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EFFECTS OF DIAZEPAM ON BIOTESTER ORGANISMS

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ABSTRACT

Benzodiazepines are the most prescribed pharmaceuticals as depresses of the nervous system. Among these, the group of benzodiazepines with special relevance to diazepam, is the most extensively studied as potential environmental contaminants. Diazepam has been detected in WWTP originating from hospitals as well as in effluents from municipal WWTP plants.

The purpose of the present study is to identify whether the diazepam solutions generate any effects at tested specie and from what concentrations these were recorded at crustaceans organism. The adults of Gammarus pulex balcanicus (Crustacea Amphipoda) were tested according to GamTox protocol for aquatic toxicity.

Diazepam injectable solutions (5 mg/mL) were purchased from Terapia SA.Cluj Napoca, Romania. For testing, four dilutions were used: 1 µg/mL, 2 µg/mL, 10 µg/mL and 20 µg/mL. The behavior and mortality were evaluated at 12, 24, 36, 48, 72 hours from start.

The lethal effects (30 %) were presented in the first 12 hours of the exposure at 20 µg/mL at diazepam concentrations. In the next time more extensive physiology reponses and the specimens mortality were gradualy recorded. LC50 was recorded at 7, 8 µg/mL, after 36 hours. The more lethal effects (LC 85 and LC100) were recorded at higher than 10 µg/mL concentrations.

In conclusions, the diazepam solutions induced changes quickly and more extensive on the physiology of the bio tester organism, which becoming in 12- 48 hours a toxic substance with effects on all vital process. These results are representative data about benzodiazepines effects at aquatic crustacean species, in current context when the effects of pharmaceuticals as potential contaminant at these aquatic communities were little known.

Keywords: *diazepam, aquatic crustacean, Gammarus, pharmaceuticals*

1. Introduction

The spectacular development of the chemical industry as well as that of the pharmaceutical industry has led to a number of questions about the harmful effects of substances spilled into the environment under various forms and conditions (accidental, wastewater, surface water, etc.). Modern techniques can detect very small amounts of pollutants or contaminants of the order ng /L-1, so GC/MS techniques have allowed the identification of some pharmaceutical or metabolite compounds in wastewater, surface water, soil and drinking water (Moldovan, 2006, Vânia, 2009).

Benzodiazepines are one of the most prescribed pharmaceuticals. In 2007, Europe registered as the Continent with the highest consumption of benzodiazepines (Vânia, 2009).

Among these, the group of benzodiazepines (with special relevance to diazepam) is the most extensively studied as potential environmental contaminants. In environmental research, benzodiazepines are among those pharmaceuticals less commonly addressed (Ferreira 2014). Diazepam has been detected in WWTP originating from hospitals as well as in effluents from municipal WWTP plants (Ferreira, 2014 Moldovan, 2006).

The present studies aimed at identifying the effects associated with the exposure to diazepam of a

common aquatic species in surface waters, Gammarus pulex balcanicus (Amphipoda, Crustacea), after GamTox model.

The GamTox test is based on several test parameters: behavior (especially locomotion and feeding) depicts rapid and sensitive early warning indicators, survival displays an indicator of severe acute stress, and biochemical biomarkers, esp. AChE inhibition, is a sensitive marker of neurotoxic xenobiotic stress (Gerhardt, 2011).

Compared to Daphnia magna, gammarids are often more sensitive to metals or different types of pesticides, such as neurotoxic substances and especially pyrethroids In 17 from 57 studies gammarids were much more sensitive than Daphnia magna (Gerhardt, 2011).

Diazepam was found to be metabolized by G. pulex and a metabolite was detected and tentatively identified as nordiazepam (Netherton, 2011).

The evaluation of these effects is aimed at identifying changes induced by the body studied by exposure to diazepam concentrations and association with action mechanisms. Identification of these effects may be a reference for knowing how environmental organisms are exposed to wastewater containing residues of pharmaceutical compounds.

It is also desirable to find alternative methods of testing for substances with applications in the neurological field to limit, as far as possible, experiments on laboratory animals (small mammals, fish, etc.).

