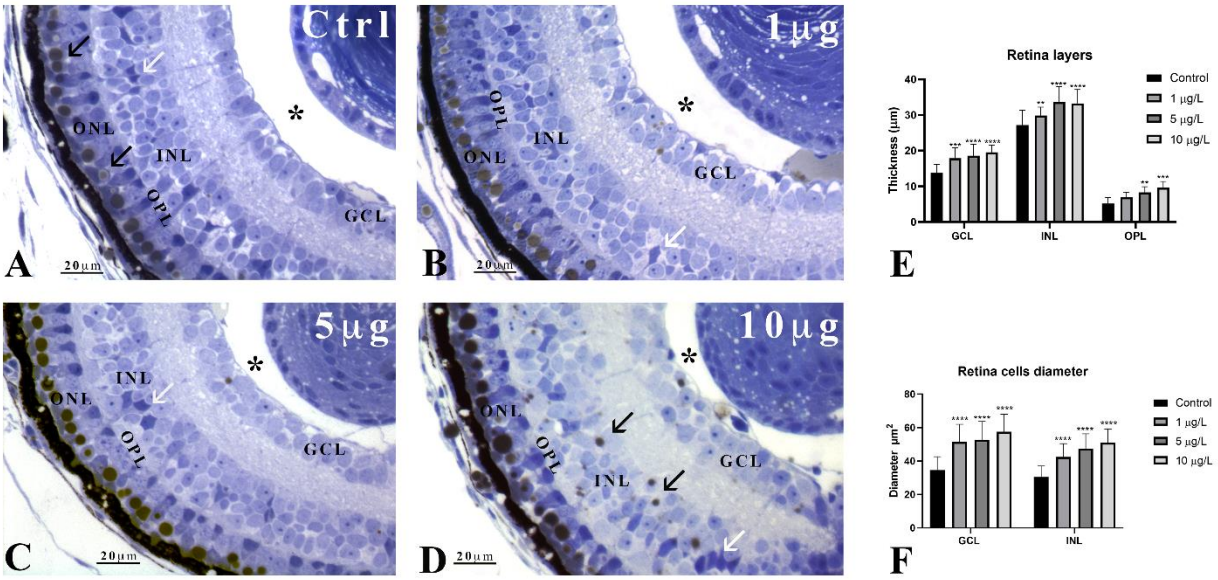


Figure 3. Histological section of the retina of *Xenopus laevis* embryos treated with delorazepam. (A) Control; typical retinal organization with a monolayered GLC, an INL with dispersed Muller cells (white arrows), and oil drops in inner segments of cones (black arrows). Vitreous chamber (asterisk). (B-C) Increase in GCL, INL, and OPL thickness and marked decrease of the vitreous chamber (*). (D) GCL is multi-layered, the INL loosely organized with increased Muller cells (white arrows) and oil droplets (black arrows). (E and F) Dose-dependent increase in retinal layers thickness and retinal cells diameters in GLC, INC, and OPL. Data are means ± SD; $^{**}p<0.01$; $^{****}p<0.0001$.

3.3 Bradycardia and impaired swimming performance

No differences were registered in the heartbeat rate between control embryos and embryos exposed to 1 µg/L DLZ: in both groups, an average frequency of 65 beats per minute was reported. A statistically significant decrease was observed in embryos exposed to 5 and 10 µg/L in which the average frequency reduced to 61 beats per minute (p <0.001; Fig. 4A). A significant decrease was also observed among the treaties (p<0.01).

Under normal conditions, swimming was inconstant, characterized by a burst in which the average speed was 2.85 ± 1.44 cm/s (Fig. 4B). The activity was followed by a short period of stasis during which the embryos were completely steady (Fig. 4B). However, when stimulated, the embryos immediately started moving. In the treated embryos, the average speed progressively and significatively decreases with increasing DLZ dosage: from 0.79 ± 0.42 cm/s at the environmental concentration (p<0.0001), to 0.62 ± 0.41 at 5 µg/L (p<0.0001) to 0.55 ± 0.36 at 10 µg/L (p<0.0001). A significant decrease was also observed between embryos treated with 1 µg/L and 10 µg/L (p<0.01). In addition, during the stasis, a prolonged stimulus was needed to restart the animal and, when swimming was finally resumed, it was slower.

Even the rest times, if compared to the controls (average 4 seconds every 60 seconds of activity), increased considerably after DLZ exposure, no matter the dose, lasting on average 41 seconds (Fig. 4C).

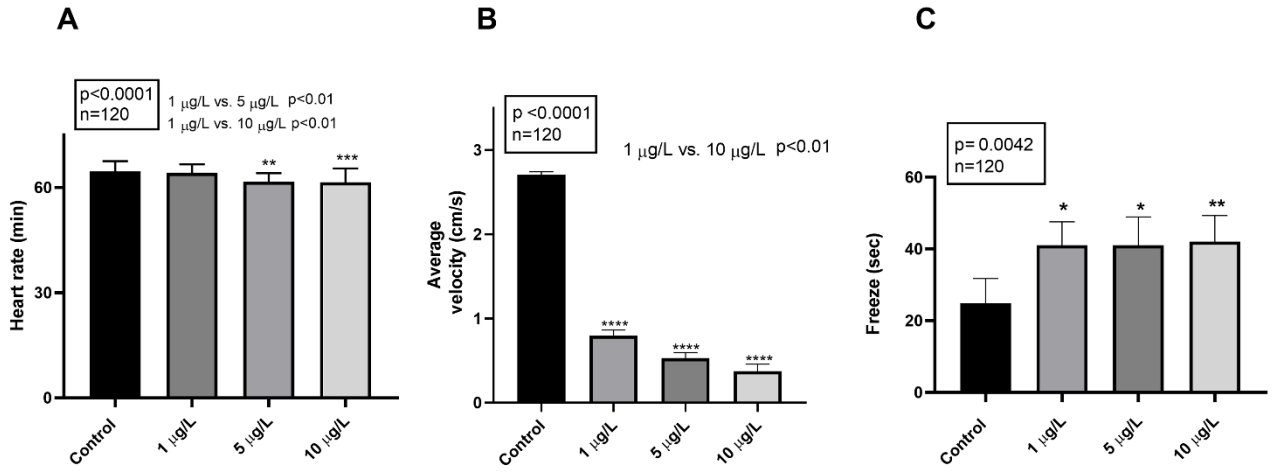


Figure 4. Heart rate and swimming performance in *Xenopus laevis* embryos exposed to delorazepam. (A) Dose-dependent bradycardia in embryos exposed at 5 and 10 µg/L. Chi-square test for trend *p<0.0001*. (B) Dose-dependent decrease in swimming speed of the treated embryos. Chi-square test for trend *p<0.0001*. (C) Significant increase in time spent resting (freeze) during the 60-second trials. Chi-square test for trend *p=0.0042*. Data are reported as means ± SD; total number of embryos examined (n); *p<0.05; **p<0.01; ****p<0.0001

3.4 Altered genes expression

Results indicated that DLZ at the environmental dose of 1 µg/L induced a significant downregulation of *bmp4* and *egr2* (p<0.001), *fgf8* (p<0.01), and *pax6* (p<0.05). At 5 µg/L, *bmp4* and *fgf8* were normally expressed while *rax1* (p<0.0001) and *egr2* (p<0.001) were overexpressed, and *sox9* and *pax6* significantly downregulated (p<0.0001). The highest dose of 10 µg/L induced overexpression of *bmp4* (p<0.0001), *egr2* and *rax1* (p<0.0001) while *sox9* and *pax6* remained downregulated (p<0.0001). *fgf8* levels were not modified (Fig. 5A).

Pro-inflammatory *tnfα* and *il1b* genes were already over-expressed at 1 µg/L (p<0.01 and p<0.0001), further increasing at the higher dosages (p<0.0001). *p65* showed a slight increase in expression at 1 and 5 µg/L, significantly raising at 10 µg/L (p<0.05). For the *abcb1* gene, the expression showed a gradual increase up to 5 µg /L (p<0.0001) and a decrease at 10 µg/L (p<0.0001) (Fig. 5B).

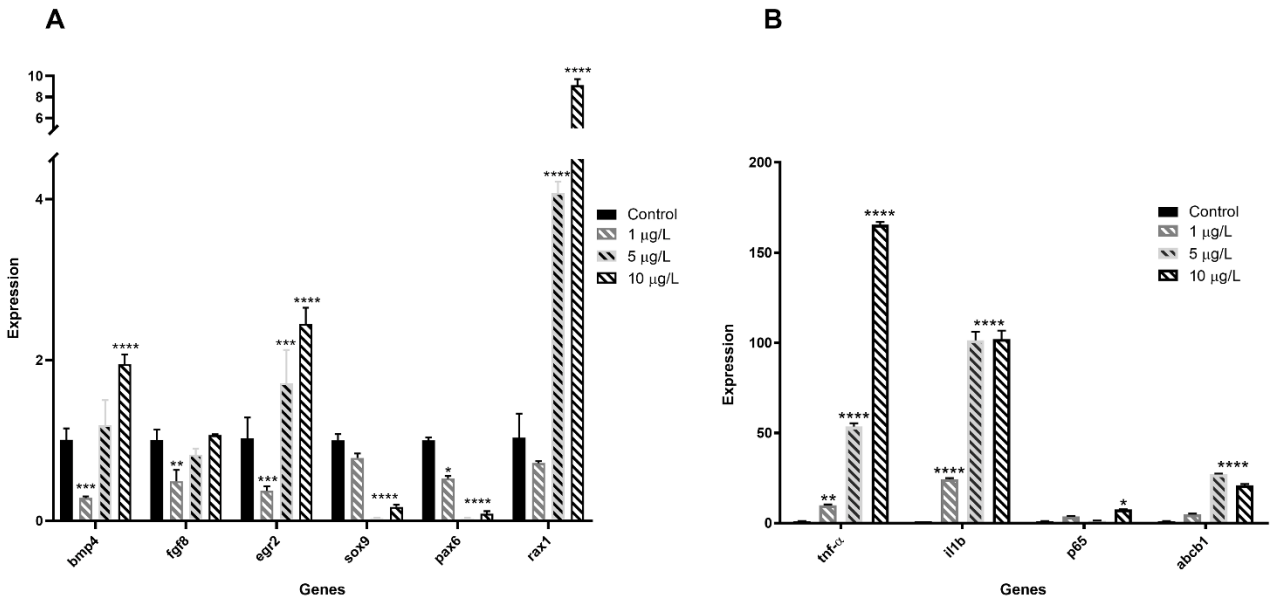


Figure 5. Changes in gene expression in *Xenopus laevis* embryos exposed to delorazepam. (A) Early developmental genes expression was always downregulated at environmental doses, and up or downregulated at higher doses if compared to control expression. (B) Pro-inflammatory cytokines and *abcb1* genes tend to be overexpressed in the treated embryos. Data are means ± SD. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

3.5 ROS content, oxidative damage of lipids, and antioxidant enzymes activity

ROS content significantly increases after exposure to delorazepam with the highest levels registered in 5 µg/L and 10 µg/L treatments (Fig. 6A). Treatments also induce a dose-dependent increase in lipid hydroperoxides levels (Fig. 6B). Both glutathione peroxidase (fig. 7A) and glutathione reductase (Fig.

7B) activities increased after DLZ exposure, raising to the highest levels at 10 µg/L. In addition, all treated groups showed increased susceptibility to oxidants, particularly in the presence of a maximum concentration of delorazepam (Fig. 7C). The cytochrome oxidase activity (COX) remained unaffected by treatment with delorazepam (Fig S1).

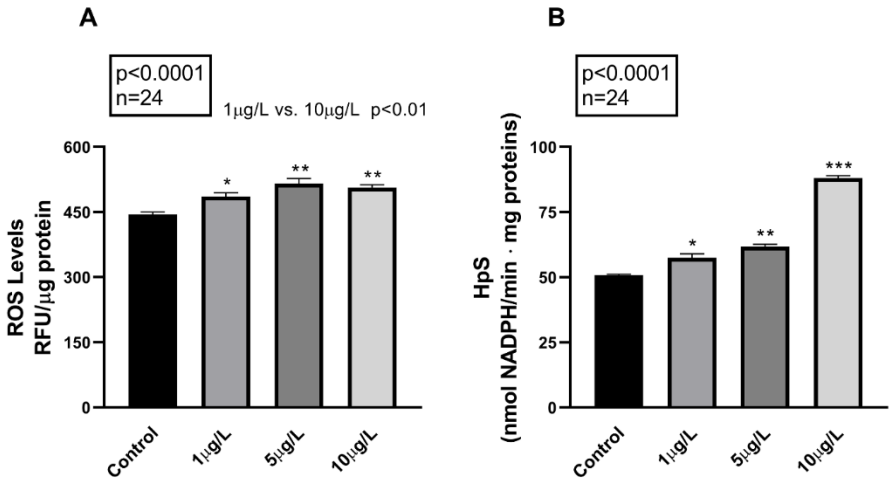


Figure 6. Analysis of ROS content and oxidative damage to lipids in *Xenopus laevis* embryos homogenates. (A) ROS production increases at the environmental dose and raises further after 5 and 10 µg/L treatments. (B) Dose-dependent increase of the lipid hydroperoxides levels. Chi-square test for trend $p<0.0001$. Data are means ± SD. Total number of embryos examined (n); Statistic Unit = 12. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

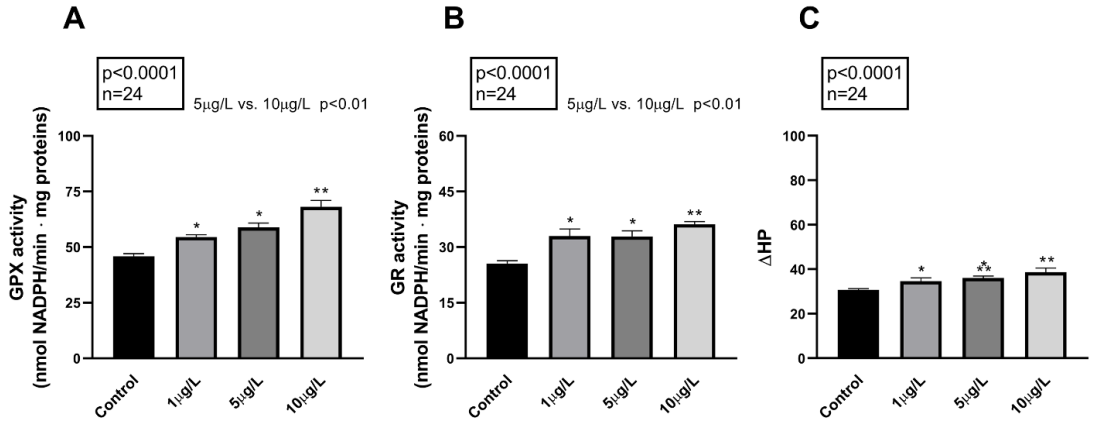


Figure 7. Activities of glutathione peroxidase GPX (A) and reductase GR (B), and *in vitro* susceptibility to oxidants ΔHP (C) in *Xenopus laevis* embryos homogenates. A dose-dependent increase is noticed for all parameters analysed. Chi-square test for trend p<0.0001. Data are means ± SD. Total number of embryos examined (n). Statistic Unit = 12. **p<0.05; **p<0.01; ***p<0.001.*

Discussion

Data collected demonstrate that DLZ profoundly influences early development in *Xenopus*. The benzodiazepine is confirmed sedative (Griffin III et al., 2013; Hollis and Boyd, 2005), as indicated by decreased heart rate and reduced locomotory performance, two effects depending on binding to GABA receptors of the central nervous system (Zanher et al., 2007; Hollis and Boyd, 2005). DLZ is also confirmed teratogenic and able to alter gene expression (McElhatton, 1994; Pasbakhsh et al., 2003) and cause oxidative stress.

Concerning morphological anomalies, in mammalian embryos, BDZs reduce birth weight and affect head development, eyes, ears, brain, and mouth in particular (Pasbakhsh et al., 2003, Tandon & Mulvihill, 2009). In *Xenopus* embryos, the head gross morphology was apparently normal but the expression of developmental genes controlling neurulation, *sox9*, *egr2*, *pax6,* and *rax1*, was altered suggesting interference with nervous system development. In particular, the altered expression of *sox9* suggests a dysregulation of neural plate cell multipotency (Scott et al., 2010) while the altered expression of *egr2* indicates an alteration in Schcellcells development (Duong and Svaren, 2019). Altered *pax6* indicates potential interference with telencephalon dorso-ventral and anterior-posterior patterning, with the specification of neuronal subtypes, neuronal migration and axonal projection (Matsumoto et al., 2008).

The hypothesis of DLZ-induced nervous system damage is supported by two further pieces of evidence. The first is the observed changes in pigmentation, a process also depending on *sox9* and *egr2*: the former controls neural crest cell differentiation (Tussellino et al., 2016) and melanocytes number while the latter controls melanocytes distribution in the skin (Aoki et al., 2003). The second piece of evidence is DLZ interference with retinal development. The downregulation of s*ox9* and *pax6* can be responsible for the observed alteration in the organization of the inner plexiform and ganglion cells layers, the two in which GABAa receptors were already expressed at the time of treatment (Soklnick et al., 1980). The action would have been exerted by interfering with retinal progenitors' fate (Hsieh and Wang, 2009). Further investigation is necessary to prove the interference and to test nervous system development and functionality; however, anomalies similar to those observed in *Xenopus* were already registered in lizard embryos exposed to cadmium. In this species, retinal damage and *pax6* dysregulation were associated with anomalies in the mesencephalic roof, due to changes in rate and time of cell proliferation (Simoniello et al., 2014).

Eye damages were not particularly severe but together with altered oil droplets size and distribution can prelude to aniridia, cataracts, or corneal defects (Nakayama et al., 2015) and impaired visual performance. Not to neglect the fact that ganglion cells normally produce a neuroactive steroid

controlling the inhibitory transmission (Guarneri et al., 1995). DLZ might have interfered with its release or function, therefore opening a number of new research questions.

Data from the retina are particularly interesting also for another aspect. *pax6* under expression accounts for the observed anticipation of retinal precursors differentiation (Philips et al., 2005) and, in particular, for the increased number of differentiating Muller cells (Zhu et al., 2013). However, it does not explain the increased thickness of the ganglion cells layer. The observed effects can be attributed to increased proliferation, but this usually relates to a *pax6* overexpression (Simoniello et al., 2014). A suggestive hypothesis comes therefore from these contrasting data: that DLZ has different, region-specific effects in the retina mimicking what is already demonstrated in the brain (Musavi and Kakkar, 1998).

Evidence of DLZ teratogenicity also comes from gut deformities. Very frequent and particularly evident, they are indicative of a delayed winding of gut loops (Chalmers and Slack, 1998), a common response in *Xenopus* embryo intoxication (Carotenuto et al., 2022). Delay can be associated with the observed over-expression of *bmp4*, a gene involved in gut specification, regionalization, and differentiation (Fu et al., 2006). Based on this evidence, exposed *Xenopus* embryos wouldn't feed properly, and the reduced size would support the hypothesis. The smaller size of treated embryos however can also depend on the altered expression of *fgf8*, a gene involved in embryonic axes determination and elongation (Dorey and Amaya, 2010).

Coming to the causes of the observed alterations, they are probably multiple and interconnected. Oxidative stress certainly has a primary role as both cause and effect. No information is available on DLZ but another benzodiazepine, diazepam, has proven pro-oxidative effects (Musavi and Kakkar, 1998). ROS production can activate the MAPK signaling pathways, which further activates several inflammatory cytokines (Park et al., 2011). In *Xenopus* embryos, overexpression of *tnfα*, *il1b*, and *p65* is registered which explains edema and developmental changes. In addition, ROS acts as a second messenger and, by regulating key transcription factors, both positively and negatively can affect cell signaling, proliferation, and death affecting embryonic development (Dennery, 2007). Is not a case that ROS represents a very early and sensitive biomarker of amphibian developmental toxicity (Rizzo et al., 2007).

Another factor must be taken into consideration: the benzodiazepine peripheral receptors or PBR/TSPO, a transmembrane protein located in the outer mitochondrial membrane. The TSPO binds benzodiazepines with micromolar affinity, is evolutionary highly conserved (Bonsack and Sukumari-Ramesh, 2018), is present in all tissues, and is already expressed in embryos (Papadopoulos et al., 1997). The receptor controls growth and differentiation, gene expression (Yasin et al., 2017), and the immune response (Betlazar et al., 2020). All these effects are compatible with the effects

observed in *X. laevis* embryos. In addition, being an oxygen sensor, TSPO can control ROS production and mitochondrial functionality (activity), thus contributing to oxidative stress production.

