BIOLOGICAL RESPONSES INDUCED BY Pb INTOXICATION ON *EUXINIA MAEOTICA* (CRUSTACEA: AMPHIPODA) IN LABORATORY CONDITIONS

SCHRÖDER Verginica, SÎRBU Rodica,
NEGREANU-PÎRJOL Ticuţa, NEGREANU-PÎRJOL Bogdan

Abstract. The study aimed at identifying behavioural and physiological changes in *Euxinia maeotica* (Crustacea, Amphipoda) in conditions of intoxication with a chemical compound, as well as at evaluating the acute and chronic toxicity of this chemical compound. The intoxication was effected with Pb acetate (50, 100, 200 ppm), given the fact that Pb is known as one of the potential pollutants of the aquatic environment. Important information was obtained regarding the organism's level of response (survival, mortality, behaviour) by age categories (adult, pre-adult and juvenile). Also, the study demonstrated clear sensitivity differences in juveniles due to the faster growth rhythm, which leads to critical levels of toxicity in a shorter timeframe.

Keywords: toxicity, *Euxinia maeotica*, lead, TL50, TL100.

Rezumat. Răspunsuri biologice induse de stress prin intoxicatia cu Pb, la *Euxinia maeotica* (Crustacea: Amphipoda) în condiţii de laborator. Lucrarea a avut ca scop identificarea modificărilor comportamentale şi fiziologice la specia *Euxinia maeotica* (Crustacea, Amphipoda), în condiţiile intoxicarii cu un compus chimic cât şi evaluarea toxicitaţii acute şi cronice a acestui compus. Intoxicatia s-a efectuat cu acetat de Pb (50, 100, 200ppm), cunoscut fiind faptul că, Pb este unul din potenţialii poluanţi din mediul acvatic. Sunt obţinute importante informaţii legate de nivelul de răspuns (supravieţuire, mortalitate, comportament) al organismului testat, pentru categorii de vârstă diferite (adulţi, preadulţi şi juvenili) şi evidenţiate modificări clare de sensibilitate a formelor din stadiul juvenil, ca urmare a ritmului de creştere mai ridicat, ceea ce duce la atingerea nivelului critic (in conditiile expunerii la toxic) într-un timp mai scurt.

Cuvinte cheie: toxicitate, *Euxinia maeotica,* plumb, TL50, TL100.

INTRODUCTION

At global level, the Worldwatch Institute estimates that no information is available on the toxic effects of 79% of the over 70,000 synthetic chemicals in everyday use (MITCHELL, 2002). Over the past decade, a number of organisms have been used as test subjects, the taxa chosen generally representing categories of maximum diversity: bacteria, algae, fish, crustaceans (MITCHELL, 2002), species of protozoa, cnideria, platyhelminthes, rotifers, anelids, insects, and molluscs (LA POINT**,** 1989). Among the crustacean, some of the taxa used for these tests are: *Mysidopsis bahia, Americanmysis bahia* (Mysidacea), *Leptocheirus plumosus* and *Ampelisca abdita* (Amphipoda) and *Hyalella azteca* (Amphipoda). These species are indicators of petrochemical pollution on beaches.

Of similar interest are studies regarding the use of marine and estuarine amphipods as biological material for testing the effects of potential contaminants in sediments. The species used for these tests are: *Rhepoxynius abronius, Eohaustorius estuarius, Ampelisca adbita, Grandidierella japonica, Leptocheirus plumosus* (ASTM, 1990) (BURGESS et. al., 2000). Similarly, amphipods are increasingly employed for the identification of the accumulation of heavy metals and more specifically as important species in aquatic biomonitoring (ZAUKE, 1998; CLASON, 2000) as well as in the study of certain pesticide toxicity (ANTONI, 1997).

The toxicity of some compounds may be powerfully influenced by various physical-chemical parameters relating to the water and the characteristics of the sediment. Amphipods, for example, have a higher sensitivity to muddy sediments than to fine sand substrates (BORJA, 2008). Likewise, reactions may differ within the same species and according to the subject's stage of development (LERA, 2008; MITCHELL, 2002).