2. Materials and methods

Gamarus pulex balcanicus were collected from Casimcea River, North of Constanta were kept for 10 days, in laboratory conditions for accommodation. The specimens were maintained in a 50 L aquarium, in water collected from river and they were fed with deshydrated leaves and mixture of aquarium crustacean fed (JBL Novo).

The static exposure was used, the organisms were transferred into fresh test solutions every 24 hours. Also, permanent aeration was assured for limit dissolved oxygen variations. Water quality parameters (pH, dissolved oxygen, temperature) were monitored throughout the experimental times (Table 1) with multiparameters Hanna HI 4521.

Diazepam injectable solutions (5 mg/ml) were purchased from Terapia SA. Cluj Napoca, Romania. For testing four dilutions were used: 1

Table 1. Experimental protocol

Diazepam con-centrations	Physical-chemical parameters (average values)	No. organisms tested (per test)	Evaluated effects (E_h)
C1 (1 µg/mL)	T=23-25^{0}C 6, 5 mg/L O_2 pH=8, 6	**10**	Gm Mp Ef
C2 (2 µg/mL)	T=24-25^{O}C 6 mg/L O_2 pH=8, 3	**10**	Gm Mp Ef
C3 (10 µg/mL)	T=24-25^{O}C 6 mg/L O_2 pH=8, 2	**10**	Gm Mp Ef
C4 (20 µg/mL)	T=25^{O}C 6, 5 mg/L O_2 pH=8, 2	**10**	Gm Mp Ef

µg/mL, 2 µg/mL, 10 µg/mL and 20 µg/mL. Two repetitions for each of the tested concentrations.

There were more effects periodically recorded (E h): general mobility (Gm) especially swimming behavior, mobility of pleopodes (Mp), appendices with respiratory physiology implications, effects of releasing feces pellets (Ef) correlated with nutritions behavior and bowel movement (Table 2), mortality recorded (< 50% lethality, LC 50, LC 80, LC 100).

The endpoint for the toxicity test was mortality. The tested period was at 72 hours (acute toxicity) For control evaluation was used sample with water.

Table 2: Quantification of recorded effects

Swimming behavior	Level of occurence
Absent	0
Slow movements	1
Normal	2
Fast	3

Statistical analysis

The Probit analysis and lethal concentration determination, LC 50 (lethal concentration of the tested substance, that kills 50% of the test animals during the experiment), were determinated from linear regression equation using the software: Stat Plus: Mac Pro, Analyst Soft - Statistical Analysis Program for Mac. OS, Version 6.

3. Results and discussions

The effects of pharmaceuticals and personal care products (PPCPs) on aquatic organisms represent a significant current concern (Gomez-Canela, 2016).

The chronic toxicity of pharmaceuticals to aquatic species has been studied for at least 65 individual compounds that comprise more than 20 pharmaceuticals classes (Whitacre, 2012).

Invertebrates sensitivity for sedatives with varying model of action were tested for acute toxicity in freshwater aquatic organisms.

The most toxic of these compounds was the benzodiazepine midazolam, which had an EC50 value of 0.2 mg/L in Daphnia magna.

Diazepam´s acute toxicity has been elucidated in several aquatic invertebrates species, with D. magna being the most sensitive specie exanimated thus far (Whitacre, 2012).

Observations made during the experiments revealed gradual responses of tested organisms to exposed diazepam concentrations.

The responses have consisted changes in general mobility (Gm), changes in movements of the pleopodes (Mp), elimination of feces pellets correlated with nutrition and movements of the digestive tract.

All of these effects were identified in the first few hours after exposure of organisms to diazepam solutions. The significant reduction was in the effects (Ef) to all concentrations.

(Fig.1. A, B, C, D).

Amphipoda exposed to 1 µg/mL of diazepam showed a significant decline of their locomotion behavior, general motility (Gm) that control sample after 36 h exposure (Fig. 1, A) while exposed to 2 and 10 µg/mL concentrations decline occurred in 24 h (Fig. 1, B, C).