TSPO, by controlling mitochondrial functionality control cell bioenergetics (Betlazar et al., 2020), and, as a consequence, is a potential responsible for reduced embryo motility (Alelwani et al., 2020). However, cytochrome oxidase activity, *in vitro* correlating to the maximum aerobic capacity of the tissues, did not change in DLZ-treated embryos. Therefore, the observed decreased heart rate and reduced locomotory performance would depend exclusively on a drop in blood pressure and sympathetic nerve activity induced by DLZ potentiation of GABAergic inhibition (Zanher et al., 2007; Snyder et al, 2000). As expected, the increase in ROS triggered a protective response.

As in other species, *Xenopus* embryos activated low molecular weight antioxidants and antioxidants enzymes, such as GPX and GR. Glutathione reductase catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell. The enzyme glutathione peroxidase utilizes reduced glutathione to neutralize hydrogen peroxide and lipid hydroperoxides (Napolitano et al., 2021). These enzymatic activities have been reported to increase after diazepam exposure (Ogueji et al., 2017). However, the antioxidant enzyme activity was not sufficient to counteract the DLZ-induced increase in ROS level. Indeed, in *Xenopus* embryos, the capacity to face *in vitro* oxidative stress was reduced in DLZ-treated animals, especially at the higher concentrations. This could explain why, though different DLZ concentrations increase ROS content to the same extent, at the higher DLZ concentrations oxidative damage was more consistent. On the other hand, the reduced capacity to face *in vitro* oxidative stress at the highest DLZ concentration can depend on the reduced expression of the *abcb1* gene, a member of the ABC cassette multi-xenobiotic pump involved in detoxifying mechanisms and, in the extrusion from the cells of unmodified exogenous compounds (Guo et al., 2020).

Conclusion

Data evidentiate that benzodiazepines such as delorazepam if released in the environment, interfere with amphibians' embryo development. Morphological, behavioral, and molecular alterations are induced, and these significantly impair embryo survival. Oxidative stress is certainly involved but up to now, it is unclear if ROS is a cause or a consequence of the observed alterations. Most probably the effects depend on a synergic action of ROS, GABA, and TSPO receptors but only further studies will fully clarify the delorazepam way of action. The relevance of the observed effects indicates that immediate attention must be paid to this class of contaminants and that they should be monitored during environmental risk assessment.

Supplementary Material

Table S1

Table S1. Primers		
Gene name	**Oligo Forward Sequence**	**Oligo Reverse Sequence**
bmp4 - bone morphogenetic protein 4	CCTCAGCAGCATTCCAGAGAA	TCCGGTGGAAACCCTCATCC
fgf8 - fibroblast growth factor 8	CGTTTGGAAGCAGAGTTCGC	GTTGCCTTGTCTTCGACCCT
sox9 - sex determining region Y-box 9	ACGGCGCAGAAAGTCTGTTA	GACATCTGTCTTGGGGGTGG
egr2 - early growth response 2	AGTAAGACCCCAGTCCACGA	GCAGTAATCGCAGGCAAAGG
pax6 - paired box protein Pax-6	CAGAACATCTTTTACCCAGGA	GAATGTGGCTGGGTGTGTTA
rax1 - retinal homeobox protein Rx1	GGAAAGACCTCAAGCGAGTG	ATACCTGCACCCTGACCTCG
tnfα – tumor necrosis factor alfa	CAAGCAATGAAAGGGGAAAA	TGCAGTCAGGACCTGTGAAG
il1b- interleukin 1 beta	TGTGCAGATAACCCATGGAA	TGCAGAGCAACAGAAGATGG
p65 – Nf-kB transcription factor family	TGGCTATTGTCTTCCGAACC	ATATGGTGGGGGTCTCCTTC
abcb1 – ATP binding cassette, subfamily B member 1	GGCTGTTGCTGAAGAGGTTC	ACCATACCAAAAGGCGAGTG
odc1 - ornithine decarboxylase	GTGGCAAGGAATCACCCGAA	TCAAAGACACATCGTGCATC

Table S2

Table S2. Embryotoxic effects of delorazepam on *Xenopus laevis* embryos				
	Control	**1 µg/L**	**5 µg/L**	**10 µg/L**
Embryos (n)	90	90	90	90
Dead embryos (n)	10	29	40	48
Living embryos (n)	80	61	50	42
Mortality (%)	11.1	32.2[b]	44.4[c]	53.3[c,d]

n=number of embryos; [b] chi-square test $p<0.001$; [c] chi square test $p<0.0001$;
[d] chi-square test for trend $p<0.0001$.

Table S3

Table S3. Malformations on living embryos after delorazepam treatment				
	Control	**1 µg/L**	**5 µg/L**	**10 µg/L**
Living embryos (n)	80	61	50	42
Total malformed embryo (n; %)	3 (3.7)	13 (21.3)[a]	31 (62.0)[c]	29 (69.0) [c,d]
Head edema	0	5 (38.5)[a]	5 (16.1)[c]	9 (31.0)[c]
Diffuse edema	0	8 (61.5)[b]	23 (74.2)[c]	20 (68.9)[c]
Bent tail	0	0	3 (9.7)[b]	0
Intestine alteration	0	13 (100)[c]	31 (100)[c]	29 (100)[c]

n=number of embryos; [a] chi-square test $p<0.01$; [b] chi-square test $p<0.001$;
[c] chi-square test $p<0.0001$; [d] chi-square test for trend $p<0.0001$.

Figure S1

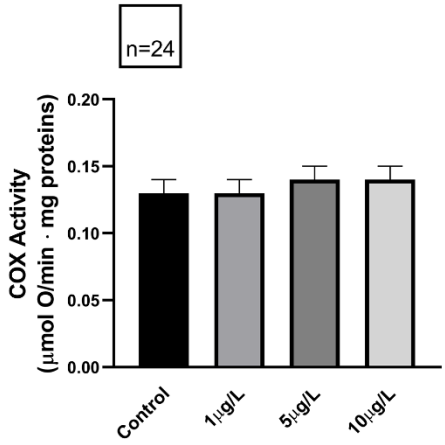


Figure S1. Cytochrome oxidase activity (COX) of *Xenopus laevis* embryos homogenates. No effects are observed for control and different treatment. Data are means ± SD. Total number of embryos examined (n).

References

Alelwani W, Elmorsy E, Kattan SW, Babteen NA, Alnajeebi AM, Al-Ghafari A, Carter WG. Carbamazepine induces a bioenergetics disruption to microvascular endothelial cells from the blood-brain barrier. Toxicol Lett. 2020 Oct 15; 333:184-191. doi: 10.1016/j.toxlet.2020.08.006.

Argyropoulos, S. V., & Nutt, D. J. (1999). The use of benzodiazepines in anxiety and other disorders. European neuropsychopharmacology: the journal of the European College of Neuropsychopharmacology, 9 Suppl 6, S407–S412. https://doi.org/10.1016/s0924-977x(99)00052-8

Aoki, Y., Saint-Germain, N., Gyda, M., Magner-Fink, E., Lee, Y. H., Credidio, C., & Saint-Jeannet, J. P. (2003). Sox10 regulates the development of neural crest-derived melanocytes in Xenopus. Developmental biology, 259(1), 19–33. https://doi.org/10.1016/s0012-1606(03)00161-1

Avallone, B., Crispino, R., Cerciello, R., Simoniello, P., Panzuto, R., Motta, C.M. (2015). Cadmium effects on the retina of adult *Danio rerio*. Comptes rendus biologies, 338(1), 40–47. https://doi.org/10.1016/j.crvi.2014.10.005

Bade, R., Ghetia, M., White, J. M., & Gerber, C. (2020). Determination of prescribed and designer benzodiazepines and metabolites in influent wastewater. Analytical methods: advancing methods and applications, 12(28), 3637–3644. https://doi.org/10.1039/d0ay00560f

Bareggi, S. R., Nielsen, N. P., Leva, S., Pirola, R., Zecca, L., & Lorini, M. (1986). Age-related multiple-dose pharmacokinetics and anxiolytic effects of delorazepam (chlordesmethyldiazepam). International journal of clinical pharmacology research, 6(4), 309–314.

Bareggi, S.R., Truci, G., Leva, S., Zecca, L., Pirola, R., Smirne, S. (1988). Pharmacokinetics and bioavailability of intravenous and oral chlordesmethyldiazepam in humans. Eur J Clin Pharmacol 34, 109–112 (1988). https://doi.org/10.1007/BF01061430.

Bernardini, G., Vismara, C., Boracchi, P., & Camatini, M. (1994). Lethality, teratogenicity and growth inhibition of heptanol in *Xenopus* assayed by a modified frog embryo teratogenesis assay-Xenopus (FETAX) procedure. The Science of the total environment, 151(1), 1–8. https://doi.org/10.1016/0048-9697(94)90480-4

Betlazar, C., Middleton, R. J., Banati, R., & Liu, G. J. (2020). The Translocator Protein (TSPO) in mitochondrial bioenergetics and immune processes. Cells, 9(2), 512. https://doi.org/10.3390/cells9020512

Bonsack, F., & Sukumari-Ramesh, S. (2018). TSPO: an evolutionarily conserved protein with elusive functions. International journal of molecular sciences, 19(6), 1694. https://doi.org/10.3390/ijms19061694

Calisto, V., & Esteves, V. I. (2009). Psychiatric pharmaceuticals in the environment. Chemosphere, 77(10), 1257–1274. https://doi.org/10.1016/j.chemosphere.2009.09.021

Carotenuto, R., Capriello, T., Cofone, R., Galdiero, G., Fogliano, C., & Ferrandino, I. (2020). Impact of copper in *Xenopus laevis* liver: Histological damages and atp7b downregulation. Ecotoxicology and environmental safety, 188, 109940. https://doi.org/10.1016/j.ecoenv.2019.109940

Carotenuto, R., Fogliano, C., Rienzi, M., Siciliano, A., Salvatore, M. M., De Tommaso, G., Benvenuto, G., Galdiero, E., & Guida, M. (2021). Comparative toxicological evaluation of tattoo inks on two model organisms. Biology, 10(12), 1308. https://doi.org/10.3390/biology10121308

Carotenuto, R., Tussellino, M., Mettivier, G., & Russo, P. (2016). Survival fraction and phenotype alterations of *Xenopus laevis* embryos at 3 Gy, 150 kV X-ray irradiation. Biochemical and biophysical research communications, 480(4), 580–585. https://doi.org/10.1016/j.bbrc.2016.10.095

Carotenuto, R., Tussellino, M., Ronca, R., Benvenuto, G., Fogliano, C., Fusco, S., & Netti, P. A. (2022). Toxic effects of SiO2NPs in early embryogenesis of *Xenopus laevis*. Chemosphere, 289, 133233. https://doi.org/10.1016/j.chemosphere.2021.133233

Cerveny, D., Brodin, T., Cisar, P., McCallum, E. S., & Fick, J. (2020). Bioconcentration and behavioral effects of four benzodiazepines and their environmentally relevant mixture in wild fish. The Science of the total environment, 702, 134780. https://doi.org/10.1016/j.scitotenv.2019.134780

Chalmers, A. D., & Slack, J. M. (1998). Development of the gut in *Xenopus laevis*. Developmental dynamics: an official publication of the American Association of Anatomists, 212(4), 509–521. https://doi.org/10.1002/(SICI)1097-0177(199808)212:4<509::AID-AJA4>3.0.CO;2-L

Dennery P. A. (2007). Effects of oxidative stress on embryonic development. Birth defects research. Part C, Embryo today: reviews, 81(3), 155–162. https://doi.org/10.1002/bdrc.20098

Dorey, K., & Amaya, E. (2010). FGF signalling: diverse roles during early vertebrate embryogenesis. Development (Cambridge, England), 137(22), 3731–3742. https://doi.org/10.1242/dev.037689

Duong, P., Svaren, J. (2019). Chromatin Signaling and Neurological Disorders. ISBN 978-0-12-813796-3

Fick, J., Brodin, T., Heynen, M., Klaminder, J., Jonsson, M., Grabicova, K., Randak, T., Grabic, R., Kodes, V., Slobodnik, J., Sweetman, A., Earnshaw, M., Barra Caracciolo, A., Lettieri, T., & Loos, R. (2017). Screening of benzodiazepines in thirty European rivers. Chemosphere, 176, 324–332. https://doi.org/10.1016/j.chemosphere.2017.02.126

Flohé, L., & Günzler, W. A. (1984). Assays of glutathione peroxidase. Methods in enzymology, 105, 114–121. https://doi.org/10.1016/s0076-6879(84)05015-1

Fu, M., Vohra, B. P., Wind, D., & Heuckeroth, R. O. (2006). BMP signaling regulates murine enteric nervous system precursor migration, neurite fasciculation, and patterning via altered Ncam1 polysialic acid addition. Developmental biology, 299(1), 137–150. https://doi.org/10.1016/j.ydbio.2006.07.016

Furuhagen, S., Fuchs, A., Lundström Belleza, E., Breitholtz, M., & Gorokhova, E. (2014). Are pharmaceuticals with evolutionary conserved molecular drug targets more potent to cause toxic effects in non-target organisms? PloS one, 9(8), e105028. https://doi.org/10.1371/journal.pone.0105028

Guarneri, P., Guarneri, R., Cascio, C., Piccoli, F., & Papadopoulos, V. (1995). gamma-Aminobutyric acid type A/benzodiazepine receptors regulate rat retina neurosteroidogenesis. Brain research, 683(1), 65–72. https://doi.org/10.1016/0006-8993(95)00343-o

Guo, B., Xu, Z., Yan, X., Buttino, I., Li, J., Zhou, C., and Qi, P. (2020) Novel ABCB1 and ABCC Transporters Are Involved in the Detoxification of Benzo(α)pyrene in Thick Shell Mussel, *Mytilus coruscus*. Front. Mar. Sci. 7:119. doi: 10.3389/fmars.2020.00119

Guthrie, G. D., Nicholson-Guthrie, C. S., & Leary, H. L., Jr (2000). A bacterial high-affinity GABA binding protein: isolation and characterization. Biochemical and biophysical research communications, 268(1), 65–68. https://doi.org/10.1006/bbrc.1999.1960

Hauptman, O., Albert, D. M., Plowman, M. C., Hopfer, S. M., & Sunderman, F. W., Jr (1993). Ocular malformations of *Xenopus laevis* exposed to nickel during embryogenesis. Annals of clinical and laboratory science, 23(6), 397–406.

Hollis, D. M., & Boyd, S. K. (2005). Distribution of GABA-like immunoreactive cell bodies in the brains of two amphibians, *Rana catesbeiana* and *Xenopus laevis*. Brain, behavior and evolution, 65(2), 127–142. https://doi.org/10.1159/000082981

Hsieh, Y. W., & Yang, X. J. (2009). Dynamic Pax6 expression during the neurogenic cell cycle influences proliferation and cell fate choices of retinal progenitors. Neural development, 4, 32. https://doi.org/10.1186/1749-8104-4-32

Klaminder, J., Brodin, T., Sundelin, A., Anderson, N. J., Fahlman, J., Jonsson, M., & Fick, J. (2015). Long-term persistence of an anxiolytic drug (oxazepam) in a large freshwater lake. Environmental science & technology, 49(17), 10406–10412. https://doi.org/10.1021/acs.est.5b01968

Lebreton, M., Malgouyres, J. M., Carayon, J. L., Bonnafé, E., & Géret, F. (2021a). Effects of the anxiolytic benzodiazepine oxazepam on freshwater gastropod reproduction: a prospective study. Ecotoxicology (London, England), 30(9), 1880–1892. https://doi.org/10.1007/s10646-021-02453-y

Lebreton, M., Sire, S., Carayon, J. L., Malgouyres, J. M., Vignet, C., Géret, F., & Bonnafé, E. (2021b). Low concentrations of oxazepam induce feeding and molecular changes in *Radix balthica* juveniles. Aquatic toxicology (Amsterdam, Netherlands), 230, 105694. https://doi.org/10.1016/j.aquatox.2020.105694

Lei, H. J., Yang, B., Ye, P., Yang, Y. Y., Zhao, J. L., Liu, Y. S., Xie, L., & Ying, G. G. (2021). Occurrence, fate and mass loading of benzodiazepines and their transformation products in eleven wastewater treatment plants in Guangdong province, China. The Science of the total environment, 755(Pt 2), 142648. https://doi.org/10.1016/j.scitotenv.2020.142648

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta C(T)) Method. Methods (San Diego, Calif.), 25(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Matsumoto, Y., & Osumi, N. (2008). Role of Pax6 in the developing central nervous system. Brain and nerve = Shinkei kenkyu no shinpo, 60(4), 365–374.

McElhatton P. R. (1994). The effects of benzodiazepine use during pregnancy and lactation. Reproductive toxicology (Elmsford, N.Y.), 8(6), 461–475. https://doi.org/10.1016/0890-6238(94)90029-9

Mendoza, A., López de Alda, M., González-Alonso, S., Mastroianni, N., Barceló, D., & Valcárcel, Y. (2014). Occurrence of drugs of abuse and benzodiazepines in river waters from the Madrid Region (Central Spain). Chemosphere, 95, 247–255. https://doi.org/10.1016/j.chemosphere.2013.08.085

Moosmann, B., & Auwärter, V. (2018). Designer Benzodiazepines: Another Class of New Psychoactive Substances. Handbook of experimental pharmacology, 252, 383–410. https://doi.org/10.1007/164_2018_154

Mouche, I., Malesic, L., & Gillardeaux, O. (2011). FETAX assay for evaluation of developmental toxicity. Methods in molecular biology (Clifton, N.J.), 691, 257–269. https://doi.org/10.1007/978-1-60761-849-2_15

Musavi, S., & Kakkar, P. (1998). Diazepam induced early oxidative changes at the subcellular level in rat brain. Molecular and cellular biochemistry, 178(1-2), 41–46. https://doi.org/10.1023/a:1006834706117

Nakayama, T., Fisher, M., Nakajima, K., Odeleye, A. O., Zimmerman, K. B., Fish, M. B., Yaoita, Y., Chojnowski, J. L., Lauderdale, J. D., Netland, P. A., & Grainger, R. M. (2015). *Xenopus* pax6 mutants affect eye development and other organ systems, and have phenotypic similarities to human aniridia patients. Developmental biology, 408(2), 328–344. https://doi.org/10.1016/j.ydbio.2015.02.012

Napolitano, G., Fasciolo, G., Magnacca, N., Goglia, F., Lombardi, A., & Venditti, P. (2022). Oxidative damage and mitochondrial functionality in hearts from KO UCP3 mice housed at thermoneutrality. Journal of physiology and biochemistry, 10.1007/s13105-022-00882-9. Advance online publication. https://doi.org/10.1007/s13105-022-00882-9

Napolitano G, Fasciolo G, Venditti P. Mitochondrial management of reactive oxygen species. Antioxidants (Basel). 2021 Nov 17;10(11):1824. doi: 10.3390/antiox10111824.

Nieuwkoop, P.D., Faber, J. (1956). Normal Table of *Xenopus laevis* (Daudin): A Systematical and Chronologica Survey of the Development from the Fertilized Egg till the End of Metamorphosis. North-Holland, Amsterdam.

Nunes, C. N., Dos Anjos, V. E., & Quináia, S. P. (2019). Are there pharmaceutical compounds in sediments or in water? Determination of the distribution coefficient of benzodiazepine drugs in aquatic environment. Environmental pollution (Barking, Essex: 1987), 251, 522–529. ttps://doi.org/10.1016/j.envpol.2019.05.015

Oggier, D. M., Weisbrod, C. J., Stoller, A. M., Zenker, A. K., & Fent, K. (2010). Effects of diazepam on gene expression and link to physiological effects in different life stages in zebrafish *Danio rerio*. Environmental science & technology, 44(19), 7685–7691. https://doi.org/10.1021/es100980r

Ogueji, E.O., Iheanacho, S.C., Nwani, C.D., Christian, E.M., Okeke, O.C., and Usman, I.B. (2017). Toxicity of diazepam on lipid peroxidation, biochemical and oxidative stress indicators on liver and gill tissues of African catfish *Clarias gariepinus* (Burchell, 1822). International Journal of Fisheries and Aquatic Studies. 5(3): 114-123.

Papadopoulos, V., Amri, H., Boujrad, N., Cascio, C., Culty, M., Garnier, M., Hardwick, M., Li, H., Vidic, B., Brown, A. S., Reversa, J. L., Bernassau, J. M., & Drieu, K. (1997). Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis. Steroids, 62(1), 21–28. https://doi.org/10.1016/s0039-128x(96)00154-7

Park, K. R., Nam, D., Yun, H. M., Lee, S. G., Jang, H. J., Sethi, G., Cho, S. K., & Ahn, K. S. (2011). β-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation. Cancer letters, 312(2), 178–188. https://doi.org/10.1016/j.canlet.2011.08.001

Pasbakhsh, P., Mehrannia, K., Barbarestani, M. (2013). The teratogenic effects of Lorazepam on the organogenesis of the rat fetus. Acta Med Iran. 1;41(1):29-32.