The majority of the toxicological studies follow the mortality rate and record performance tests (respiratory system, the organism's mobility, oxygen consumption etc.). Behavioural and physiological changes are also monitored as responses to stress (BEATON et. al., 1999). Research is also being conducted into the effects of toxic substances at cellular and molecular level (DAILIANIS, 2003; EDITA MAZUROVA, 2010), as well as the effects of these xenobiontic substances at the supra-individual level (LERA, 2008; ALVARO, 2010). The results are applied to the wider macroscopic context**.**

Amphipods are an important component of freshwater and brackish ecosystems since they play a key role in the detritus breakdown process and constitute an important source of food for predators. They can be found in high densities and may be very sensitive to a wide range of toxicants. These elements explain why amphipods are often used in ecotoxicology. The most widely employed amphipod is the common species *Gammarus pulex* (Crustacea, Amphipoda) mainly because it has a wide distribution area (ALVARO, 2010). Also, with regard to freshwater Crustacea, a large toxicity database is available only for *Daphnia magna.*

The study seeks to identify behavioural and physiological changes in *Euxinia maeotica* (Crustacea, Amphipoda), in conditions of intoxication with a chemical compound (Pb acetate), as well as to evaluate the acute and chronic toxicity of this chemical compound. *Euxinia maeotica* fulfils the criteria of a biotester species: ecological importance, availability and ease of care in the laboratory (CLASON, 2000; MITCHELL, 2002; ZAUKE, 1998). Also, another criterion is that it has already been used in a series of tests for detection of heavy metal toxicity from potentially toxic substances on the Romanian coast (ŞERBAN et. al., 2001, 2004; PALICI et. al., 2001, NEGREANU et al., 2002).

Intoxication was effected with Pb, given the fact that Pb is known as one of the potential pollutants of the aquatic environment. The retention and circulation of this substance in the organism is influenced by ions of calcium, iron, phosphate, iodine, chelation agents and vitamin D. No previous data is available regarding the sensitivity of *Euxinia maeotica* to this metal.

MATERIALS AND METHODS

Invertebrates were collected using hand dredge (0.2 mm mesh size) from the bottom sand north of Constantza. Amphipods were transferred to the laboratory using plastic containers. In the laboratory they were kept in aerated aquaria for accommodation. Animals were fed with dry *Daphnia* and algal fragments (*Ceramium* and *Enteromorpha*). The marine water was filtrated for the natural simulated conditions design. The specimens with body length between 4-13 mm were selected and used to test the effects of chemical solution toxicants. The organisms were placed in a 10-liter glass vessel. For each treatment, a group of 10 individuals was used, therefore, two replicates.

Table 1. Experimental design for the bioassays with *Euxinia maeotica.*
Tabel 1. Model experimental pentru biotestele cu *Euxinia maeotica.*

Tested substance	**Animals stages**	**Body length intervals (mm)**	**Toxicant concentrations (ppm)**	**Solution volume**	**Individuals per vessel**
$Pb(CH_3COOH)_2$	adult	10-13	C1=50	10 L	10
	pre-adult	7-8	C2=100	10L	10
	juvenile	4-6	C3=200	10L	10

The use of the substrate in containers was avoided in order to prevent interactions with the Pb acetate and a possible bacterial development.

The physical and chemical (dissolved oxygen, temperature, pH, salinity) water parameters were daily monitored. These parameters were measured in the test vessels and are shown in Table 2.

Table 2. Mean, Standard Deviation (SD), Confidence level (95%) –water physical and chemical parameters for bioassay.
Tabel 2. Media, deviația standard şi nivelul de confidență –parametri fizico-chimici ai apei pentru biotestare.

Parameters	**Mean and SD**	**Confidence level (95%)**
Water temperature (^{0}C)	19.5±0.137	0.82
pH	8.32±0.177	0.11
Salinity (‰)	17.51±1.56	0.99
Dissolved oxygen (mg/L)	7.7±1.25	0.12
Biological oxygen demand (BOD) (mg/L/h)	0.42±0.22	0.35

Oxygen consumption was measured for both adults and juveniles. This parameter was measured using hermetically sealed containers (10 ml) into which an electrode connected to a WTW oxygen meter was introduced (ProfiLine Oxi 197 with ±0.5% oxygen concentration accuracy). Following each measurement, the organisms were weighed and oxygen consumption was recorded against the weight of the organism at intervals of 1 h. Consumption through respiration was calculated using Gnaiger's conversion factor for units of oxygen consumption (GNAIGER, 1983), so that the variations in oxygen consumption were evaluated as oxygen µmol.