The effect of diazepam was obvious after 12 h to higher tested concentrations, 20 µg/mL, the amphipods motility (Gm) was lower (Fig. 1. D).

The pleopod's motility (Mp) has been maintained at a constant level, with slow movements (Fig. 1, A, B, C) after 12 h exposure. Mp was close to immobility after 72 h exposure in 20 µg/mL diazepam concentrations (Fig. 1. D).

This results suggested that increase diazepam concentrations establish slowly pleopodal velocity.

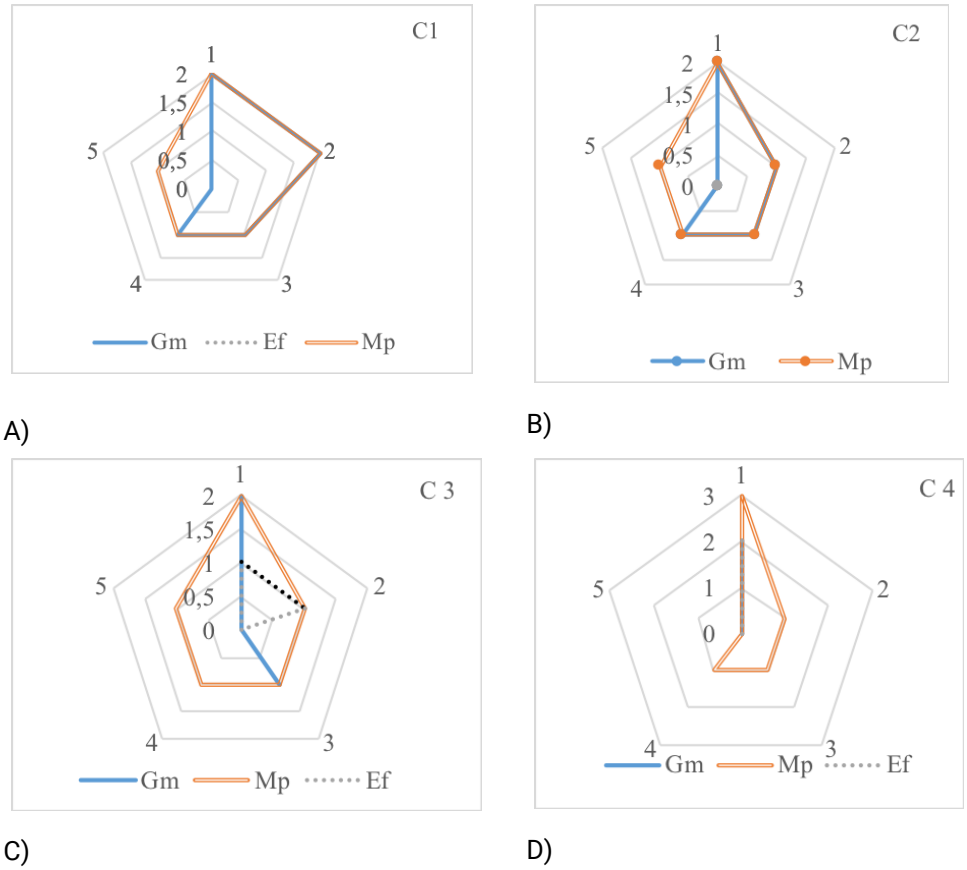

Figure 1. The effects of motility recorded in experimental time (1-12 h, 2-24 h, 3-36 h, 4-48 h, 5-72 h) Gm, Mp, Ef after diazepam exposuse A) C1 = 1 µg/mL, B) C2= 2 µg/mL, C) C3 = 10 µg/mL, D) C4 = 20 µg/mL