Patel, M., Kumar, R., Kishor, K., Mlsna, T., Pittman, C. U., Jr, & Mohan, D. (2019). Pharmaceuticals of emerging concern in aquatic systems: chemistry, occurrence, effects, and removal methods. Chemical reviews, 119(6), 3510–3673. https://doi.org/10.1021/acs.chemrev.8b00299

Philips, G. T., Stair, C. N., Young Lee, H., Wroblewski, E., Berberoglu, M. A., Brown, N. L., & Mastick, G. S. (2005). Precocious retinal neurons: Pax6 controls timing of differentiation and determination of cell type. Developmental biology, 279(2), 308–321. https://doi.org/10.1016/j.ydbio.2004.12.018

Rizzo, A. M., Adorni, L., Montorfano, G., Rossi, F., & Berra, B. (2007). Antioxidant metabolism of Xenopus laevis embryos during the first days of development. Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology, 146(1), 94–100. https://doi.org/10.1016/j.cbpb.2006.09.009

Schmitz, A. (2016). Benzodiazepine use, misuse, and abuse: A review. The mental health clinician, 6(3), 120–126. https://doi.org/10.9740/mhc.2016.05.120

Scott, C. E., Wynn, S. L., Sesay, A., Cruz, C., Cheung, M., Gomez Gaviro, M. V., Booth, S., Gao, B., Cheah, K. S., Lovell-Badge, R., & Briscoe, J. (2010). SOX9 induces and maintains neural stem cells. Nature neuroscience, 13(10), 1181–1189. https://doi.org/10.1038/nn.2646

Silva, A., Nilin, J., Loureiro, S., & Costa-Lotufo, L. V. (2020). Acute and chronic toxicity of the benzodiazepine diazepam to the tropical crustacean *Mysidopsis juniae*. Anais da Academia Brasileira de Ciencias, 92(1), e20180595. https://doi.org/10.1590/0001-3765202020180595

Simoniello, P., Trinchella, F., Filosa, S., Scudiero, R., Magnani, D., Theil, T., & Motta, C. M. (2014). Cadmium contaminated soil affects retinogenesis in lizard embryos. Journal of experimental zoology. Part A, Ecological genetics and physiology, 321(4), 207–219. https://doi.org/10.1002/jez.1852

Snyder, M.J, Watson, S., Peeke, H.V.S. (2000). Lobster locomotor activity as a measure of GABAA receptor modulation. Marine and Freshwater Behaviour and Physiology, 34(1), 37–51. doi:10.1080/10236240009379058

Soklnick, P., Paul, S., Zatz, M., & Eskay, R. (1980). 'Brain-specific'benzodiazepine receptors are localized in the inner plexiform layer of rat retina. European journal of pharmacology, 66(1), 133-136.

Tandon, A., Mulvihill, A. (2009). Ocular teratogens: old acquaintances and new dangers. Eye 23, 1269–1274. https://doi.org/10.1038/eye.2009.30

Tussellino, M., Ronca, R., Carotenuto, R., Pallotta, M. M., Furia, M., & Capriglione, T. (2016). Chlorpyrifos exposure affects fgf8, sox9, and bmp4 expression required for cranial neural crest morphogenesis and chondrogenesis in *Xenopus laevis* embryos. Environmental and molecular mutagenesis, 57(8), 630–640. https://doi.org/10.1002/em.22057

Venditti P, Napolitano G, Barone D, Pervito E, Di Meo S. Vitamin E-enriched diet reduces adaptive responses to training determining respiratory capacity and redox homeostasis in rat heart. Free Radic Res. 2016;50(1):56-67. doi: 10.3109/10715762.2015.1106530.

Votaw, V. R., Geyer, R., Rieselbach, M. M., & McHugh, R. K. (2019). The epidemiology of benzodiazepine misuse: A systematic review. Drug and alcohol dependence, 200, 95–114. https://doi.org/10.1016/j.drugalcdep.2019.02.033

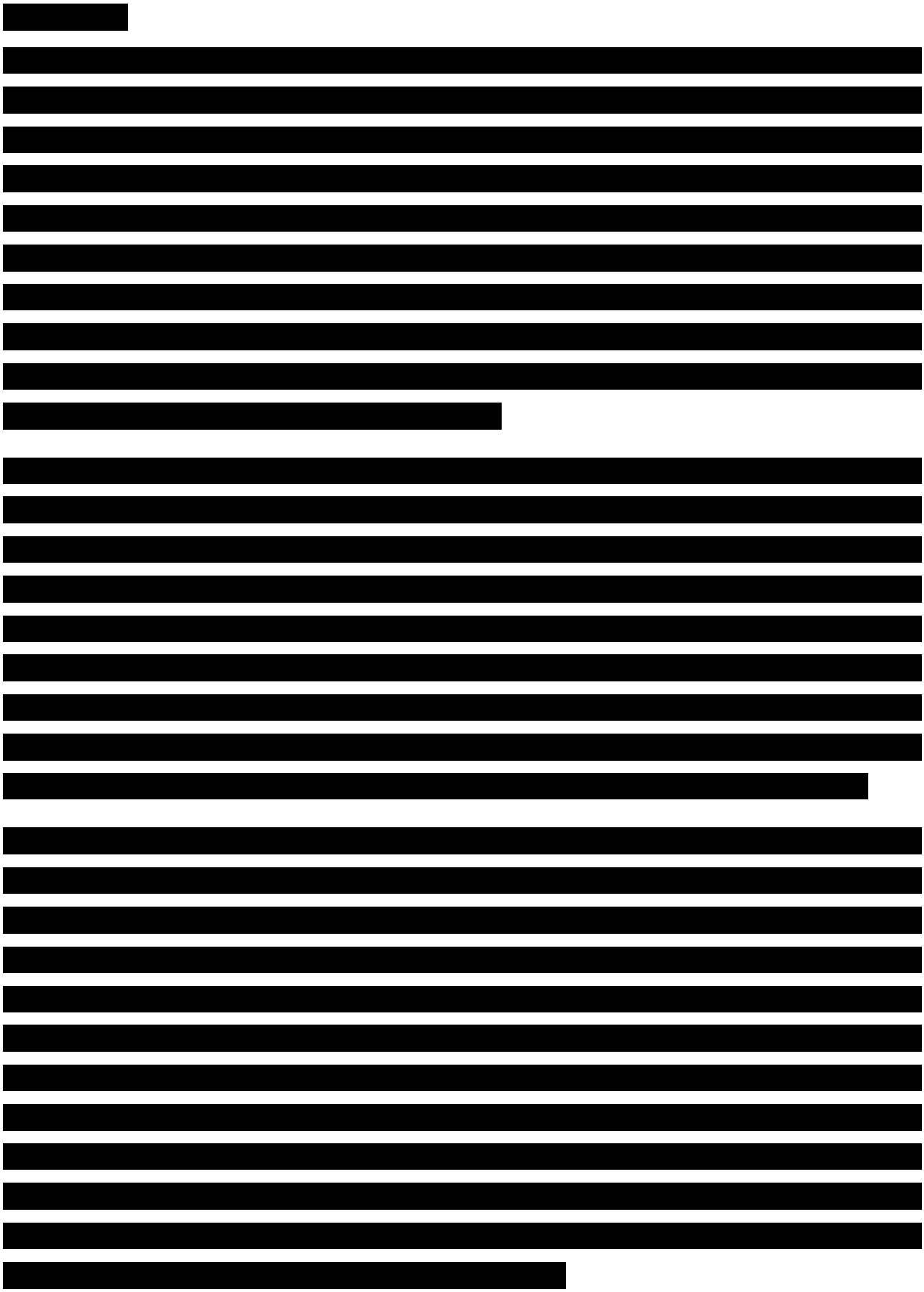
Yasin, N., Veenman, L., Singh, S., Azrad, M., Bode, J., Vainshtein, A., Caballero, B., Marek, I., & Gavish, M. (2017). Classical and Novel TSPO Ligands for the Mitochondrial TSPO Can Modulate Nuclear Gene Expression: Implications for Mitochondrial Retrograde Signaling. International journal of molecular sciences, 18(4), 786. https://doi.org/10.3390/ijms18040786

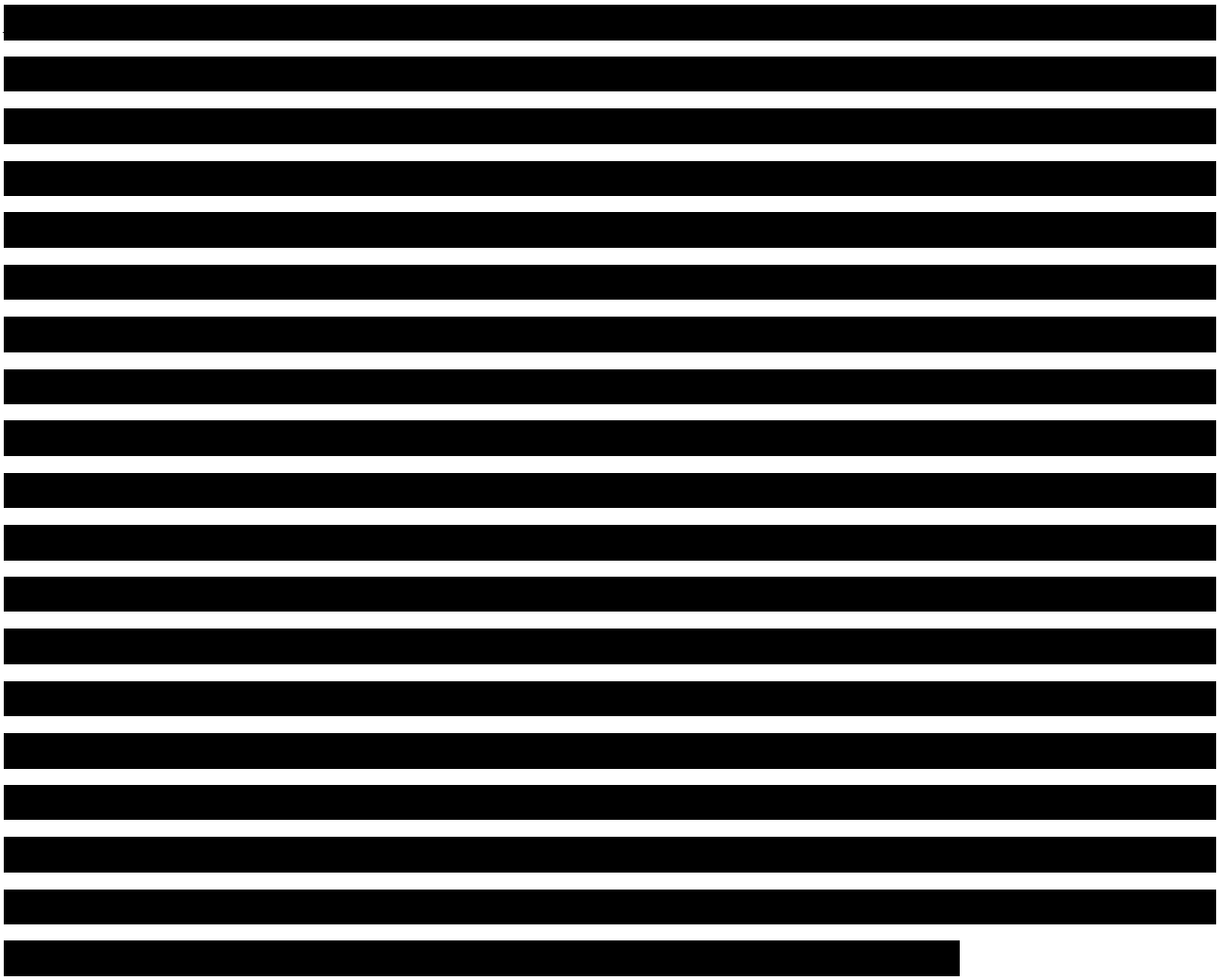
Zahner, M. R., Li, D. P., & Pan, H. L. (2007). Benzodiazepine inhibits hypothalamic presympathetic neurons by potentiation of GABAergic synaptic input. Neuropharmacology, 52(2), 467–475. https://doi.org/10.1016/j.neuropharm.2006.08.024

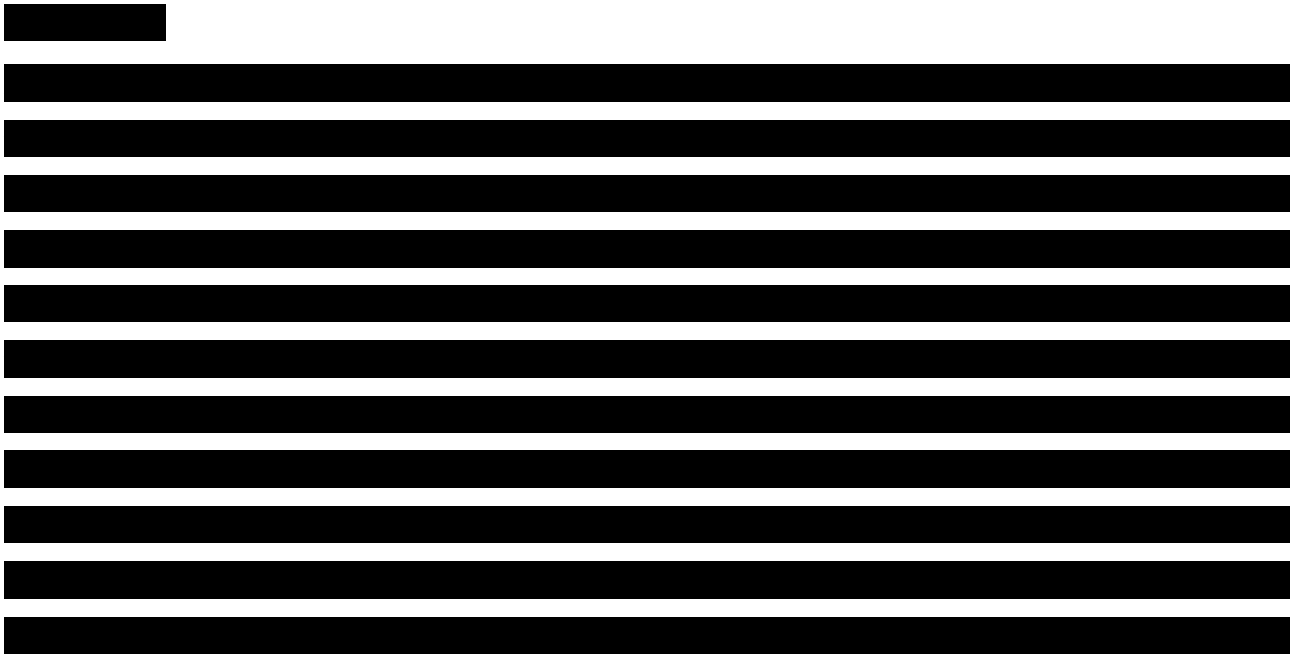
Zhu, Y., Carido, M., Meinhardt, A., Kurth, T., Karl, M. O., Ader, M., & Tanaka, E. M. (2013). Three-dimensional neuroepithelial culture from human embryonic stem cells and its use for quantitative conversion to retinal pigment epithelium. PloS one, 8(1), e54552. https://doi.org/10.1371/journal.pone.0054552

2.2

Water contamination by delorazepam induces epigenetic defects and genomic instability in the embryos of the clawed frog *Xenopus laevis*







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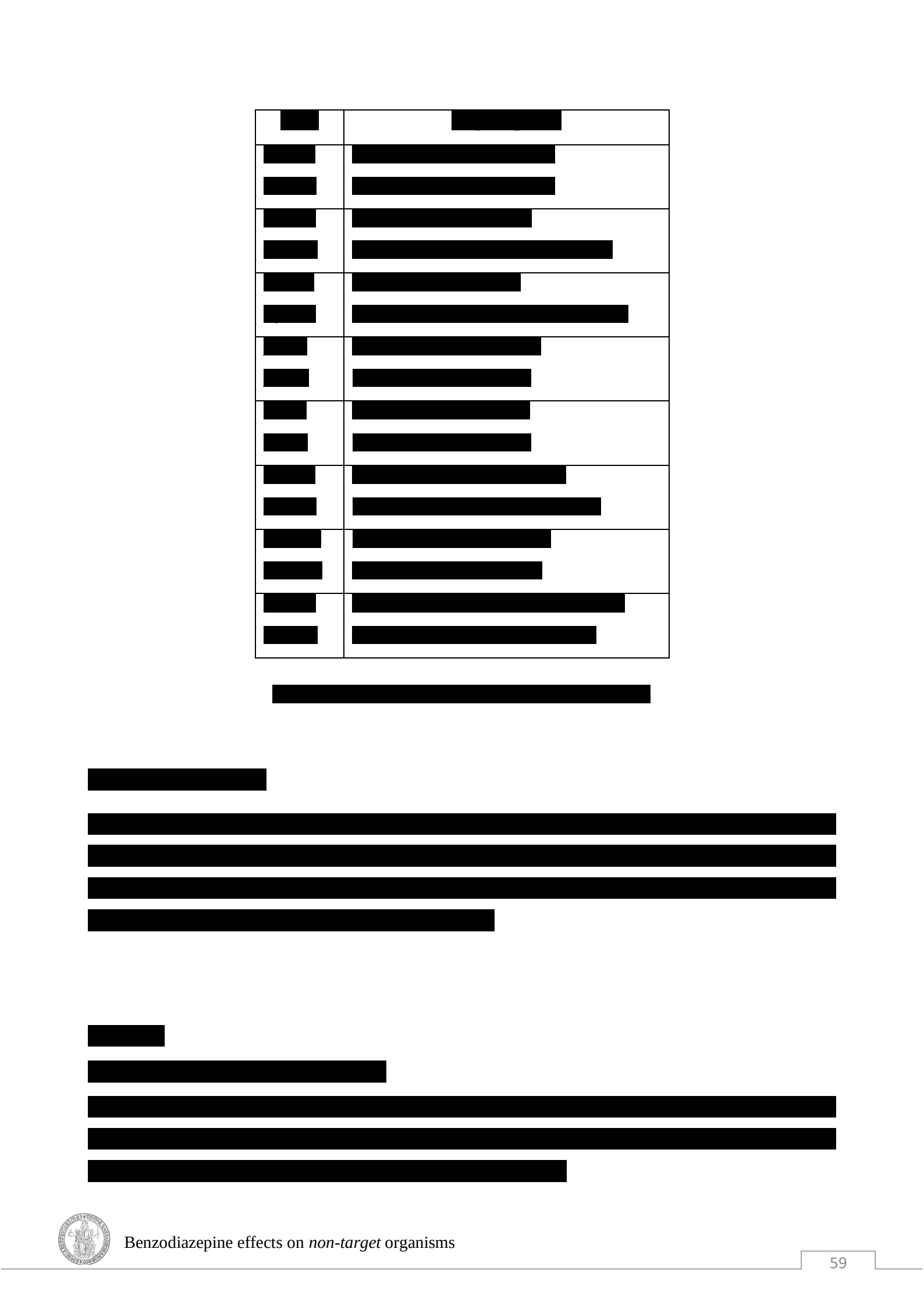

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2.3

Structural and functional damage to the retina, intestine, and skeletal muscle in *Xenopus laevis* embryos exposed to the commonly used psychotropic benzodiazepine delorazepam

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N-Acetyl-glucosamine content in the intestine of *Xenopus laevis* embryos exposed to delorazepam.