For each water test, with toxic agents and without (control), biological oxygen consumption was recorded at intervals of 1h. The purpose was to calculate the difference between measurements with animals and biological oxygen consumption in order to obtain the most precise consumption rate possible for organisms.

In order to process these results, the following were calculated: survival rate (Rs) measured in percentage / experiment day. TL50 and TL100 toxicity were calculated and the results represented graphically. Where significant mortality rates occurred in the control vessels, the Abbott correction factor for mortality in experimental vessels (Pcor) applies.

P cor. = (P ob.-C)/(1-C)x100
Pcor. = corrected proportion
Pob. = actual proportion (observed) in vessels
C = mortality in control vessels

Statistical analysis was achieved using linear regression for oxygen consumption, the Anova test for the evaluation of repeated experiments.

RESULTS AND DISCUSSIONS

Limited data is available on lead toxicity in an aquatic environment, more precisely in the case of marine organisms (MANCE, 1987). Of all lead compounds, it appears that tetramethyl and tetraethyl lead are more toxic than inorganic compounds. Marine organisms are highly sensitive and it appears that this ion causes rapid effects in the case of larval forms. Thus, molluscs and Decapoda larvae display abnormal developments in 48 hours of exposure to solutions of 45 ppm Pb^{+2} (MARTIN et. al., 1981).

The toxicity of this substance for organisms is due to the fact that it inhibits, by blockage, the thiolic groups of certain enzyme systems. It deactivates enzymes following the movement of biometals within the structure of metalloenzymes or enzymes activated by metals, due to a higher affinity towards ligands with aminic, carboxylic groups, including those of amino acids (lysine, glutamic acid, aspartic acid, imidazole, tyrosine phenoxy groups) (GRECU, 1982). Thus, some adenosine triphosphates are sensitive to small concentrations of lead, which also strongly inhibits lipoamide dehydrogenase, an enzyme of great importance in cell oxidation.

Thus, when molecular and biochemical responses are accentuated and mechanisms of homeostasis cease to appear, the toxic substances begin to affect the organism's normal physiological functions. Organisms do have strategies to survive and to counteract the impact of the chemical compound. Some of the best-known of these, in crustaceans, consist of shedding toxins via excretion or their isolation in inactive tissues such as the exoskeleton.

Similarly, the synthesis of specific proteins such as metallothionein or stress proteins may be initiated to protect the organism. Normally, increases in such syntheses are directly proportional to the accumulation level of the toxic compound. For example, the accumulation of metallothionein in bivalves is conditioned by the absorption of heavy metals in the animal's body. This enzymatic system in molluscs is broadly similar to that of vertebrates. Changes in the respiration rate are one of the universal physiological responses to intoxication.

Numerous studies have shown that animals and plants can manifest increases or decreases in their respiration rate (consumption of oxygen) as a response to various toxins (metals, phenols, pesticides). It is important to observe that increases in the synthesis of proteins in conditions of intoxication are accompanied by increases in the respiration rate. Additionally, energy consumed is used for metabolism, excretion, storage of the toxin, and physiological and behavioural compensation. In practice, the increase in protein synthesis and energy consumption would be reflected in the increase of the respiration rate.

Euxinia maeotica possesses hereditary osmoregulatory mechanisms, which begin to be brought into play immediately upon the onset of physiological discomfort. Through these processes the organism attempts to adapt, and according to the resulting changes (induced through contamination and which depend on the toxin as compound or on its concentration), the organism resorts to methods of partial compensation of the toxic effects through the modification of vital functions such as: mobility, feeding, respiration and excretion.

Physiological and lethality effects have been followed in three categories of organisms according to size and developmental state (adult, pre-adult and juvenile - see Table 1), noting differences in responses to the toxin used and in correlation with the concentration level.

Regarding the behavior of these animals and the short-term physiological or compensatory effects, the following is observed: in the first 30-60 minutes, upon initial impact of the contaminant, the organism modifies its respiratory rhythm. This animal has an exoskeleton impregnated with calcium carbonate, which renders its surface impermeable, so that the only route of ingress for the contaminant from an aqueous solution is via the branchial system. The recorded values for this stress parameter, the first physiological parameter employed in this type of test, indicate a direct correlation between the compound toxicity and the degree of reaction compared to the control, i.e. the physiological response in normal conditions.