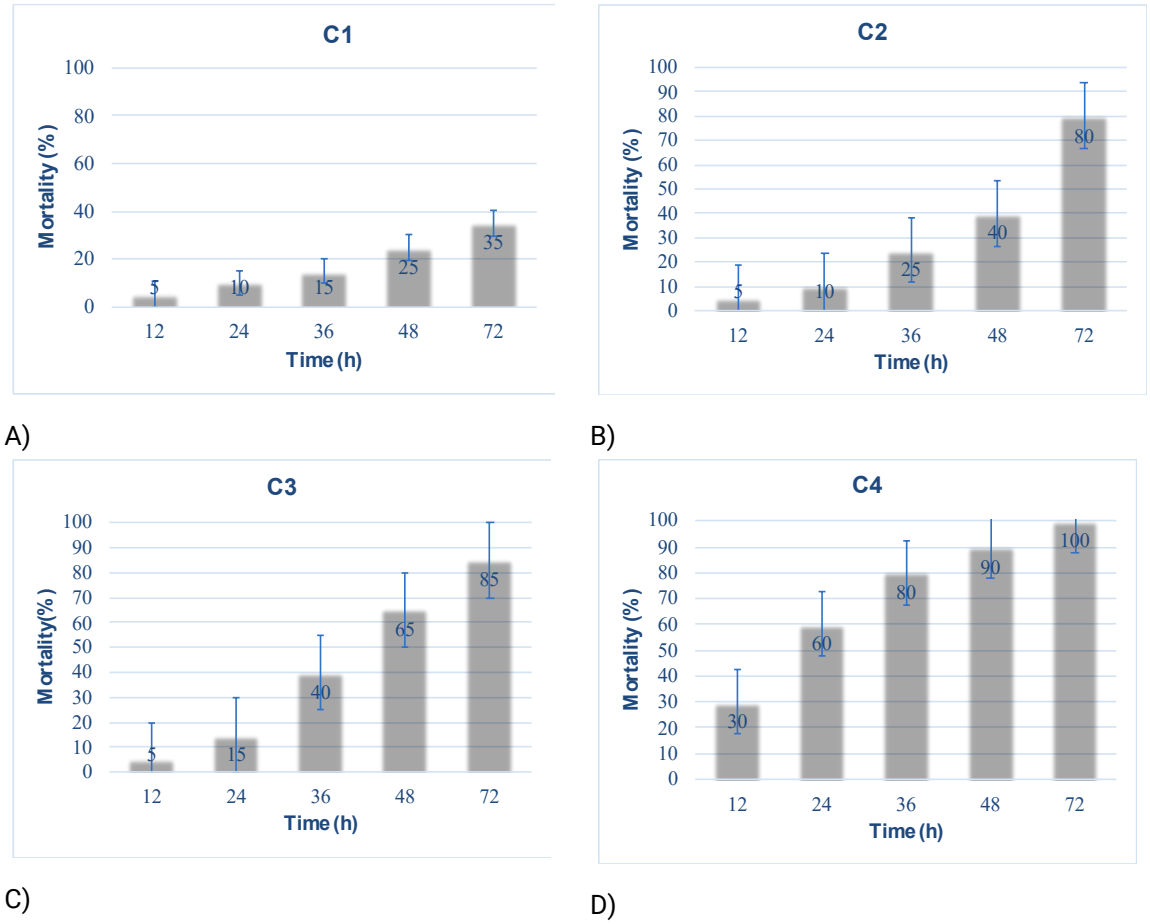

Figure 2. The mortality (%) average values of Gammarus pulex balcanicus after diazepam exposure, recorded in 12, 24, 36, 48, 72 hours after start; A) C1 = 1 µg/mL, B) C2= 2 µg/mL, C) C3 = 10 µg/mL, D) C4 = 20 µg/mL; standards errors (bar)