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Chapter 3

Mytilus galloprovincialis

3.1 Behavioral alterations and gills damage in *Mytilus galloprovincialis* exposed to an environmental concentration of delorazepam

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3.1

Behavioral alterations and gills damage in *Mytilus galloprovincialis* exposed to an environmental concentration of delorazepam

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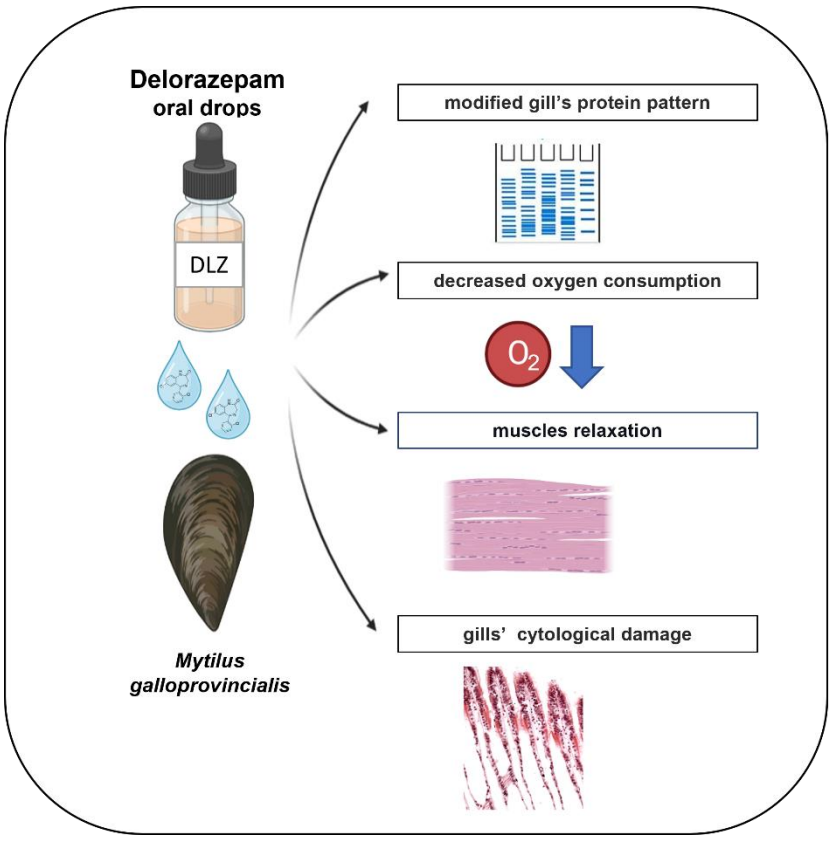

Highlights

- An environmental concentration of delorazepam was tested on *Mytilus galloprovincialis*
- Delorazepam affected valve movements by inducing muscles relaxation
- Damaged gills' lamellae and decreased oxygen consumption were observed
- Delorazepam treatment modified gills protein pattern
- The exposition to the psychotropic delorazepam would severely impair animal fitness

Abstract

Psychoactive compounds, and benzodiazepines (BZPs) in particular, represent an important class of emerging pollutants due to their large (ab)use and high resistance to degradation. Nowadays it is known that sewage treatment does not completely eliminate these substances and, therefore, BZPs and their metabolites reach concern levels in most aquatic environments all over Europe, ranging from µg/L to ng/L. In this study, we investigated the effects of delorazepam on *Mytilus galloprovincialis*, a model organism in toxicity testing and a key species in coastal marine ecosystems. Given its psychoactive activity, the study primarily addressed discovering the effects on behavior, by conventional valve opening and closure tests. Possible cytotoxic activity was also investigated by analyzing valve abductor muscles, gills histology, and correlated oxygen consumption. Results demonstrate negative effects on mussel behavior, interference with metabolism, and alteration of gill morphology and protein content. In conclusion, delorazepam confirms its toxicity to aquatic environments, highlighting the possibility that BZDs can ultimately affect the structure of the food web and the functions of the coastal ecosystems.

Introduction

In the last decades, it has become clear that most pharmaceutical residues released in wastewater are not degraded during their passage through the waste plants (Fatta-Kassinos et al., 2011; Kot-Wasik et al., 2016). As a consequence, they are dispersed in the environment, accumulating in superficial waters at nanogram to microgram concentrations (Hernando et al., 2006).

Psychoactive compounds and benzodiazepines (BZDs), in particular, are very common among these contaminants (Lei et al., 2020) due to their extensive (ab)use and high resistance to degradation, especially in sediments (Kosjek et al., 2012; Klaminder et al., 2015). BZDs and their metabolites have reached concerning levels in most aquatic environments all over Europe (Fick et al., 2017), and negative effects on aquatic species have been already reported even at low concentrations (Brodin et al., 2014; Silva et al., 2020). In addition, direct exposure effects might be further magnified by bioaccumulation in the food web chain (Gomez et al., 2012; Cerveny et al., 2020).

BZDs cause sedation by potentiating the effect of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) on $GABA_A$ receptors, ligand-gated chloride channels (Sieghart et al., 2012). Benzodiazepines bind between the α1 and γ2 subunits, at a site distinct from that of GABA and, by acting as allosteric modulators, increase ligand-receptor affinity potentiating the response (Manchester et al., 2018). This results in significant mitigation of signal at the synaptic level corresponding to

reduced excitability. A second mechanism of action is exerted via peripheral benzodiazepine receptors (TSPO) located on mitochondrial membranes (Papadopoulos et al., 2006). On mitochondria, TSPO regulates cell energy metabolism, transmembrane potential, and sensitivity to reactive oxygen species (Casellas et al., 2002).

Improper activation of GABA receptors may induce potentially relevant consequences on the behavior of *non-target* aquatic species, while the effects on TSPO receptors may alter mitochondrial function and energy availability. The consequent impairment of animal fitness includes, for example, reduced feeding success, inefficiency to elude predators or altered reproductive behavior.

The present work aimed to investigate the effects of the benzodiazepine delorazepam (DLZ) on *Mytilus galloprovincialis*, model organisms in toxicity testing (Prud'homme et al., 2020) and key species in coastal marine ecosystems (Maggi et al., 2009). Mussels are filter feeders preferring eutrophicated waters, rich in plankton or suspended particulate matter as occurring in richly populated basins. A drawback however is the concomitant exposure to high levels of contaminants, potentially endangering their survival (Sampaio et al., 2022). Consequences may be severe considering that mussels contribute to the circulation of matter and nutrients, provide shelter for reproducers and protect from erosion. Not less important, they are of conspicuous commercial relevance (Seitz et al., 2014; Alegria et al., 2017).

Therefore, to mimic an environmental condition, mussels were exposed to delorazepam at 1 µg/L (Fogliano et al., 2022), under static conditions in order to determine the potentially toxic effects of the active principle and its biotransformed products.

Treatment effects were determined by analyzing valve opening and closing, a conventional behavioral test (Ayad et al., 2011), and the condition of the valve abductor muscles. To correlate changes in valve and/or muscle activity with changes in metabolic activity, oxygen consumption was determined. Finally, cytological and SDS-PAGE analyses were performed to test DLZ cytotoxicity on the gills, directly exposed to the drug and probable absorption site (Ogueji et al., 2017).

2. Materials and methods

2.1 Animals handling and maintenance

Adult samples of *Mytilus galloprovincialis* (valve range 6-8 cm) were obtained from a commercial supplier and maintained in 50 L tanks with artificial seawater (Instant Ocean Sea Salt, salinity 36 ± 1‰), maintained at 16 ± 1 °C under a natural photothermal regime (Motta et al., 2018). They were

fed with unicellular algae, homogenized mussel tissues, and food for filter feeders (Coral Diet, Filtrator, Xaqua, Italy).

After two weeks of acclimation (Motta et al., 2018), animals were randomly divided into two groups (n=30 controls; n=45 DLZ-treated) and transferred into 30 L aquaria filled so to maintain a ratio of 2.5 L/animal. The first group was left untreated (control); the second received a largely consumed pharmaceutical product containing the active principle delorazepam at 1 mg/ml concentration and excipients in unspecified quantities (purified water, ethanol, glycasol N, glycerol, propylene glycol, sodium saccharin). The solution was prepared by dissolving the drug, already dissolved in oral drops (1mg/ml) in artificial seawater to a final concentration of 1 µg/L of delorazepam (Fogliano et al., 2022).

Treatments, carried out in triplicates, were static (solutions remained unchanged throughout the test, Avallone et al., 2015; Fogliano et al., 2022) so to expose the animals to both the drug and the metabolites. The experiments lasted for 21 days and the water level was maintained by adding pure distilled water to adjust for evaporation loss. Oxygen levels and water circulation were granted by an external pump connected to an oxygenator for aquaria. No biological or chemical filter was used so to avoid drug absorption and/or metabolization in filtering materials. Experiments were carried out between November and February, in a non-reproductive period, to avoid any possible interference from changes in behavior and physiology related to spawning.

2.2 Behavioral tests

Animal tanks were placed under stable conditions of light and temperature (16 ± 1 °C). On days 1, 3, 7, 14, and 21 the animals with open valves were gently touched with a glass stick in order to induce their closure. The operation was always performed between 11.00 and 12.00 am, so to reduce possible interferences of circadian rhythm (Gnyubkin, 2010). In addition, care was taken to avoid touching the same animal more than twice during the observation period. Proper manipulation and animal response were verified by filming the operations with a camera. Video obtained were used to calculate the average time employed by animals to close the valves after stimulation and the average time employed by animals to reopen the valve by at least 5 mm, after the induced closure. Measurements were carried out on a total of 150 control mussels and 225 DLZ-treated mussels, in three different experiments. After the operation, a few animals were extracted from the tank and processed for histological and biochemical analyses. The water level was reduced accordingly to maintain the volume at 2.5 L/mussel.

2.3 Oxygen consumption determination

The respiratory oxygen consumption (rMO_2) was measured in a closed system, using a Clark electrode, as described by Uliano et al. (2010), and expressed as µg O_2 h^{-1} k^{-1} total weight. On days 1, 3, 7, 14, and 21 after the beginning of the experiments, 5 control and 5 treated animals were randomly collected from the tanks and individually placed in 300 mL respiratometric chambers connected with a recirculation pump and a flush pump. The chambers were placed in a larger seawater bath and maintained at 20°C via thermocriostat. Animals were left to adapt to the chamber for at least 15 min; during measurements, valves were constantly open.

2.4 Histological analyses

For each experiment, on days 1, 3, 7, 14, and 21, 9 control and 9 treated animals were opened, and samples of gills and abductor muscles were dissected and processed for microscopy.

The gills were fixed in Bouin's solution for 6 hours, dehydrated in a graded series of ethanol, and embedded in wax. Sections were stained with Galgano's trichrome or hemalum-eosin to show the general morphology (Motta et al., 2018), with the methyl blue to show the gills chitinous support (Atkins, 1943) or Alcian blue 1% to show goblet cells and mucous (Motta et al, 2022).

To characterize glycans, a panel of three FITC-lectins was used (Vector Laboratories Inc; 2 mg/ml). In particular, WGA (*Triticum vulgaris* agglutinin) was used to detect N-acetyl-glucosamine (glcNAc), DBA (*Dolichos biflorus* agglutinin) for N-acetyl-galactosamine (galNAc) and PNA (*Arachis hypogaea* agglutinin) for terminal galactose (gal). Sections were covered with 1µl of lectin diluted in 19 µl of PBS, placed in a dark moist chamber at room temperature for 15 min, rinsed with PBS, and observed under a UV microscope (excitation maximum at 495 nm and emission maximum at 515 nm). Negative controls were prepared by incubating slides with the lectin and the specific competing sugar or by omitting the lectin (Motta et al., 2005).

Valve abductor muscles were processed for resin embedding (Avallone et al., 2015). Briefly, for each experiment, on days 1, 3, 7, 14, and 21, 3 control and 3 treated animals were opened and samples of muscles were fixed in 2.5 % glutaraldehyde + 4 % paraformaldehyde in 0.1 M PBS pH 7.4 for 4h at 4°C, and post-fixed in 1% osmium tetroxide. After washing in 0.1 M PBS pH 7.4 at 4 °C, samples were dehydrated in an ascending series of ethyl alcohol and embedded in Epon 812. Semi-thin sections (1,5 µm) were cut with a glass knife and stained with 1% toluidine blue solution prepared in 1% sodium tetraborate buffer. Sections obtained were analyzed with a Zeiss Axiocam microscope camera applied to a Zeiss Axioskop microscope.

2.5 Protein analysis by SDS-PAGE

For each experiment, on days 1, 3, 7, 14, and 21, gill samples were collected from 3 control and 3 treated animals, pooled, rapidly frozen, and stored at -80 °C until processed. For protein extraction, the frozen tissue was dissolved in 50 mM PBS, pH 7.5 with protease inhibitor cocktail (Sigma), sonicated for 2.5 min, and centrifuged at 13,000 rpm for 20 min (Coscia et al., 2014). The supernatant was collected, and the extracted proteins were run on a 12.5% acrylamide gel, in Tris-glycine buffer, at 60 mA (Motta et al., 2017).

2.6 Statistical analysis of data

Data on behavioral performance and oxygen consumption are presented as means ± SE of three separate determinations. A two-way ANOVA was used to compare means with Šídák's multiple comparisons *post hoc* test. Statistics were performed with GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Probability was considered statistically significant at $p< 0.05$ (*) and very significant at $p< 0.01$ (**).

3. Results

3.1 Behavioral effect of delorazepam on mussels' valve closure and opening

After mechanical stimulations, about 2 seconds is the average time taken by the control mussels to close the valves (fig. 1A); at the different experimental times, values do not change significantly ($p=0.59$), indicating that mussels do not undergo habituation to the mechanical stress. After exposition to the DLZ, responses show a significant delay on days 1 and 3 ($p<0.01$) and on day 7 ($p<0.05$), while on days 14 and 21 no significant differences are observed compared to control animals. Valves reopening in control mussels occur, on average, after 100 seconds, with a significant decrease on day 21 (fig. 1B; $p=0.02$). After DLZ exposure, valve reopening is significantly anticipated on days 1 and 3 ($p<0.01$), with average values reducing to about 60 seconds. On days 7, 14, and 21 similar responses to the control group were observed.

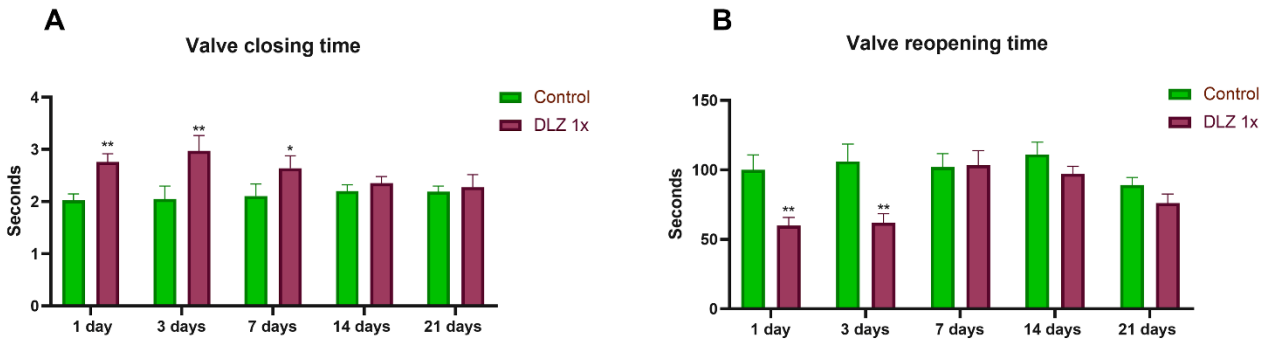

Figure 1. Behavioral response to the benzodiazepine delorazepam (1 µg/L) in *M. galloprovincialis*. A) Valves closure after mechanical stimulation is significantly delayed in animals exposed for 1, 3, and 7 days. B) Valves reopening after closure induced by mechanical stimulation is significantly anticipated in treated animals, on days 1 and 3. Data are means ± SD. *, $p < 0.05$; **, $p < 0.01$.

3.2 Cytological effects of delorazepam on valve abductor muscles

Abductor muscles of animals exposed to DLZ for 1, 3, or 7 days do not show evidence of relaxation or injury compared to control muscles. In all samples, muscular bundles are distinct and well-defined, myofilaments show a regular and compact arrangement with the alternation of smooth and curled fibers with peripheral nuclei, divided by thin connective tissue that forms the myosepta. Fiber dimensions do not differ, except on day 7 of treatment, when the muscle is more compact. After 3 days of treatment a slight increase in myosepta metachromasia is noticed (fig. 2). No changes were highlighted for muscles after 14 and 21 days of treatment (data not shown).

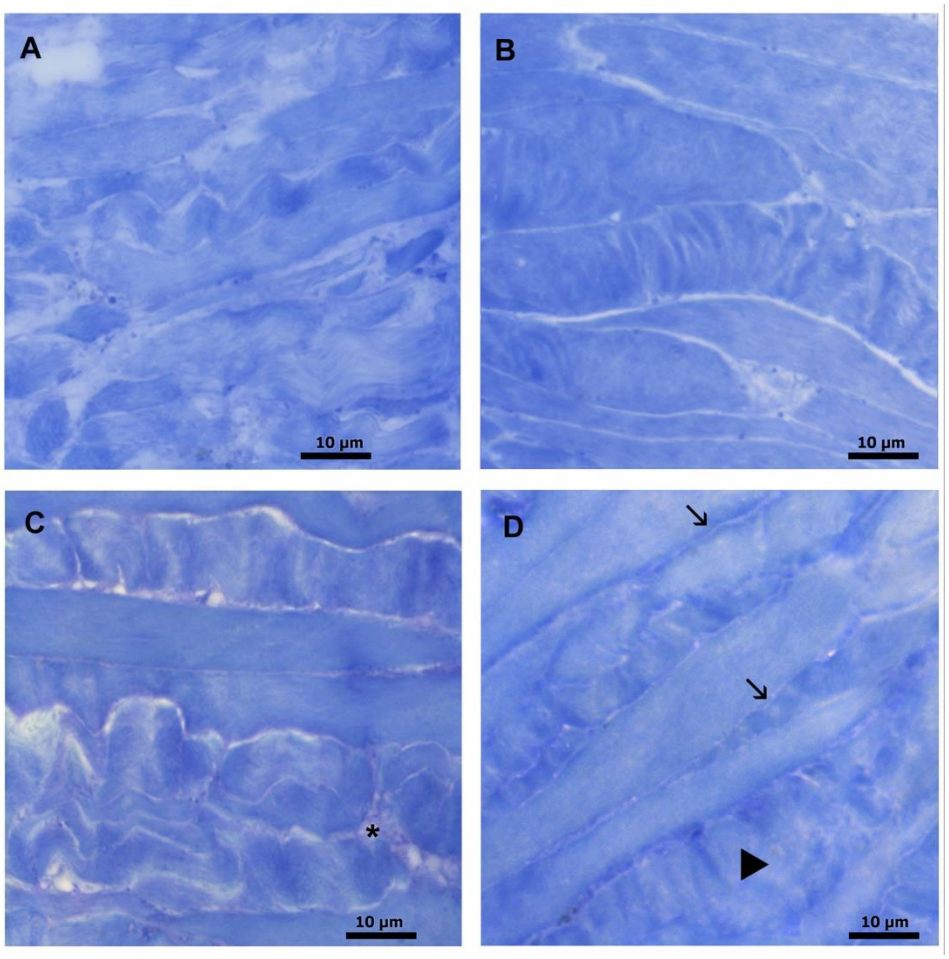

Figure 2. Effects of the benzodiazepine delorazepam (1 µg/L) in *M. galloprovincialis* valve abductor muscles. Longitudinal semi-thin sections. A) Control: normal morphology with smooth and curled fibers. B) 1-day exposure muscle with no difference compared to control. C) 3 days exposure muscle with a slight increase of metachromasia in myosepta (asterisk). D) 7 days exposure muscle, with reduction of space between muscle bundles (arrows) which looks enlarged (arrowhead). Toluidine blue staining, 40x magnification.

3.3 Effects of DLZ on oxygen consumption

The oxygen consumption of *M. galloprovincialis* was significantly affected by the treatment with DLZ (repeated measure 2-way ANOVA, $p = 0.0052$). rMO_2 tended to be lower than the control (Fig. 3). However, *post-hoc* test indicated that this effect was statistically significant at 14 days only ($p<0.05$).

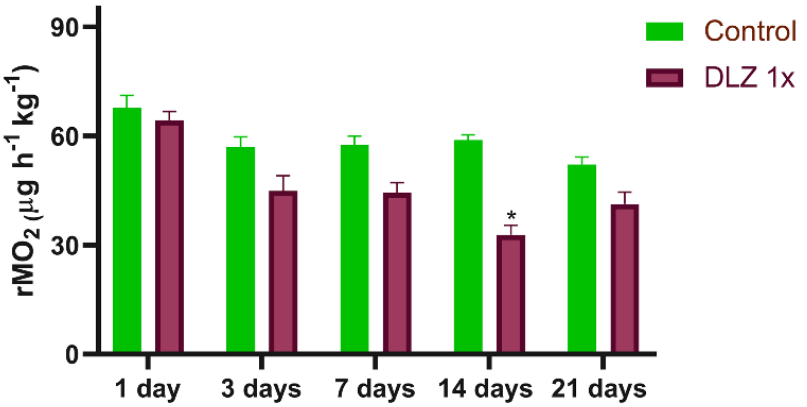

Figure 3. Effects of benzodiazepine delorazepam (1 µg/L) on oxygen consumption (VO2) in *M. galloprovincialis*. Two-way ANOVA indicated a significant effect of treatment ($p < 0.01$). The ˇSíd´ak's *post hoc* test indicated a significant reduction respect to control at day 14. Data are means ± SD.*, $p < 0.05$

3.4 Effects of DLZ on gill tissues morphology

In controls, gill filaments are ordinarily arranged (fig. 4A), the apical epithelium is rich in frontal and lateral cilia and is underlined by a thin, hardly visible chitinous support (fig. 4B-C). This latter significantly thickens in DLZ-exposed mussels as evident in 14- and 21-day samples (fig. 4D-E). In the same samples, the epithelial cells contain several eosinophilic bodies (fig. 4E-F). Goblet cells in control gills form two distinct groups, one on the top of the lamellae and one at the base of the epithelium (fig. 4G). DLZ exposure causes a temporary decrease in their mucous content on day 14 (fig. 4H) but by day 21 differences are no longer evident (fig. 4F).