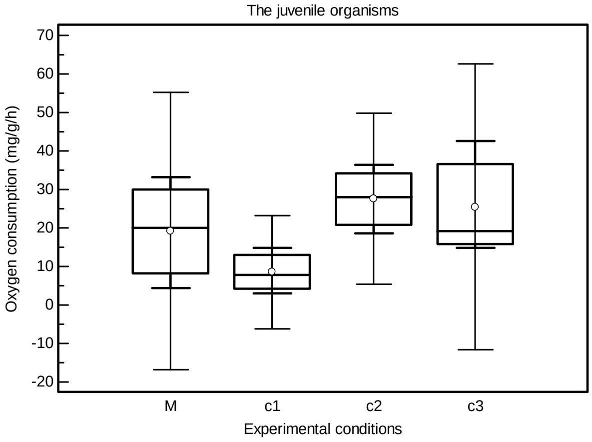


Figure 1. Oxygen consumption in adult organisms (mg $g^{-1}h^{-1}$) recorded at intervals of 15, 30, and 60 minutes, in the control vessel (M) and experimental vessel, with C1, C2, and C3 solutions.

Figura 1. Consumul de oxigen (mg $g^{-1}h^{-1}$) înregistrat la intervalele de timp 15´, 30´ şi respectiv 60´, la organismele adulte din vasele control (M) şi vasele experimentale cu C1, C2, C3.

Thus, oxygen consumption is modified, relative to the control, upon introduction of Pb and according to the increase in the concentration of metals in the water. This also differs among juvenile subjects compared to adults (Figs. 1 and 2). A significant drop in the respiration rate of juveniles is observed in conditions of contamination with C1 (50 ppm), while at C2 concentrations (100 ppm) and C3 (200ppm), a rise occurs.

Reactions to the toxin in water are to be noted. Adult organisms accelerate their respiratory rhythm at lower concentrations, while a significant reduction in consumption - even total cessation - is seen at maximum concentration, at measurement intervals of 15 to 60 minutes, but at a level closely comparable to control subjects in terms of the maximum figure recorded (Fig. 1).

This suggests a change in defensive strategies; the inhibition of respiration in order to limit the impact of the toxin. The correlation coefficient (r^2), linear regression parameters has different values for adults and juveniles (Fig. 3). Lower values of the regression factor were registered in the adults (Fig. 3).

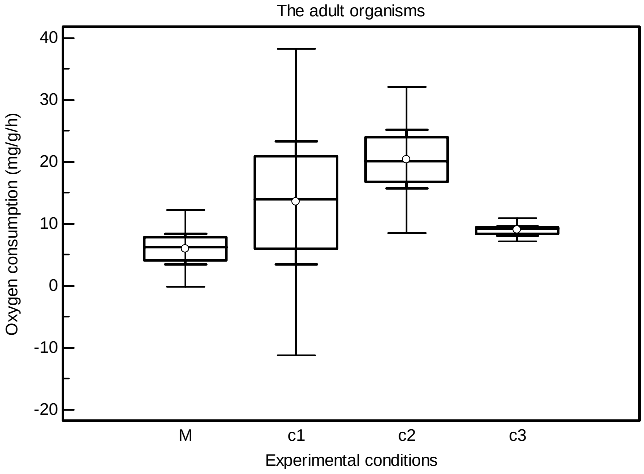


Figure 2. Oxygen consumption in juvenile organisms (mg $g^{-1}h^{-1}$) recorded at intervals of 15, 30 and 60 minutes, in the control vessel (M) and experimental vessel, with C1, C2, and C3 solutions.
Figura 2. Consumul de oxigen (mg $g^{-1}h^{-1}$) înregistrat la intervalele de timp 15´, 30´ şi respectiv 60´, la organismele juvenile din vasele control (M) şi vasele experimentale cu solutiile C1, C2, C3.