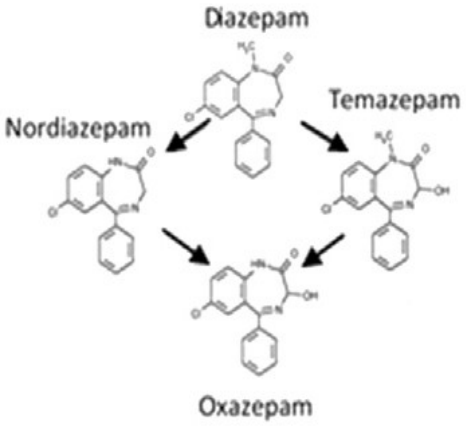

Figure 3. Diazepam metabolic pathway

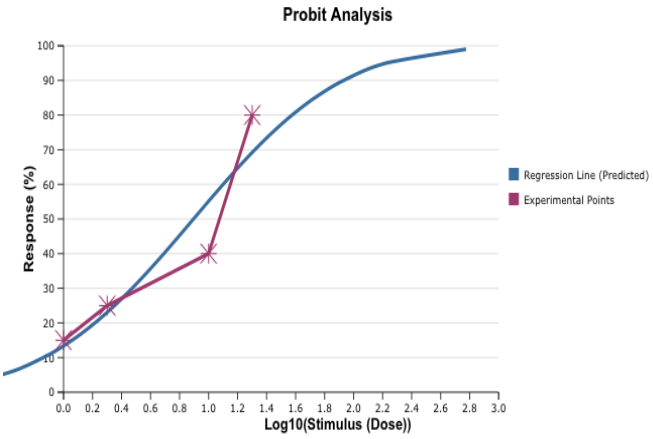

Figure 4. Stimulus -response correlations (Probit analysis) after 36 h exposure in diazepam solutions

Exposure-induced toxicity shows that the first effects measured by mortality are highlighted 12 hours after the start of the experiment.

The lethal effects (30 %) were presented in the first 12 hours of the exposure at 20 µg/mL at diazepam concentrations (Fig. 2, D). Thereafter broad physiological responses and specimen's mortality were gradually recorded.

Explanations were suggested by diazepam's biotransformation. Miller, in recent study, showed that the biotransformation of diazepam to nordiazepam is the major metabolic pathway (Fig. 3) in contrast to the conversion of diazepam to temazepam (Miller et al. 2017).

G. pulex were shown to metabolize diazepam into several different biotransformation products, indicating the conservation of cytochrome P450 enzymes in this species (Miller et al. 2017).

The effects of immobility and subsequent mortality are correlated with the extension of the exposure time (Fig. 2, A, B, C, D). LC 50 was recorded at 7, 8 µg/mL (Fig.4), after 36 hours, comparable data to other literature results (Table 3).

The more lethal effects (LC 85 and LC 100) were recorded at higher than 10 µg/mL concentrations (Fig. 2, C, D).

These can be explained by accelerating the phenomenon of metabolism and accumulation of metabolites with lethal effects.

Table 3. Acute effective concentrations in our experiment comparative with other species taken from published literature

Specie	Effect recorded	Predicted concentration	Reference
Gammarus pulex balcanicus (Crustacea)	LC 50 acute 36 hour	7,87 ±2,79 µg/mL	our study
	acute 48-72 hour LC85	22,5 µg/mL	
	acute 48-72 hour LC100	28 µg/mL	
Daphnia magna (Crustacea)	Acute 48 hour EC50	4,25 µmol/L (mg/L)	Lijnus et al., 1995, cited by Netherton, 2011)
Hydra vulgaris	Sublethal effects	10 µg/mL	Pascoe, 2003
Artemia partenoge-netica (Crustacea)	Acute 96 hour LC50	>12 mg/L	http://sitem.herts.ac.uk/
Giradia (Dugesia) tigrina (PlatyherminthesTur	Acute 24-48 hours	>10 mg/L	Alvez & Melo, 2012

Similar studies showed that alterations in metabolite concentrations were observed, which could be involved in several different pathways relating to protein synthesis oxidative stress and signaling cascades (Gomez-Canela, 2016). This observations were provided after of invertebrate crustacean *Gammarus pulex,* exposure at pharmaceuticals compound.

Conclusions

In conclusions, the diazepam solutions induced changes quickly and more extensive on the physiology of the bio tester organism, which becoming in 12- 48 hours a toxic substance with effects on all vital process.

These results are representative data about benzodiazepines toxicity effects at aquatic G.pulex balcanicus, from S-E Romanian waters, in current context when the effects of pharmaceuticals as potential contaminant at these aquatic communities were little known.

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