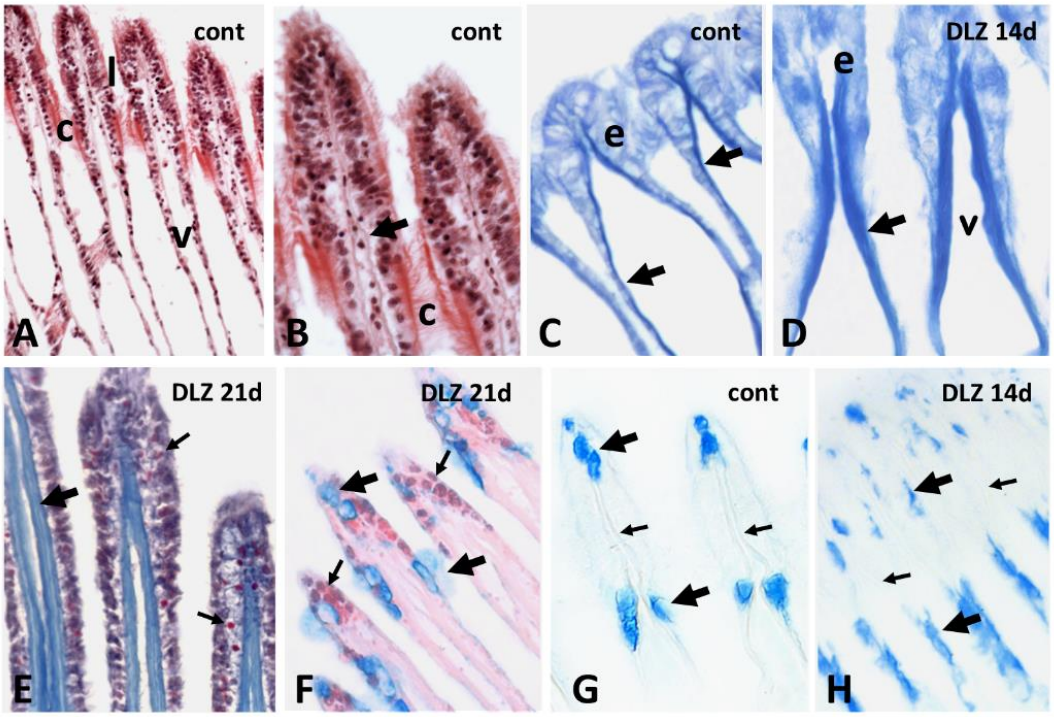

Figure 4. Effect of benzodiazepine delorazepam (1 µg/L) on *Mytilus galloprovincialis* gill tissue. A) Regularly arranged gill filaments (l) showing a central vessel (v) and groups of cilia (c). B) Detail of the epithelium showing the cilia (c) and the thin chitinous layer (arrows). C-D) Thickening of the chitinous layer (arrows) underlying the epithelium. (E) Epithelium with eosinophilic bodies (small arrows) and an underlying thick chitinous layer (arrow). F) Goblet cells (arrows) and epithelial cells with eosinophilic bodies (small arrows). G) Goblet cells (arrows) in lamellae with thin chitinous support (small arrow). H) Goblet cells in basal and apical lamellae (arrows); unstained chitinous support. Hemalum-eosin staining (A-B); methyl blue staining (C, D); Galgano's trichrome staining (E); Alcian blue (F-H) with eosin contrast (F). Bars: 40 µm.

3.5 Effects of DLZ on protein pattern of gills

SDS-PAGE highlights the existence of significant differences in protein patterns between control and DLZ-treated gills tissues, indicating a time-dependent relationship (fig. 5). All DLZ-treated samples lack a very high and a 100 kDa band; at lower MWs, several bands disappear or reduce in intensity. An apparent and partial recovery is observed from day 14, a time in which, however, a further high MW band disappears. Bands below about 30 kDa do not undergo noticeable variations.

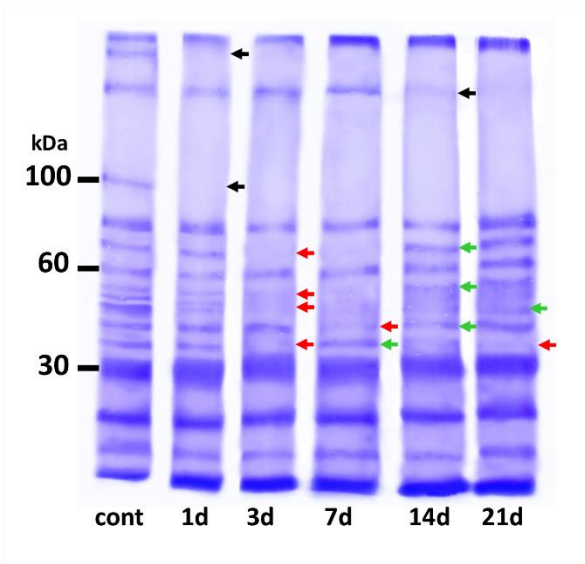

Figure 5. Effects of delorazepam (1 µg/L) on gill's protein pattern. Significant differences are observed in the different lanes, at all molecular weights. Notice that three high MW bands disappear (black arrows) while, at lower MW, several bands become weaker or disappear (red arrows) often reappearing (green arrows) at later experimental times.

3.6 Effects of DLZ on gill tissues glycan content

WGA stains goblet cells in control (fig. 6A) and DLZ-treated animals highlighting a progressive increase in cell number and mucus content (fig. 6B-D). PNA stains chitin support of gill lamella in controls and in DLZ-treated animals up to day 7 (fig. 6E) but from day 14 (fig. 6F) labeling disappears. DBA also stains the chitinous support in control (fig. 6G) and in DLZ-treated gills but only up to day 3. In later samples, gill lamellae are always unstained (fig. 6H).

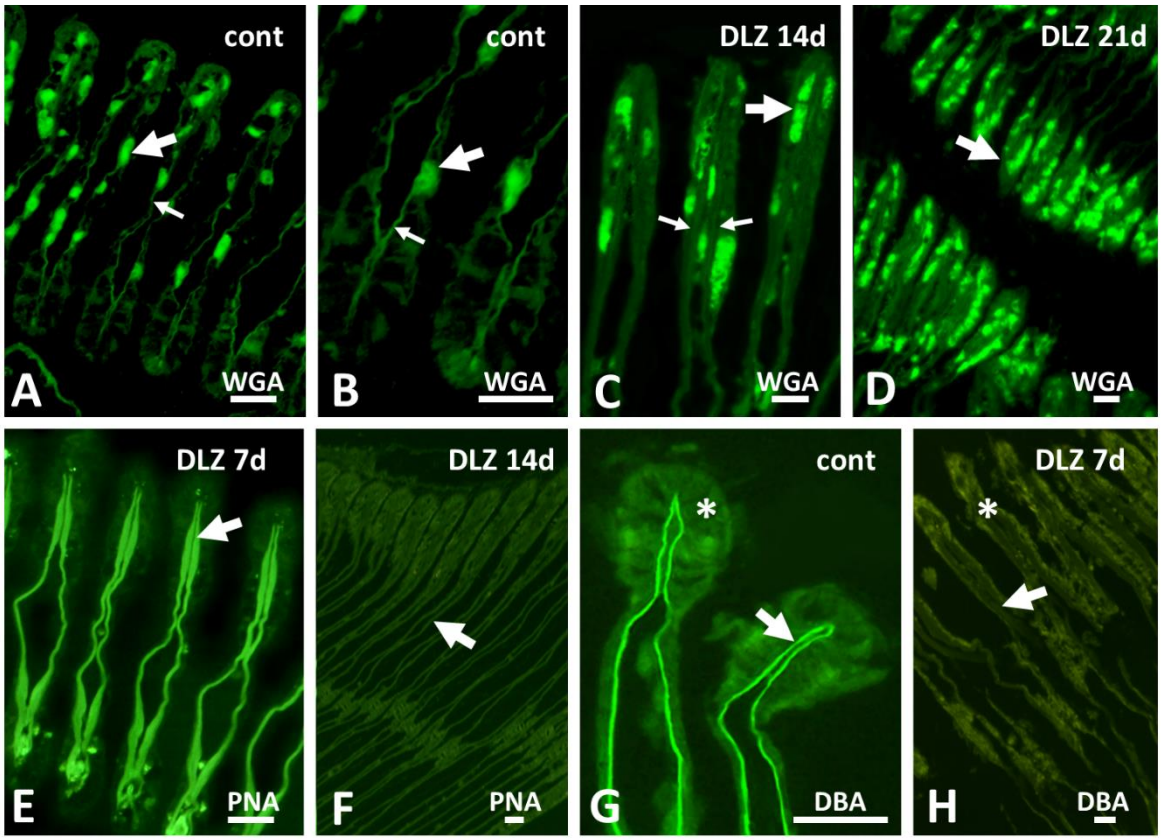

Figure 6. Effects of delorazepam (1 µg/L) on gill's glycan residues distribution: N-acetyl-glucosamine (WGA lectin), terminal galactose (PNA lectin), and N-acetyl-galactosamine (DBA lectin). A-D) Time-dependent increase in stained goblet cells (arrows). Chitinous support (small arrows). E-F and G-H) Disappearance of labeling on chitinous gill support (arrows) and epithelial cells (asterisks). Bars: 40 µm.

4. Discussion

The results indicate that delorazepam has a sedative effect on the mussel and that, in particular, it induced relaxation of the shell abductor muscles. In *Mytilus*, valve closure depends on the active contraction of the abductor muscles that counteract the action of an elastic ligament (Gnyubkin, 2015). The abductor muscle relaxation induced by DLZ explains the increase in the time necessary for valve closure and why valves reopen earlier than expected. This is a foreseeable result considering that *Mytilus* possesses GABA receptors (Betti et al., 2003) and that benzodiazepines reduce the intensity of the action potential by opening GABA-A ligand-gated chloride channels on the postsynaptic neurons (Gallager et al., 1991).

Delorazepam, however, like all benzodiazepines (Nothdurfter et al., 2012), also binds to the TSPO peripheral receptor, in the outer mitochondrial membrane (Jaremko et al., 2015). By controlling

mitochondrial functionality, the receptor regulates oxygen-mediated metabolism (Fan et al., 2012), respiration (Austin et al., 2013), and, eventually, cell bioenergetics (Betlazar et al., 2020). The abundance of TSPO receptors in muscle fibers (Beavis, 1989; Larcher et al., 1989) suggests that the reduced performance in valve tests might depend on interference with mitochondrial functionality rather than on a direct effect of benzodiazepine on GABA. This putative effect would explain the reduced oxygen consumption observed at the whole-body level (rMO_2), which, on the other hand, is likely to depend on the altered and probably functionally impaired branchial system. The first evidence of gills damage is goblet cell hyperplasia. Mucus is released as the first line of defense (Garcia-Reyero et al., 2015), and contributes to detoxification by absorbing contaminants and by preventing contact with the cell surface (Dimitriadis et al., 2003; Marigomez et al., 2002). However, hypersecretion may induce asphyxiation (Kent et al., 1995) and affect feeding, and by increasing the formation of pseudofeces, decreases metabolic efficiency (Salas-Yanquin et al., 2018).

Further confirmation of gills toxicity of DLZ is represented by the changes induced in the chitinous support, a preferred target of toxicity since rich in positively charged glucosamide residues (Einsporn and Koehler, 2008). The observed thickening could reduce the diffusion rate of O2 (Jayakumar et al., 2011; McDonnell et al., 2016) with consequent respiratory failure, and a decrease in metabolic efficiency and energy production. Chitinous thickening may also affect the innate defense (Willoughby and Tomlinson, 1999) and, by deceiving the immunocompetent circulating hemocytes, alter the lamellar robust line of defense against potential pathogens and chemical contaminants (Venier et al., 2011). Staining with lectins indicates that thickening is accompanied by a loss in galactose and galNAc. Unfortunately, no data on chitinous support composition could be found in mussels; however, in fungi, the chitinous cell wall is reported to contain 6% of insoluble residues including mannose, galactose, and uronic acid (Skujins et al., 1965). Their role in the chitinous support is unclear, and so are the consequences of the observed changes.

Another indication of altered branchial function is the appearance of intracytoplasmic eosinophilic bodies in lamellar epithelial cells. These are protein structures already associated with exposure to various xenobiotics (Camargo and Matinez, 2007; Van Dyk et al., 2007), a direct consequence of cell damage (Abdel-Moneim et al., 2012), and associated with lysosomal activity in macrophages (Nagy et al., 1989). Of particular significance is the evidence that DLZ toxic effects are induced very rapidly. Staining with DBA lectin shows a decrease in galNAc in the chitinous support already on day 3 and SDS-PAGE indicates that alterations are even more immediate, being already appreciable at 24 hours. In addition, the protein analysis reveals a very dynamic situation during the 21 days of observation, with several bands disappearing and reappearing at different times and others simply disappearing. At

the moment, no attempt has been made to identify the different proteins, but their characterization will give important information on the toxicodynamics of delorazepam.

Another important point concerns the toxicokinetic of the drug. The liquid drop formulation used in this study is reported to be absorbed quickly and to have good bioavailability. Early responses confirm that DLZ penetrates rapidly in gills epithelial cells. Delorazepam is a long-acting benzodiazepine, between 1 and 7 days (Morgan et al., 1990), which makes it superior to lorazepam, its major metabolite. So, with this respect, it could be speculated that the combination of delorazepam/lorazepam, which is relatively potent (1 mg of delorazepam being the equivalent of 10 mg of diazepam) (Cosci et al, 2016) could bring long-lasting effects or, simply, that active principle gave rise to very stable metabolite in the first 7 days, which continue to exert secondary effect until day 21. It's reported that metabolites can accumulate more than the parent drug, even without being present in water (Miller et al., 2017) reaching concentration levels sufficient to exert a pharmacological effect (Cerveny et al., 2021a). Because of their biological activity on *non-target* organisms, metabolites of many drugs should be considered pharmaceutically active compounds (PhACs) and be included in ecotoxicology studies (Celiz et al., 2009). For these reasons, delorazepam/lorazepam bioconcentrations in tissues, half-life, and catabolite production are currently under investigation.

Conclusions

Results obtained show that DLZs and/or metabolites, at environmental concentrations, have a profound impact on mussel behavior. More significantly, they are toxic to gill cells affecting respiratory function in a time-dependent manner. As far as we are aware, this is the first report of a direct cytotoxic effect of benzodiazepine, a drug considered essentially safe for tissues.

Relapses are relevant as they open to a reconsideration of the evidence collected so far. The consequences of exposing the aquatic ecosystems to benzodiazepines appear now more severe than previously postulated. Future studies are essential to evaluate if damages observed in mussels are exerted also on other models and to accurately evaluate the impact that these effects can have on the survival of the shore communities, the more exposed to anthropic pollution.

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References

Abdel-Moneim, A. M., Al-Kahtani, M. A., & Elmenshawy, O. M. (2012). Histopathological biomarkers in gills and liver of *Oreochromis niloticus* from polluted wetland environments, Saudi Arabia. Chemosphere, 88(8), 1028-1035. https://doi.org/10.1016/j.chemosphere.2012.04.001

Atkins, D. (1943). On the Ciliary Mechanisms and Interrelationships of Lamellibranches: PART VIII: Notes on Gill Musculature in the Microcilio branchia. Journal of Cell Science, 2(334), 187-256.

Austin, C. J., Kahlert, J., Kassiou, M., & Rendina, L. M. (2013). The translocator protein (TSPO): a novel target for cancer chemotherapy. The international journal of biochemistry & cell biology, 45(7), 1212–1216. https://doi.org/10.1016/j.biocel.2013.03.004

Avallone, B., Agnisola, C., Cerciello, R., Panzuto, R., Simoniello, P., Cretì, P., Motta, C.M., 2015. Structural and functional changes in the zebrafish (*Danio rerio*) skeletal muscle after cadmium exposure. Cell Biol. Toxicol. 31 (6), 273e283. https://doi.org/10.1007/s10565-015-9310-0.

Ayad, M. A., Fdil, M. A., Mouabad, A. (2011). Effects of cypermethrin (pyrethroid insecticide) on the valve activity behavior, byssal thread formation, and survival in air of the marine mussel *Mytilus galloprovincialis*. Archives of environmental contamination and toxicology, 60(3), 462-470. https://doi.org/10.1007/s00244-010-9549-7

Beavis, A.D. (1989). On the inhibition of the mitochondrial inner membrane anion uniporter by cationic amphiphiles and other drugs. J. Biol. Chem. 264.

Betlazar, C., Middleton, R. J., Banati, R., Liu, G. J. (2020). The Translocator Protein (TSPO) in Mitochondrial Bioenergetics and Immune Processes. Cells, 9(2), 512. https://doi.org/10.3390/cells9020512

Betti, L., Giannaccini, G., Nigro, M., Dianda, S., Gremigni, V., & Lucacchini, A. (2003). Studies of peripheral benzodiazepine receptors in mussels: comparison between a polluted and a nonpolluted site. Ecotoxicology and environmental safety, 54(1), 36–42. https://doi.org/10.1016/s0147-6513(02)00014-3

Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M., & Jonsson, M. (2014). Ecological effects of pharmaceuticals in aquatic systems--impacts through behavioural alterations. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 369(1656), 20130580. https://doi.org/10.1098/rstb.2013.0580

Camargo, M.M.P., Matinez, C.B.R., 2007. Histopathology of gills, kidney and liver of a neotropical fish caged in an urban stream. Neotrop. Ichthyol. 5, 327–336. https://doi.org/10.1590/S1679-62252007000300013

Casellas, P., Galiegue, S., Basile, A. S. (2002). Peripheral benzodiazepine receptors and mitochondrial function. Neurochemistry international, 40(6), 475–486. https://doi.org/10.1016/s0197-0186(01)00118-8

Celiz, M. D., Tso, J., Aga, D. S. (2009). Pharmaceutical metabolites in the environment: analytical challenges and ecological risks. Environmental toxicology and chemistry, 28(12), 2473–2484. https://doi.org/10.1897/09-173.1

Cerveny, D., Brodin, T., Cisar, P., McCallum, E. S., & Fick, J. (2020). Bioconcentration and behavioral effects of four benzodiazepines and their environmentally relevant mixture in wild fish. The Science of the total environment, 702, 134780. https://doi.org/10.1016/j.scitotenv.2019.134780

Cosci, F., Mansueto, G., Faccini, M., Casari, R., Lugoboni, F. (2016). Socio-demographic and clinical characteristics of benzodiazepine long-term users: Results from a tertiary care center. Comprehensive psychiatry, 69, 211–215. https://doi.org/10.1016/j.comppsych.2016.06.008

Coscia, M. R., Simoniello, P., Giacomelli, S., Oreste, U., & Motta, C. M. (2014). Investigation of immunoglobulins in skin of the Antarctic teleost *Trematomus bernacchii*. Fish & shellfish immunology, 39(2), 206–214. https://doi.org/10.1016/j.fsi.2014.04.019

Dimitriadis, V.K., Domouhtsidou, G.P., Raftopoulou, E. (2003). Environmental Pollution, 125, pp. 345-353

Einsporn, S., & Koehler, A. (2008). Immuno-localisations (GSSP) of subcellular accumulation sites of phenanthrene, aroclor 1254 and lead (Pb) in relation to cytopathologies in the gills and digestive gland of the mussel Mytilus edulis. Marine environmental research, 66(1), 185–186. https://doi.org/10.1016/j.marenvres.2008.02.053

Fan, J., Lindemann, P., Feuilloley, M. G., Papadopoulos, V. (2012). Structural and functional evolution of the translocator protein (18 kDa). Current molecular medicine, 12(4), 369–386. https://doi.org/10.2174/1566524011207040369

Fatta-Kassinos, D., Meric, S., & Nikolaou, A. (2011). Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. Analytical and bioanalytical chemistry, 399(1), 251–275. https://doi.org/10.1007/s00216-010-4300-9

Fick, J., Brodin, T., Heynen, M., Klaminder, J., Jonsson, M., Grabicova, K., Randak, T., Grabic, R., Kodes, V., Slobodnik, J., Sweetman, A., Earnshaw, M., Barra Caracciolo, A., Lettieri, T., Loos, R. (2017). Screening of benzodiazepines in thirty European rivers. Chemosphere, 176, 324–332. https://doi.org/10.1016/j.chemosphere.2017.02.126

Fogliano, C., Motta, C. M., Venditti, P., Fasciolo, G., Napolitano, G., Avallone, B., & Carotenuto, R. (2022). Environmental concentrations of a delorazepam-based drug impact on embryonic development of non-target *Xenopus laevis*. Aquatic toxicology (Amsterdam, Netherlands), 250, 106244. https://doi.org/10.1016/j.aquatox.2022.106244

Gallager D.W., Marley R.J., Hernandez T.D. (1991). Biochemical and Electrophysiological Mechanisms Underlying Benzodiazepine Tolerance and Dependence, J. Pratt (ed.). The Biological Basis of Drug Tolerance and Dependence London: Academic Press.

Garcia-Reyero, N., Thornton, C., Hawkins, A. D., Escalon, L., Kennedy, A. J., Steevens, J. A., Willett, K. L. (2015). Assessing the exposure to nanosilver and silver nitrate on fathead minnow gill gene expression and mucus production. Environmental nanotechnology, monitoring & management, 4, 58-66. https://doi.org/10.1016/j.enmm.2015.06.001

Gnyubkin, V.F. (2010). The circadian rhythms of valve movements in the mussel *Mytilus galloprovincialis*. Russ J Mar Biol 36, 419–428. https://doi.org/10.1134/S1063074010060039

Gnyubkin, V.F. The valve-movement model for the mediterranean mussel, *Mytilus galloprovincialis* Lamarck, 1819 (Bivalvia: Mytilidae). Russ J Mar Biol 41, 40–50 (2015). https://doi.org/10.1134/S1063074015010034

Gomez, E., Bachelot, M., Boillot, C., Munaron, D., Chiron, S., Casellas, C., & Fenet, H. (2011). Bioconcentration of two pharmaceuticals (benzodiazepines) and two personal care products (UV filters) in marine mussels (*Mytilus galloprovincialis*) under controlled laboratory conditions. Environmental science and pollution research international, 19(7), 2561–2569. https://doi.org/10.1007/s11356-012-0964-3

Hernando, M. D., Mezcua, M., Fernández-Alba, A. R., & Barceló, D. (2006). Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. Talanta, 69(2), 334–342. https://doi.org/10.1016/j.talanta.2005.09.037

Jaremko, M., Jaremko, Ł., Jaipuria, G., Becker, S., & Zweckstetter, M. (2015). Structure of the mammalian TSPO/PBR protein. Biochemical Society transactions, 43(4), 566–571. https://doi.org/10.1042/BST20150029

Jayakumar, R., Prabaharan, M., Sudheesh Kumar, P. T., Nair, S. V., & Tamura, H. (2011). Biomaterials based on chitin and chitosan in wound dressing applications. Biotechnology advances, 29(3), 322–337. https://doi.org/10.1016/j.biotechadv.2011.01.005

Kent M.L., Whyte J.N.C., LaTrace C. Gill lesions and mortality in seawater pen-reared Atlantic salmon Salmo salar associated with a dense bloom of *Skeletonema costatum* and *Thalassiosira* species. Dis. Aquat. Org. 1995; 22:77–81. https://doi.org/ 10.3354/dao022077.

Klaminder J., Jonsson M., Fick J., Sunderlin A., Brodin T. (2014). The conceptual imperfection of aquatic risk assessment tests: Highlighting the need for tests designed to detect therapeutic effects of pharmaceutical contaminants. Environ. Res. Lett. 9. https://doi.org/10.1088/1748-9326/9/8/084003

Kosjek, T., Perko, S., Zupanc, M., Zanoški Hren, M., Landeka Dragičević, T., Zigon, D., Kompare, B., Heath, E. (2012). Environmental occurrence, fate and transformation of benzodiazepines in water treatment. Water research, 46(2), 355–368. https://doi.org/10.1016/j.watres.2011.10.056

Kot-Wasik, A., Jakimska, A., & Śliwka-Kaszyńska, M. (2016). Occurrence and seasonal variations of 25 pharmaceutical residues in wastewater and drinking water treatment plants. Environmental monitoring and assessment, 188(12), 661. https://doi.org/10.1007/s10661-016-5637-0

Larcher, J. C., Vayssiere, J. L., Le Marquer, F. J., Cordeau, L. R., Keane, P. E., Bachy, A., Gros, F., & Croizat, B. P. (1989). Effects of peripheral benzodiazepines upon the O2 consumption of neuroblastoma cells. European journal of pharmacology, 161(2-3), 197–202. https://doi.org/10.1016/0014-2999(89)90843-1

Lei, H. J., Yang, B., Ye, P., Yang, Y. Y., Zhao, J. L., Liu, Y. S., Xie, L., & Ying, G. G. (2021). Occurrence, fate and mass loading of benzodiazepines and their transformation products in eleven wastewater treatment plants in Guangdong province, China. The Science of the total environment, 755(Pt 2), 142648. https://doi.org/10.1016/j.scitotenv.2020.142648

Maggi, E., Bertocci, I., Vaselli, S., & Benedetti-Cecchi, L. (2009). Effects of changes in number, identity and abundance of habitat-forming species on assemblages of rocky seashores. Marine ecology progress series, 381, 39-49. https://doi.org/ 10.3354/meps07949

Manchester, K. R., Lomas, E. C., Waters, L., Dempsey, F. C., & Maskell, P. D. (2018). The emergence of new psychoactive substance (NPS) benzodiazepines: A review. Drug testing and analysis, 10(1), 37–53. https://doi.org/10.1002/dta.2211

Marigomez, I., Soto, M., Cajaraville, M., Angulo, E. (2002). Microscopy Research and Technique, 56 pp. 358-392.

McDonnell, M. T., Greeley, D. A., Kit, K. M., & Keffer, D. J. (2016). Molecular Dynamics Simulations of Hydration Effects on Solvation, Diffusivity, and Permeability in Chitosan/Chitin Films. The journal of physical chemistry. B, 120(34), 8997–9010. https://doi.org/10.1021/acs.jpcb.6b05999

Miller, T. H., Bury, N. R., Owen, S. F., & Barron, L. P. (2017). Uptake, biotransformation and elimination of selected pharmaceuticals in a freshwater invertebrate measured using liquid chromatography tandem mass spectrometry. Chemosphere, 183, 389–400. https://doi.org/10.1016/j.chemosphere.2017.05.083

Morgan, W.W. (1990). Abuse Liability of Barbiturates and Other Sedative-Hypnotics. Advances in Alcohol and Substance. Abuse 9.

Motta, C. M., Califano, E., Scudiero, R., Avallone, B., Fogliano, C., De Bonis, S., Raggio, A., Simoniello, P. (2022). Effects of Cadmium Exposure on Gut Villi in *Danio rerio*. International journal of molecular sciences, 23(4), 1927. https://doi.org/10.3390/ijms23041927

Motta, C. M., Cerciello, R., De Bonis, S., Mazzella, V., Cirino, P., Panzuto, R., Ciaravolo, M., Simoniello, P., Toscanesi, M., Trifuoggi, M., Avallone, B. (2016). Potential toxicity of improperly discarded exhausted photovoltaic cells. Environmental pollution (Barking, Essex: 1987), 216, 786–792. https://doi.org/10.1016/j.envpol.2016.06.048

Motta, C. M., Simoniello, P., Di Lorenzo, M., Migliaccio, V., Panzuto, R., Califano, E., & Santovito, G. (2021). Endocrine disrupting effects of copper and cadmium in the oocytes of the Antarctic Emerald rockcod *Trematomus bernacchii*. Chemosphere, 268, 129282. https://doi.org/10.1016/j.chemosphere.2020.129282

Motta, C. M., Tizzano, M., Tagliafierro, A. M., Simoniello, P., Panzuto, R., Esposito, L., Migliaccio, V., Rosati, L., & Avallone, B. (2018). Biocide triclosan impairs byssus formation in marine mussels *Mytilus galloprovincialis*. Environmental pollution (Barking, Essex: 1987), 241, 388–396. https://doi.org/10.1016/j.envpol.2018.05.064

Motta, C.M., Tammaro, S., Simoniello, P., Prisco, M., Ricchiari, L., Andreuccetti, P. and Filosa, S. (2005), Characterization of cortical alveoli content in several species of Antarctic notothenioids. Journal of Fish Biology, 66: 442-453. https://doi.org/10.1111/j.0022-1112.2005.00613.x

Nagy, G. K., Jacobs, J. B., Mason-Savas, A., Pomerantz, S. N., & DeCiero, G. J. (1989). Intracytoplasmic eosinophilic inclusion bodies in breast cyst fluids are giant lysosomes. Acta cytologica, 33(1), 99–103.

Nothdurfter, C., Rammes, G., Baghai, T. C., Schüle, C., Schumacher, M., Papadopoulos, V., Rupprecht, R. (2012). Translocator protein (18 kDa) as a target for novel anxiolytics with a favourable side-effect profile. Journal of neuroendocrinology, 24(1), 82–92. https://doi.org/10.1111/j.1365-2826.2011.02166.x

Ogueji, E. O., Iheanacho, S. C., Nwani, C. D., Mbah, C. E., Okeke, O. C., Ibrahim, B. U. (2017). Toxicity of diazepam on lipid peroxidation, biochemical and oxidative stress indicators on liver and gill tissues of African catfish *Clarias gariepinus* (Burchell, 1822). International Journal of Fisheries and Aquatic Studies, 5(3), 114-123.

Papadopoulos, V., Baraldi, M., Guilarte, T. R., Knudsen, T. B., Lacapère, J. J., Lindemann, P., Norenberg, M. D., Nutt, D., Weizman, A., Zhang, M. R., Gavish, M. (2006). Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends in pharmacological sciences, 27(8), 402–409. https://doi.org/10.1016/j.tips.2006.06.005

Prud'homme, S. M., Hani, Y. M. I., Cox, N., Lippens, G., Nuzillard, J. M., & Geffard, A. (2020). The Zebra Mussel (*Dreissena polymorpha*) as a Model Organism for Ecotoxicological Studies: A Prior 1H NMR spectrum interpretation of a whole body extract for metabolism monitoring. Metabolites, 10(6), 256. https://doi.org/10.3390/metabo10060256

Salas-Yanquin, L. P., Navarro, J. M., Pechenik, J. A., Montory, J. A., & Chaparro, O. R. (2018). Volcanic ash in the water column: Physiological impact on the suspension-feeding bivalve *Mytilus chilensis*. Marine pollution bulletin, 127, 342–351. https://doi.org/10.1016/j.marpolbul.2017.12.024

Sampaio, L., Moreira, J., Rubal, M., Guerrero-Meseguer, L., & Veiga, P. (2022). A Review of Coastal Anthropogenic Impacts on Mytilid Mussel Beds: Effects on mussels and their associated assemblages. Diversity, 14(5), 409. https://doi.org/10.3390/d14050409

Sieghart, W., Ramerstorfer, J., Sarto-Jackson, I., Varagic, Z., & Ernst, M. (2012). A novel GABA(A) receptor pharmacology: drugs interacting with the α(+) β(-) interface. British journal of pharmacology, 166(2), 476–485. https://doi.org/10.1111/j.1476-5381.2011.01779.x

Silva, A., Nilin, J., Loureiro, S., & Costa-Lotufo, L. V. (2020). Acute and chronic toxicity of the benzodiazepine diazepam to the tropical crustacean *Mysidopsis juniae*. Anais da Academia Brasileira de Ciencias, 92(1), e20180595. https://doi.org/10.1590/0001-3765202020180595

Skujins, J. J., Potgieter, H. J., & Alexander, M. (1965). Dissolution of fungal cell walls by a streptomycete chitinase and beta-(1-3) glucanase. Archives of biochemistry and biophysics, 111(2), 358–364. https://doi.org/10.1016/0003-9861(65)90197-9

Uliano, E., Cataldi, M., Carella, F., Migliaccio, O., Iaccarino, D., & Agnisola, C. (2010). Effects of acute changes in salinity and temperature on routine metabolism and nitrogen excretion in gambusia (Gambusia affinis) and zebrafish (*Danio rerio*). Comparative biochemistry and physiology. Part A, Molecular & integrative physiology, 157(3), 283–290. https://doi.org/10.1016/j.cbpa.2010.07.019

van Dyk, J. C., Pieterse, G. M., van Vuren, J. H. (2007). Histological changes in the liver of *Oreochromis mossambicus* (Cichlidae) after exposure to cadmium and zinc. Ecotoxicology and environmental safety, 66(3), 432–440. https://doi.org/10.1016/j.ecoenv.2005.10.012

Venier, P., Varotto, L., Rosani, U., Millino, C., Celegato, B., Bernante, F., Lanfranchi, G., Novoa, B., Roch, P., Figueras, A., Pallavicini, A. (2011). Insights into the innate immunity of the Mediterranean mussel *Mytilus galloprovincialis*. BMC genomics, 12, 69. https://doi.org/10.1186/1471-2164-12-69

Willoughby, D.A., Tomlinson, A. Inducible Enzymes in the Inflammatory Response. 1422-7746, IX, 214. https://doi.org/10.1007/978-3-0348-8747-2

Chapter 4

Planorbarius corneus

3.1 Benzodiazepine delorazepam inhibits feeding behavior while inducing hyperactivity and altering pedal mucus characteristics in the freshwater gastropod *Planorbarius corneus*

Article in preparation

4.1

Benzodiazepine delorazepam inhibits feeding behavior while inducing hyperactivity and altering pedal mucus characteristics in the freshwater gastropod *Planorbarius corneus*

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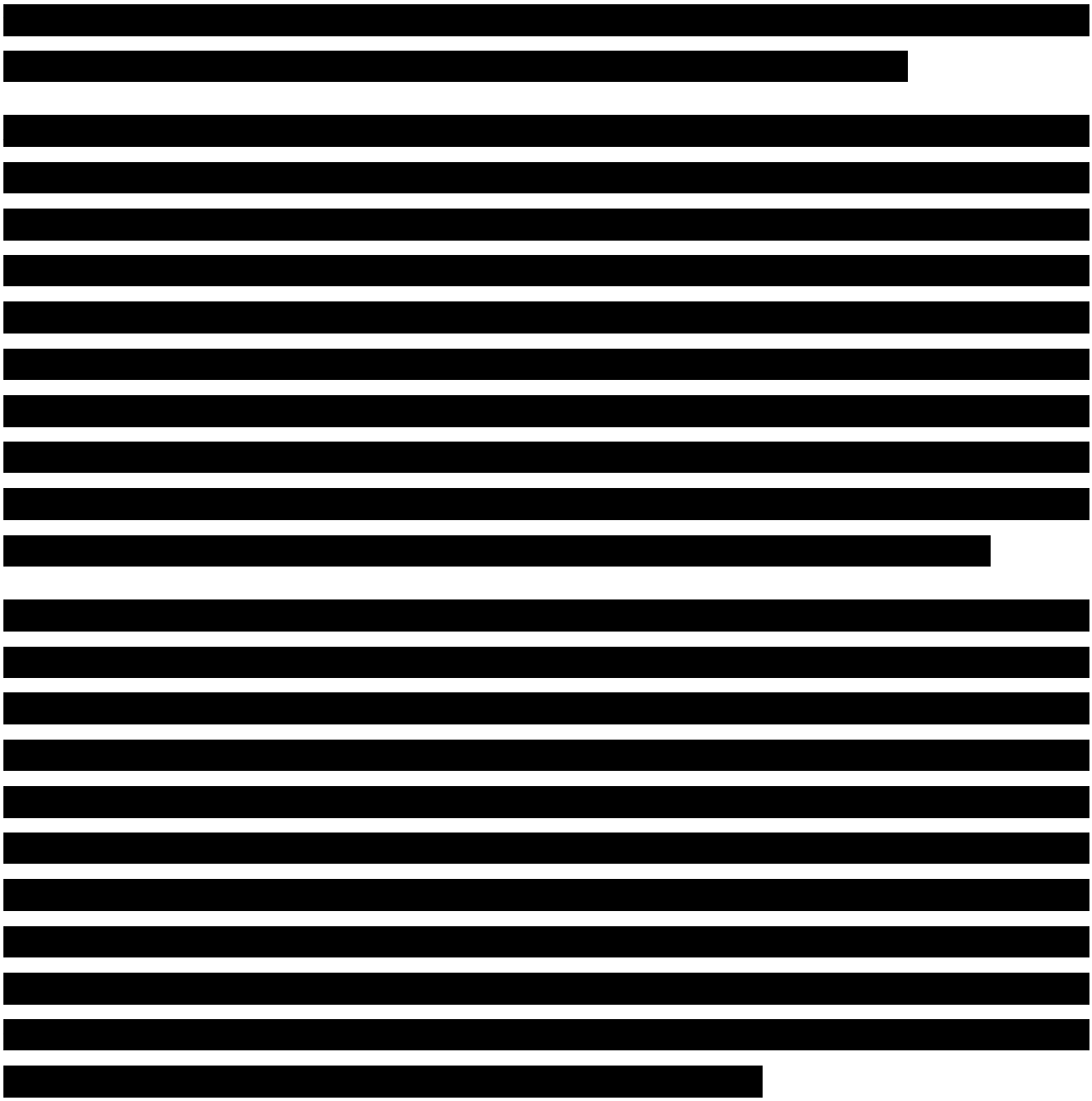

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Chapter 5
Paracentrotus lividus

5.1 Effects of benzodiazepine delorazepam on fertilization and early development in the sea urchin *Paracentrotus lividus*

Article in preparation

5.1

Effects of benzodiazepine delorazepam on fertilization and early development in the sea urchin *Paracentrotus lividus*

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present features (i.e. wide distribution, easily accessible samples, abundance, low mobility, life cycle, feeding behavior, ecological and economic importance) which render them suitable as sentinel species for aquatic environment biomonitoring.

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Chapter 6

Artemia salina

6.1 Toxic effects of the anxiolytic benzodiazepine delorazepam on *Artemia salina* development

Article in preparation

6.1

Toxic effects of the anxiolytic benzodiazepine delorazepam on *Artemia salina* development

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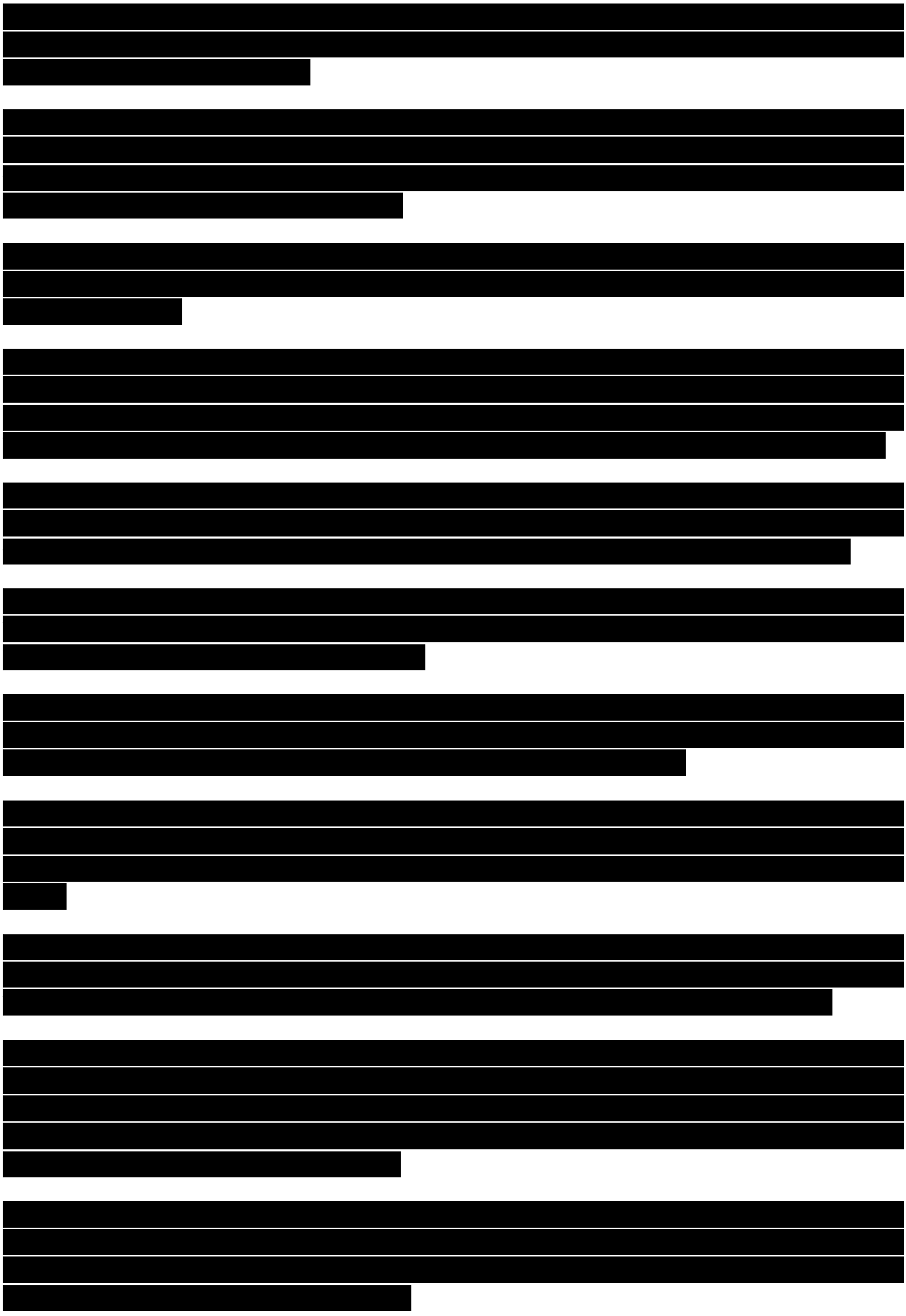

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Chapter 7
Cucumis sativus

7.1 Effects of benzodiazepine delorazepam on Cucumis sativus: a preliminary investigation

Article in progress

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Effects of benzodiazepine delorazepam on Cucumis sativus: a preliminary investigation

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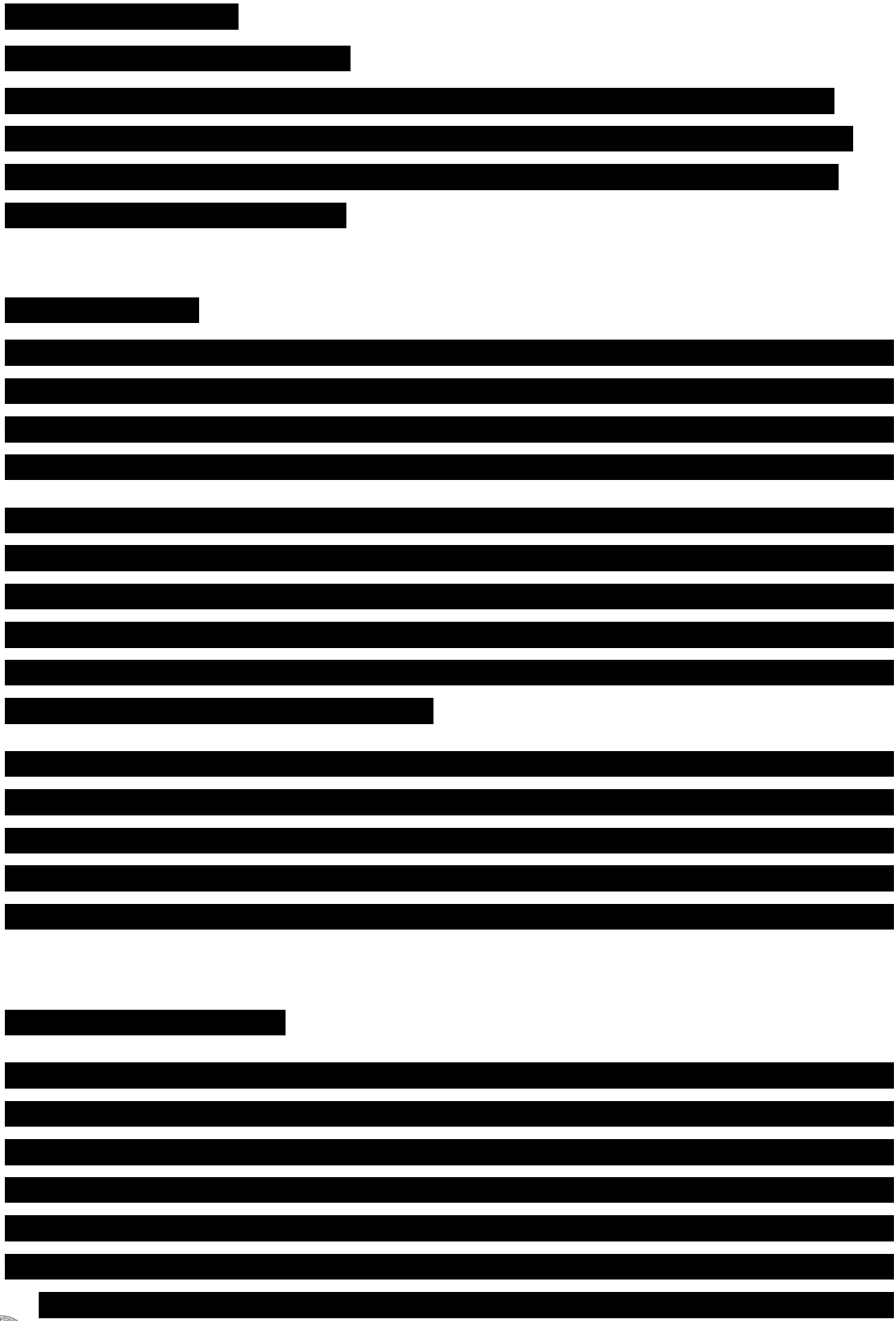

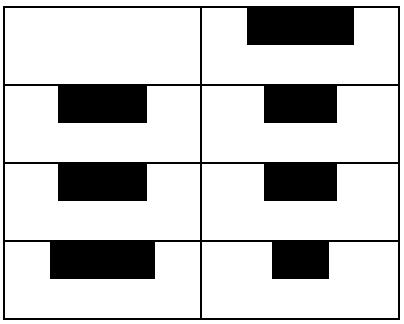

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General conclusions

In conclusion, this multidisciplinary study proved, without a doubt, the negative impact of delorazepam on a wide range of mechanisms and functions, in all the six models examined. The effects were mostly dose-dependent and already observable at the lower, environmental concentration, thus indicating that interference with natural ecosystems is more severe than considered so far.

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Publications

1. Carotenuto, R., Capriello, T., Cofone, R., Galdiero, G., **Fogliano, C**., & Ferrandino, I. (2020). Impact of copper in Xenopus laevis liver: Histological damages and atp7b downregulation. Ecotoxicology and environmental safety, 188, 109940. doi.org/10.1016/j.ecoenv.2019.109940

2. Cimmino, F., Catapano, A., Trinchese, G., Cavaliere, G., Culurciello, R., **Fogliano, C.**, Penna, E., Lucci, V., Crispino, M., Avallone, B., Pizzo, E., & Mollica, M. P. (2021). Dietary Micronutrient Management to Treat Mitochondrial Dysfunction in Diet-Induced Obese Mice. International journal of molecular sciences, 22(6), 2862. doi.org/10.3390/ijms22062862

3. Olanrewaju, O.S., Tommonaro, G., Guerriero, G., **Fogliano, C.**, Iodice, C., Velotto, G., Tramice, A. New Insight into Marine Biotechnology: Carrageenans Chemical Features and Acetylcholinesterase (AChE) Inhibition Activity of Two Edible Seaweeds of the Genus Kappaphycus. Recent Advances in Environmental Science from the Euro-Mediterranean and Surrounding Regions (2nd Edition). EMCEI 2019. Environmental Science and Engineering. Springer, Cham. doi.org/10.1007/978-3-030-51210-1_345

4. Sodano, F., Avallone, B., Tizzano, M., **Fogliano, C**., Rolando, B., Gazzano, E., Riganti, C., Magliocca, S., Cuozzo, M., Albrizio, S., Calignano, A., Cristiano, C., Russo, R., & Rimoli, M. G. (2021). Ketogal Safety Profile in Human Primary Colonic Epithelial Cells and in Mice. Pharmaceuticals (Basel, Switzerland), 14(11), 1149. doi.org/10.3390/ph14111149

5. Carotenuto, R., Tussellino, M., Ronca, R., Benvenuto, G., **Fogliano, C.**, Fusco, S., & Netti, P. A. (2022). Toxic effects of SiO2NPs in early embryogenesis of *Xenopus laevis*. Chemosphere, 289, 133233. doi.org/10.1016/j.chemosphere.2021.133233

6. Carotenuto, R., **Fogliano, C.**, Rienzi, M., Siciliano, A., Salvatore, M. M., De Tommaso, G., Benvenuto, G., Galdiero, E., & Guida, M. (2021). Comparative Toxicological Evaluation of Tattoo Inks on Two Model Organisms. Biology, 10(12), 1308. doi.org/10.3390/biology10121308

7. Motta, C. M., Califano, E., Scudiero, R., Avallone, B., **Fogliano, C**., De Bonis, S., Raggio, A., & Simoniello, P. (2022). Effects of Cadmium Exposure on Gut Villi in *Danio rerio*. International journal of molecular sciences, 23(4), 1927. doi.org/10.3390/ijms23041927

8. Avallone, B., Arena, C., Simoniello, P., Di Lorenzo, M., Vitale, E., Capriello, T., Ferrandino, I., Raggio, A., Sasso, M., Napolitano, G., De Bonis, S., **Fogliano, C**., Agnisola, C., Motta, C.M. (2022). Comparative Toxicity of Vegan Red, E124, and E120 Food Dyes on Three Rapidly Proliferating Model Systems. Environments. 9, 89. doi.org/10.3390/environments9070089

9. **Fogliano, C.**, Motta, C. M., Venditti, P., Fasciolo, G., Napolitano, G., Avallone, B., & Carotenuto, R. (2022). Environmental concentrations of a delorazepam-based drug impact on embryonic development of non-target *Xenopus laevis*. Aquatic toxicology (Amsterdam, Netherlands), 250, 106244. doi.org/10.1016/j.aquatox.2022.106244

10. Trinchese, G., Cimmino, F., Cavaliere, G., Catapano, A., **Fogliano, C**., Lama, A., Pirozzi, C., Cristiano, C., Russo, R., Petrella, L., Meli, R., Mattace Raso, G., Crispino, M., Avallone, B., & Mollica, M. P. (2022). The Hepatic Mitochondrial Alterations Exacerbate Meta-Inflammation in Autism Spectrum Disorders. Antioxidants (Basel, Switzerland), 11(10), 1990. doi.org/10.3390/antiox11101990

11. Fogliano, C., Motta, C.M., & Avallone, B. (2022). Salicylate attenuates gentamicin-induced ototoxicity and facilitates the recovery in the basilar papilla of the lizard Podarcis siculus. Neurotoxicology. 3, 301-310. doi.org/10.1016/j.neuro.2022.10.013

12. Pallotta, M. M., **Fogliano, C.**, & Carotenuto, R. (2022). Temperature Incubation Influences Gonadal Gene Expression during Leopard Gecko Development. Animals: an open access journal from MDPI, 12(22), 3186. doi.org/10.3390/ani12223186

13. **Fogliano, C.**, Carotenuto, R., Panzuto, R., Spennato, V., De Bonis, S., Simoniello, P., Raggio, A., Avallone, B., Agnisola, C., & Motta, C. M. (2023). Behavioral alterations and gills damage in *Mytilus galloprovincialis* exposed to an environmental concentration of delorazepam. Environmental toxicology and pharmacology, 97, 104030. doi.org/10.1016/j.etap.2022.104030

Conference attendance

- 32nd Congress **ESCPB**: Benzodiazepine inhibits feeding behavior while inducing hyperactivity and altering pedal mucus characteristics in the freshwater gastropod *Planorbarius corneus*

- 66° Convegno **GEI-SIBSC**: Delorazepam impairs the embryonic development of *Xenopus laevis*

- **MUNA** Summer School: Effects of benzodiazepine delorazepam on *Mytilus galloprovincialis.* (Awarded as best presentation)

- **ICEPTP** 2022 (7th International Conference on Environmental Pollution, Treatment and Protection) : Influence of benzodiazepine Delorazepam on *Xenopus laevis* embryogenesis

- 67° Convegno **GEI-SIBSC**: Psychotropic Delorazepam induces epigenetic changes and retinal disorders in *Xenopus laevis* embryos (Awarded as best presentation)

Other conference topics

- **NANO-DAY** IV Edizione: Nanoparticles modify embryonic genes expression in *Xenopus laevis. (Poster/abstract)*

- 66° Convegno **GEI-SIBSC:** Erbium affects the Xenopus laevis development. (*Poster/abstract)*

- 3rd Italian **Zebrafish Meeting:** Effects of three food dyes on motor activity in Zebrafish embryos. (*Poster)*

- VII Convegno **CUCS** Napoli : Vegan food pigments: not a safe solution. (*Poster/abstract)*

- 67° Convegno **GEI-SIBSC**: Yolk consumption/degradation: a novel target for microplastic toxicity? (*Poster/abstract)*

- 67° Convegno **GEI-SIBSC**: The effects of gadolinium on Xenopus laevis embryonic development. (*Poster/abstract)*

Bibliography

Abrahamsson, T., & Hakansson, A. (2015). Nonmedical prescription drug use (NMPDU) in the Swedish general population--correlates of analgesic and sedative use. Substance use & misuse, 50(2), 148–155. https://doi.org/10.3109/10826084.2014.962047

Altamura, A. C., Moliterno, D., Paletta, S., Maffini, M., Mauri, M. C., & Bareggi, S. (2013). Understanding the pharmacokinetics of anxiolytic drugs. Expert opinion on drug metabolism & toxicology, 9(4), 423–440. https://doi.org/10.1517/17425255.2013.759209

Anholt, R.R., Pedersen, P.L., De Souza, E.B., Snyder, S.H. The peripheral-type benzodiazepine receptor. Localization to the mitochondrial outer membrane. *J. Biol. Chem.*, **1986**, *261*, 576-583.

Antkiewicz-Michaluk, L., Guidotti, A., & Krueger, K. E. (1988). Molecular characterization and mitochondrial density of a recognition site for peripheral-type benzodiazepine ligands. Molecular pharmacology, 34(3), 272–278.

Argyropoulos, S.V., Nutt, D.J., 1999. The use of benzodiazepines in anxiety and other disorders. Eur. Neuropsychopharmacol. 9 (6), S407–S412. https://doi.org/10.1016/ s0924-977x(99)00052-8. Suppl

Arria, A. M., Caldeira, K. M., O'Grady, K. E., Vincent, K. B., Fitzelle, D. B., Johnson, E. P., & Wish, E. D. (2008). Drug exposure opportunities and use patterns among college students: results of a longitudinal prospective cohort study. Substance abuse, 29(4), 19–38. https://doi.org/10.1080/08897070802418451

Ashton, H.C. Benzodiazepine abuse. Drugs Depend. 2002, 197–212

aus der Beek, T., Weber, F. A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., & Küster, A. (2016). Pharmaceuticals in the environment--Global occurrences and perspectives. Environmental toxicology and chemistry, 35(4), 823–835. https://doi.org/10.1002/etc.3339

Austin, C. J., Kahlert, J., Kassiou, M., & Rendina, L. M. (2013). The translocator protein (TSPO): a novel target for cancer chemotherapy. The international journal of biochemistry & cell biology, 45(7), 1212–1216. https://doi.org/10.1016/j.biocel.2013.03.004

Bachhuber, M. A., Hennessy, S., Cunningham, C. O., & Starrels, J. L. (2016). Increasing Benzodiazepine Prescriptions and Overdose Mortality in the United States, 1996-2013. American journal of public health, 106(4), 686–688. https://doi.org/10.2105/AJPH.2016.303061

Bade, R., Ghetia, M., White, J.M., Gerber, C., 2020. Determination of prescribed and designer benzodiazepines and metabolites in influent wastewater. Anal. Methods 12 (28), 3637–3644. https://doi.org/10.1039/d0ay00560f.

Batt, A. L., Kincaid, T. M., Kostich, M. S., Lazorchak, J. M., & Olsen, A. R. (2016). Evaluating the extent of pharmaceuticals in surface waters of the United States using a National-scale Rivers and Streams Assessment survey. Environmental toxicology and chemistry, 35(4), 874–881. https://doi.org/10.1002/etc.3161

Bernassau, J.M.; Reversat, J.L.; Ferrara, P.; Caput, D.; Le Fur, G. A 3D model of the peripheral benzodiazepine receptor and its implication in intra mitochondrial cholesterol transport. J. Mol.

Bonete, G. P., Martínez, C. P., López, L. F., & Luna, A. (2018). Strichnyne and Delorazepam detection in bone human remains. A case report. Romanian Journal of Legal Medicine, 26(3), 298-301. http://doi.org/10.4323/rjlm.2018.298

Boyd, C. J., West, B., & McCabe, S. E. (2018). Does misuse lead to a disorder? The misuse of prescription tranquilizer and sedative medications and subsequent substance use disorders in a U.S. longitudinal sample. Addictive behaviors, 79, 17–23. https://doi.org/10.1016/j.addbeh.2017.11.042

Calisto, V., Esteves, V.I., 2009. Psychiatric pharmaceuticals in the environment. Chemosphere 77 (10), 1257–1274. https://doi.org/10.1016/j. chemosphere.2009.09.021.

Carotenuto, R., Fogliano, C., Rienzi, M., Siciliano, A., Salvatore, M.M., De Tommaso, G., Benvenuto, G., Galdiero, E., Guida, M., 2021. Comparative toxicological evaluation of tattoo inks on two model organisms. Biology 10 (12), 1308. https://doi.org/ 10.3390/biology10121308.

Carotenuto, R., Tussellino, M., Mettivier, G., Russo, P., 2016. Survival fraction and phenotype alterations of Xenopus laevis embryos at 3 Gy, 150 kV X-ray irradiation. Biochem. Biophys. Res. Commun. 480 (4), 580–585. https://doi.org/10.1016/j. bbrc.2016.10.095.

Carrasco-Garrido, P., Jiménez-Trujillo, I., Hernández-Barrera, V., García-Gómez-Heras, S., Alonso-Fernández, N., & Palacios-Ceña, D. (2018). Trends in the Misuse of Tranquilizers, Sedatives, and Sleeping Pills by Adolescents in Spain, 2004-2014. The Journal of adolescent health : official publication of the Society for Adolescent Medicine, 63(6), 709–716. https://doi.org/10.1016/j.jadohealth.2018.04.003

Carter, L. J., Williams, M., Martin, S., Kamaludeen, S. P. B., & Kookana, R. S. (2018). Sorption, plant uptake and metabolism of benzodiazepines. The Science of the total environment, 628-629, 18–25. https://doi.org/10.1016/j.scitotenv.2018.01.337

Cascade, E., & Kalali, A. H. (2008). Use of benzodiazepines in the treatment of anxiety. *Psychiatry (Edgmont (Pa. : Township))*, *5*(9), 21–22.

Casellas, P., Galiegue, S., Basile, A.S., 2002. Peripheral benzodiazepine receptors and mitochondrial function. Neurochem. Int. 40 (6), 475–486. https://doi.org/10.1016/ s0197-0186(01)00118-8.

Cerveny, D., Brodin, T., Cisar, P., McCallum, E.S., Fick, J., 2020. Bioconcentration and behavioral effects of four benzodiazepines and their environmentally relevant mixture in wild fish. Sci. Total Environ. 702, 134780 https://doi.org/10.1016/j. scitotenv.2019.134780.

Chen, K. W., Berger, C. C., Forde, D. P., D'Adamo, C., Weintraub, E., & Gandhi, D. (2011). Benzodiazepine use and misuse among patients in a methadone program. BMC psychiatry, 11, 90. https://doi.org/10.1186/1471-244X-11-90

Cunha, D. L., de Araujo, F. G., & Marques, M. (2017). Psychoactive drugs: occurrence in aquatic environment, analytical methods, and ecotoxicity-a review. *Environmental science and pollution research international*, *24*(31), 24076–24091. https://doi.org/10.1007/s11356-017-0170-4

Danza, A.; Cristiani, F.; Tamosiunas, G. Benzodiazepine-related risks. Arch. Med. Intern. 2009, 31, 103–107

Daughton, C. G. Non-Regulated Water Contaminants: Emerging Research. Environ. Impact Assess. Rev. 2004, 24, 711−732.

Daughton, Christian G. (2007). [Comprehensive Analytical Chemistry] Analysis, Fate and Removal of Pharmaceuticals in the Water Cycle Volume 50 || Chapter 1 Pharmaceuticals in the environment: sources and their management. , (), 1–58. doi:10.1016/s0166-526x(07)50001-2

Esteban, S., Valcárcel, Y., Catalá, M., & Castromil, M. G. (2012). Psychoactive pharmaceutical residues in the watersheds of Galicia (Spain). Gaceta sanitaria, 26(5), 457–459. https://doi.org/10.1016/j.gaceta.2012.01.018

European Drug Report 2021: Trends and Developments. EMCDDA. Pages: 60 ISBN: 978-92-9497-588-1 ISSN: 2314-9086 Pub. DOI: 10.2810/18539

European Monitoring Centre for Drugs and Drug Addiction (2021), New benzodiazepines in Europe – a review, Publications Office of the European Union, Luxembourg doi:10.2810/725973

Fatta-Kassinos, D., Meric, S. & Nikolaou, A. Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. Anal Bioanal Chem 399, 251–275 (2011). https://doi.org/10.1007/s00216-010-4300-9

Fekadu, S., Alemayehu, E., Dewil, R., & Van der Bruggen, B. (2019). Pharmaceuticals in freshwater aquatic environments: A comparison of the African and European challenge. The Science of the total environment, 654, 324–337. https://doi.org/10.1016/j.scitotenv.2018.11.072

Fick, J., Brodin, T., Heynen, M., Klaminder, J., Jonsson, M., Grabicova, K., Randak, T., Grabic, R., Kodes, V., Slobodnik, J., Sweetman, A., Earnshaw, M., Barra Caracciolo, A., Lettieri, T., Loos, R., 2017. Screening of benzodiazepines in thirty European rivers. Chemosphere 176, 324–332. https://doi.org/10.1016/j. chemosphere.2017.02.126.

Fukasawa, T., Suzuki, A., & Otani, K. (2007). Effects of genetic polymorphism of cytochrome P450 enzymes on the pharmacokinetics of benzodiazepines. Journal of clinical pharmacy and therapeutics, 32(4), 333–341. https://doi.org/10.1111/j.1365-2710.2007.00829.x

Furuhagen, S., Fuchs, A., Lundström Belleza, E., Breitholtz, M., & Gorokhova, E. (2014). Are pharmaceuticals with evolutionary conserved molecular drug targets more potent to cause toxic effects in non-target organisms?. PloS one, 9(8), e105028. https://doi.org/10.1371/journal.pone.0105028

Gafni, I., Busto, U. E., Tyndale, R. F., Kaplan, H. L., & Sellers, E. M. (2003). The role of cytochrome P450 2C19 activity in flunitrazepam metabolism in vivo. Journal of clinical psychopharmacology, 23(2), 169–175. https://doi.org/10.1097/00004714-200304000-00009

Galduróz, J. C., Noto, A. R., Nappo, S. A., & Carlini, E. A. (2005). Household survey on drug abuse in Brazil: study involving the 107 major cities of the country--2001. Addictive behaviors, 30(3), 545–556. https://doi.org/10.1016/j.addbeh.2004.08.004

Gerace, E., Salomone, A., Di Corcia, D., Mazzucco, P., & Vincenti, M. (2015). Postmortem redistribution of triazolam, alprazolam, delorazepam (chlordesmethyldiazepam) and zolpidem in a suicide case. Toxicologie Analytique et Clinique, 27(4), 233-238. https://doi.org/10.1016/j.toxac.2015.10.001

Griffin, C. E., 3rd, Kaye, A. M., Bueno, F. R., & Kaye, A. D. (2013). Benzodiazepine pharmacology and central nervous system-mediated effects. The Ochsner journal, 13(2), 214–223.

Guina, J., & Merrill, B. (2018). Benzodiazepines II: Waking Up on Sedatives: Providing Optimal Care When Inheriting Benzodiazepine Prescriptions in Transfer Patients. Journal of clinical medicine, 7(2), 20. https://doi.org/10.3390/jcm7020020

Guthrie, G.D., Nicholson-Guthrie, C.S., Leary Jr, H.L., 2000. A bacterial high-affinity GABA binding protein: isolation and characterization. Biochem. Biophys. Res. Commun. 268 (1), 65–68. https://doi.org/10.1006/bbrc.1999.1960.

Hall, W., Teesson, M., Lynskey, M., & Degenhardt, L. (1999). The 12-month prevalence of substance use and ICD-10 substance use disorders in Australian adults: findings from the National Survey of Mental Health and Well-Being. Addiction (Abingdon, England), 94(10), 1541–1550.

Herbert, L. T., Cossi, P. F., Painefilú, J. C., Mengoni Goñalons, C., Luquet, C. M., & Kristoff, G. (2021). Acute neurotoxicity evaluation of two anticholinesterasic insecticides, independently and in mixtures, and a neonicotinoid on a freshwater gastropod. Chemosphere, 265, 129107. https://doi.org/10.1016/j.chemosphere.2020.129107

Hester, J. B., Jr, & Von Voigtlander, P. (1979). 6-Aryl-4H-s-triazolo[4,3-a][1,4]benzodiazepines. Influence of 1-substitution on pharmacological activity. Journal of medicinal chemistry, 22(11), 1390–1398. https://doi.org/10.1021/jm00197a021

Isjanovski, V., Kacarska, M. Screening of the use of benzodiazepines during COVID-19 pandemic in the general population . Macedonian pharmaceutical bulletin, 67 (1) 9 - 16 (2021) DOI: 10.33320/maced.pharm.bull.2021.67.01.002

ISPRA (2011) Linee Guida 67/2011 - https://www.isprambiente.gov.it/files/pubblicazioni/manuali-lineeguida/mlg-67-2011-n.pdf

Johnson, A.C., Jurgens, M.D., Williams, R.J., Kummerer, K., Kortenkamp, A., Sumpter, J.P., 2008. Do cytotoxic chemotherapy drugs discharged into rivers pose a risk to the environment and human health? An overview and UK case study. Journal of Hydrology 348 (1, 2), 167e175.

Klaminder, J., Brodin, T., Sundelin, A., Anderson, N. J., Fahlman, J., Jonsson, M., & Fick, J. (2015). Long-Term Persistence of an Anxiolytic Drug (Oxazepam) in a Large Freshwater Lake. Environmental science & technology, 49(17), 10406–10412. https://doi.org/10.1021/acs.est.5b01968

Kosjek T, Perko S, Zupanc M, Zanoški Hren M, Landeka Dragičević T, Zigon D, Kompare B, Heath E. Environmental occurrence, fate and transformation of benzodiazepines in water treatment. Water Res. 2012 Feb 1;46(2):355-68. doi: 10.1016/j.watres.2011.10.056. Epub 2011 Nov 7. PMID: 22115636.

Lader M. (1991). History of benzodiazepine dependence. Journal of substance abuse treatment, 8(1-2), 53–59. https://doi.org/10.1016/0740-5472(91)90027-8

Lang S. (2002). The role of peripheral benzodiazepine receptors (PBRs) in CNS pathophysiology. *Current medicinal chemistry*, *9*(15),1411–1415.

Langer, E.; Einat, H.; Stukalin, Y. Similarities and dissimilarities in the effects of benzodiazepines and specific serotonin reuptake inhibitors (SSRIs) in the defensive marble burying test: A systematic review and meta-analysis. Eur. Neuropsychopharmacol. 2020, 36, 38–49

Lebreton, M., Malgouyres, J.M., Carayon, J.L., Bonnaf´e, E., G´eret, F., 2021a. Effects of the anxiolytic benzodiazepine oxazepam on freshwater gastropod reproduction: a prospective study. Ecotoxicology 30 (9), 1880–1892. https://doi.org/10.1007/ s10646-021-02453-y.

Lebreton, M., Sire, S., Carayon, J.L., Malgouyres, J.M., Vignet, C., G´eret, F., Bonnaf´e, E., 2021b. Low concentrations of oxazepam induce feeding and molecular changes in Radix balthica juveniles. Aquat. Toxicol. 230, 105694 https://doi.org/10.1016/j. aquatox.2020.105694.

Lechuga, G.; Indart, C. Selection of benzodiazepines: Bases for its use in the hospital. Farm. Hosp. 1996, 21, 117–122.

Lei, H.J., Yang, B., Ye, P., Yang, Y.Y., Zhao, J.L., Liu, Y.S., Xie, L., Ying, G.G., 2021. Occurrence, fate and mass loading of benzodiazepines and their transformation products in eleven wastewater treatment plants in Guangdong province, China. Sci. Total Environ. 755 (Pt 2), 142648 https://doi.org/10.1016/j. scitotenv.2020.142648.

Lembke, A., Papac, J., & Humphreys, K. (2018). Our Other Prescription Drug Problem. The New England journal of medicine, 378(8), 693–695. https://doi.org/10.1056/NEJMp1715050

Li, F., Liu, J., Liu, N., Kuhn, L. A., Garavito, R. M., & Ferguson-Miller, S. (2016). Translocator Protein 18 kDa (TSPO): An Old Protein with New Functions?. Biochemistry, 55(20), 2821–2831. https://doi.org/10.1021/acs.biochem.6b00142

Lombardi, N., Bettiol, A., Crescioli, G., Ravaldi, C., Bonaiuti, R., Venegoni, M., Vighi, G. D., Mugelli, A., Mannaioni, G., Vannacci, A., & MEREAFaPS Study group (2020). Risk of hospitalisation associated with benzodiazepines and z-drugs in Italy: a nationwide multicentre study in emergency departments. Internal and emergency medicine, 15(7), 1291–1302. https://doi.org/10.1007/s11739-020-02339-7

Lugoboni, F., Mirijello, A., Morbioli, L., Arzenton, E., Leone, R., Faccini, M., Casari, R., De Cosmo, S., Gasbarrini, A., & Addolorato, G. (2018). Does high-dose benzodiazepine abuse really produce liver toxicity? Results from a series of 201 benzodiazepine monoabusers. Expert opinion on drug safety, 17(5), 451–456. https://doi.org/10.1080/14740338.2018.1457646

Luján R, Shigemoto R, López-Bendito G. Glutamate and GABA receptor signalling in the developing brain. Neuroscience. 2005;130(3):567-80. doi: 10.1016/j.neuroscience.2004.09.042. PMID: 15590141.

Magnaghi, V., Ballabio, M., Consoli, A., Lambert, J. J., Roglio, I., & Melcangi, R. C. (2006). GABA receptor-mediated effects in the peripheral nervous system: A cross-interaction with neuroactive steroids. Journal of molecular neuroscience : MN, 28(1), 89–102. https://doi.org/10.1385/jmn:28:1:89

Magrini, N., Vaccheri, A., Parma, E., D'Alessandro, R., Bottoni, A., Occhionero, M., & Montanaro, N. (1996). Use of benzodiazepines in the Italian general population: prevalence, pattern of use and risk factors for use. European journal of clinical pharmacology, 50(1-2), 19–25. https://doi.org/10.1007/s002280050063

Mattiuzzi, C., Sanchis-Gomar, F., & Lippi, G. (2022). Benzodiazepines consumption may have increased during the COVID-19 pandemic. Journal of affective disorders, 314, 124–125. https://doi.org/10.1016/j.jad.2022.07.017

Milić, M., Divljaković, J., Rallapalli, S., van Linn, M. L., Timić, T., Cook, J. M., & Savić, M. M. (2012). The role of α1 and α5 subunit-containing GABAA receptors in motor impairment induced by benzodiazepines in rats. Behavioural pharmacology, 23(2), 191–197. https://doi.org/10.1097/FBP.0b013e3283512c85

Mizuno, K., Katoh, M., Okumura, H., Nakagawa, N., Negishi, T., Hashizume, T., Nakajima, M., & Yokoi, T. (2009). Metabolic activation of benzodiazepines by CYP3A4. Drug metabolism and disposition: the biological fate of chemicals, 37(2), 345–351. https://doi.org/10.1124/dmd.108.024521

Moosmann, B., & Auwärter, V. (2018). Designer Benzodiazepines: Another Class of New Psychoactive Substances. Handbook of experimental pharmacology, 252, 383–410. https://doi.org/10.1007/164_2018_154

Moss, G. P.. "Nomenclature of fused and bridged fused ring systems (IUPAC Recommendations 1998)" Pure and Applied Chemistry, vol. 70, no. 1, 1998, pp. 143-216. https://doi.org/10.1351/pac199870010143

Motta, C. M., Cerciello, R., De Bonis, S., Mazzella, V., Cirino, P., Panzuto, R., Ciaravolo, M., Simoniello, P., Toscanesi, M., Trifuoggi, M., & Avallone, B. (2016). Potential toxicity of improperly discarded exhausted photovoltaic cells. Environmental pollution (Barking, Essex : 1987), 216, 786–792. https://doi.org/10.1016/j.envpol.2016.06.048

Motta, C. M., Simoniello, P., Arena, C., Capriello, T., Panzuto, R., Vitale, E., Agnisola, C., Tizzano, M., Avallone, B., & Ferrandino, I. (2019). Effects of four food dyes on development of three model species, Cucumis sativus, Artemia salina and Danio rerio: Assessment of potential risk for the environment. Environmental pollution (Barking, Essex : 1987), 253, 1126–1135. https://doi.org/10.1016/j.envpol.2019.06.018

New Poll: COVID-19 Impacting Mental Well-Being: Americans Feeling Anxious, Especially for Loved Ones; Older Adults are Less Anxious. https://www.psychiatry.org/newsroom/news-releases/new-pollcovid-19-impacting-mental-well-being-americans-feeling-anxiousespecially-for-loved-ones-older-adults-are-less-anxious. Accessed October 4, 2020

Nunes, B. S., Carvalho, F. D., Guilhermino, L. M., & Van Stappen, G. (2006). Use of the genus Artemia in ecotoxicity testing. Environmental pollution (Barking, Essex : 1987), 144(2), 453–462. https://doi.org/10.1016/j.envpol.2005.12.037

Nutt, D., & Malizia, A. (2001). New insights into the role of the GABAA–benzodiazepine receptor in psychiatric disorder. British Journal of Psychiatry, 179(5), 390-396. doi:10.1192/bjp.179.5.390

Oggier, D.M., Weisbrod, C.J., Stoller, A.M., Zenker, A.K., Fent, K., 2010. Effects of diazepam on gene expression and link to physiological effects in different life stages in zebrafish Danio rerio. Environ. Sci. Technol. 44 (19), 7685–7691. https://doi.org/ 10.1021/es100980r.

Ogueji, E.O., Iheanacho, S.C., Nwani, C.D., Christian, E.M., Okeke, O.C., Usman, I.B., 2017. Toxicity of diazepam on lipid peroxidation, biochemical and oxidative stress indicators on liver and gill tissues of African catfish Clarias gariepinus (Burchell, 1822). Int. J. Fish. Aquat. Stud. 5 (3), 114–123.

Palamar, J. J., Han, B. H., & Martins, S. S. (2019). Shifting characteristics of nonmedical prescription tranquilizer users in the United States, 2005-2014. Drug and alcohol dependence, 195, 1–5. https://doi.org/10.1016/j.drugalcdep.2018.11.015

Papadopoulos, V., Amri, H., Boujrad, N., Cascio, C., Culty, M., Garnier, M., Hardwick, M., Li, H., Vidic, B., Brown, A. S., Reversa, J. L., Bernassau, J. M., & Drieu, K. (1997). Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis. Steroids, 62(1), 21–28. https://doi.org/10.1016/s0039-128x(96)00154-7

Papadopoulos, V., Baraldi, M., Guilarte, T.R., Knudsen, T.B., Lacap`ere, J.J., Lindemann, P., Norenberg, M.D., Nutt, D., Weizman, A., Zhang, M.R., Gavish, M., 2006. Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol. Sci. 27 (8), 402–409. https://doi.org/10.1016/j.tips.2006.06.005

Patel, M., Kumar, R., Kishor, K., Mlsna, T., Pittman, C. U., Jr, & Mohan, D. (2019). Pharmaceuticals of Emerging Concern in Aquatic Systems: Chemistry, Occurrence, Effects, and Removal Methods. Chemical reviews, 119(6), 3510–3673. https://doi.org/10.1021/acs.chemrev.8b00299

Puangkot, S., Laohasiriwong, W., Saengsuwan, J., & Chiawiriyabunya, I. (2010). Benzodiazepines misuse: the study community level Thailand. Indian journal of psychological medicine, 32(2), 128–130. https://doi.org/10.4103/0253-7176.78510

Raffa, R. and Pergolizzi, J. (2019) Commentary: Benzodiazepine (BZD) and Related BZD-Receptor Agonists: Basic Science Reasons to Limit to Four Weeks or Less. Pharmacology & Pharmacy, 10, 357-364. doi: 10.4236/pp.2019.108029.

Report of the International Narcotics Control Board (INCB) for 2021 on the Implementation of Article 12 of the United Nations Convention against Illicit Traffic in Narcotic Drugs and Psychotropic Substances of 1988, UNITED NATIONS PUBLICATION, ISBN: 978-92-1-148372-7

Ribeiro, S., Torres, T., Martins, R., & Santos, M. M. (2015). Toxicity screening of diclofenac, propranolol, sertraline and simvastatin using Danio rerio and Paracentrotus lividus embryo bioassays. Ecotoxicology and environmental safety, 114, 67–74. https://doi.org/10.1016/j.ecoenv.2015.01.008

Richards, S. M., & Cole, S. E. (2006). A toxicity and hazard assessment of fourteen pharmaceuticals to Xenopus laevis larvae. Ecotoxicology (London, England), 15(8), 647–656. https://doi.org/10.1007/s10646-006-0102-4

Richardson, S. D.; Ternes, T. A. Water Analysis: Emerging Contaminants and Current Issues. Anal. Chem. 2014, 86, 2813−2848.

Roig, B.; D'Aco, V. Distribution of pharmaceutical residues in the environment. In Issues in Environmental Science and Technology; Hester, R.E., Harrison, R.M., Eds.; The Royal Society of Chemistry: London, UK, 2016; pp. 34–69. ISBN 9781782621898.

Rudge, A. D., Chase, J. G., Shaw, G. M., & Lee, D. (2006). Physiological modelling of agitation-sedation dynamics including endogenous agitation reduction. Medical engineering & physics, 28(7), 629–638. https://doi.org/10.1016/j.medengphy.2005.10.008

Schmitz, A., 2016. Benzodiazepine use, misuse, and abuse: a review. Mental Health Clin.6 (3), 120–126. https://doi.org/10.9740/mhc.2016.05.120

Sieghart W. (2006). Structure, pharmacology, and function of GABAA receptor subtypes. Advances in pharmacology (San Diego, Calif.), 54, 231–263. https://doi.org/10.1016/s1054-3589(06)54010-4

Silva, A., Nilin, J., Loureiro, S., Costa-Lotufo, L.V., 2020. Acute and chronic toxicity of the benzodiazepine diazepam to the tropical crustacean Mysidopsis juniae. An. Acad. Bras. Cienc. 92 (1), e20180595 https://doi.org/10.1590/0001-3765202020180595.

Taliani, S., Da Settimo, F., Da Pozzo, E., Chelli, B., & Martini, C. (2009). Translocator protein ligands as promising therapeutic tools for anxiety disorders. Current medicinal chemistry, 16(26), 3359–3380. https://doi.org/10.2174/092986709789057653

The Medicines Utilisation Monitoring Centre. National Report on Medicines use in Italy. Year 2021. Rome: Italian Medicines Agency, 2022

Turna, J., Zhang, J., Lamberti, N., Patterson, B., Simpson, W., Francisco, A. P., Bergmann, C. G., & Ameringen, M. V. (2021). Anxiety, depression and stress during the COVID-19 pandemic: Results from a cross-sectional survey. Journal of psychiatric research, 137, 96–103. https://doi.org/10.1016/j.jpsychires.2021.02.059

Twenge, J. M., & Joiner, T. E. (2020). U.S. Census Bureau-assessed prevalence of anxiety and depressive symptoms in 2019 and during the 2020 COVID-19 pandemic. Depression and anxiety, 37(10), 954–956. https://doi.org/10.1002/da.23077

Vicens, C., Socias, I., Mateu, C., Leiva, A., Bejarano, F., Sempere, E., Basora, J., Palop, V., Mengual, M., Beltran, J. L., Aragonès, E., Lera, G., Folch, S., Piñol, J. L., Esteva, M., Roca, M., Arenas, A., Del Mar Sureda, M., Campoamor, F., & Fiol, F. (2011). Comparative efficacy of two primary care interventions to assist withdrawal from long term benzodiazepine use: a protocol for a clustered, randomized clinical trial. BMC family practice, 12, 23. https://doi.org/10.1186/1471-2296-12-23

Votaw, V. R., Geyer, R., Rieselbach, M. M., & McHugh, R. K. (2019). The epidemiology of benzodiazepine misuse: A systematic review. Drug and alcohol dependence, 200, 95–114. https://doi.org/10.1016/j.drugalcdep.2019.02.033

Wang, C., Hou, L., Li, J., Xu, Z., Gao, T., Yang, J., Zhang, H., Li, X., & Du, P. (2017). Occurrence of diazepam and its metabolites in wastewater and surface waters in Beijing. Environmental science and pollution research international, 24(18), 15379–15389. https://doi.org/10.1007/s11356-017-8922-8

Wang, Z., Gao, S., Dai, Q., Zhao, M., & Yang, F. (2020). Occurrence and risk assessment of psychoactive substances in tap water from China. Environmental pollution (Barking, Essex : 1987), 261, 114163. https://doi.org/10.1016/j.envpol.2020.114163

Watanabe, M., Maemura, K., Kanbara, K., Tamayama, T., & Hayasaki, H. (2002). GABA and GABA receptors in the central nervous system and other organs. International review of cytology, 213, 1–47. https://doi.org/10.1016/s0074-7696(02)13011-7

Wick J. Y. (2013). The history of benzodiazepines. The Consultant pharmacist : the journal of the American Society of Consultant Pharmacists, 28(9), 538–548. https://doi.org/10.4140/TCP.n.2013.538

Zandonai T, Peiró AM, Fusina F, Lugoboni F and Zamboni L (2022) Benzodiazepines in sport, an underestimated problem: Recommendations for sports medicine physicians' practice. Front. Psychiatry 13:1066330. doi: 10.3389/fpsyt.2022.1066330

Zuccato, E., Castiglioni, S., Fanelli, R., Reitano, G., Bagnati, R., Chiabrando, C., Pomati, F., Rossetti, C., & Calamari, D. (2006). Pharmaceuticals in the environment in Italy: causes, occurrence, effects and control. *Environmental science and pollution research international*, *13*(1), 15–21. https://doi.org/10.1065/espr2006.01.004