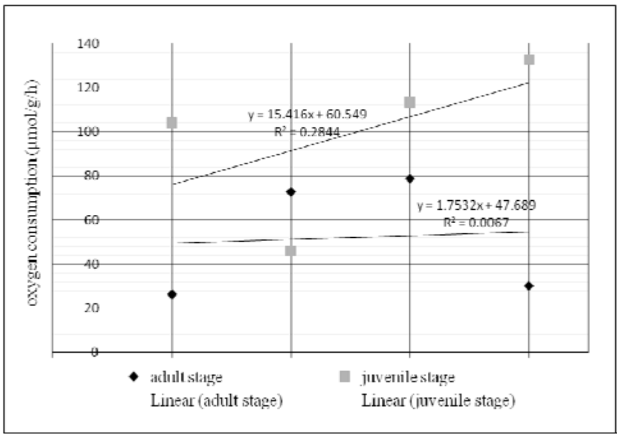


Figure 3. Respiration rate (oxygen consumption expressed in μmol O_2 g^{-1} h^{-1}) by category of size and the linear regression between dose and respiration.
Figura 3. Rata respirației (consumul de oxigen exprimat in μmol O_2 g^{-1} h^{-1}) pe categorii de mărime şi regresia liniara între doza şi respirație.

Other studies accomplished on this species also emphasize modifications in respiration. Upon contamination with methanol and biguanide, two solvents known to have a high toxicity level, an acceleration was observed; oxygen consumption doubled or even tripled compared to the control, which indicates intense cellular metabolic effort. The same effect was also observed in contamination with Ce $(NO_3)_3$, lower in the case of lanthanum salt (NEGREANU et. al., 2002).

Another physiological aspect monitored in the first minutes is mobility of the organism. In natural conditions, *Euxinia maeotica* is a poor swimmer which spends a large part of its time in or on the surface of sediment, where it feeds. The swimming reflex is only triggered by the need for self-preservation, fluctuations in environmental factors (waves, hypoxia, exhaustion of food sources, variations in salinity and temperature and so on).

In the first minutes following introduction of the contaminants, the organisms become highly agitated, periodically rising towards the surface. These effects wane after approximately one hour and the organisms retreat to the bottom of the vessel. Following the introduction of Pb, the organisms reach a state of immobility in 24 hours. Reduction in muscular function is also evident in all organisms 24 hours prior to death.

Stress continues to affect net physiological equilibrium, so that in 24-96 hours the observed effects spread to the nutritional function. Their appetites decline (Fig. 4) until they are unable to feed themselves any longer. This is a clear indication of physiological stress, given the otherwise voracious appetite of this crustacean, whose metabolism is very fast (SOLDATOVA, 1986).

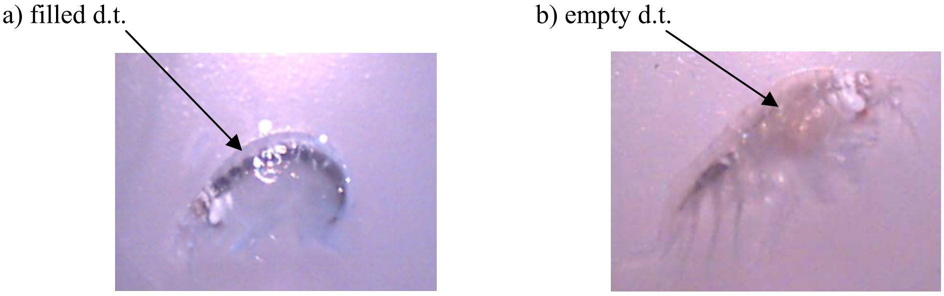


Figure 4. Details of the digestive tract (d.t.) at *Euxinia maeotica* (original photos) (a – in normal physiological conditions; b - following contamination).
Figura 4. Detalii privind încărcătura tubului digestiv (t.d.) la *Euxinia maeotica* (original) (a –in condiţii fiziologice normale; B - după contaminare).

Reduction and even cessation of feeding is a response which already indicates a critical state, when the balance tips towards irreversible balancing mechanisms. In other words, the organism uses its last reserves of energy for survival, but is no longer able to compensate for the toxic effects.

In what regards sublethal effects on *Euxinia maeotica*, Pb contamination is characterized by the cessation of feeding within 48 hours, also noted in the case of Cd, Cu ions contamination (SERBAN et. al., 2001; PALICI et. al., 2001). This effect is closely related to the lack of mobility, the majority of organisms lying still, with their ventral area upwards, while ventilation movements generated by the pleopoda (abdominal appendages) undergo a considerable reduction.

These reactions can also be observed in the case of cerium and lanthanum contamination, as well as in the case of contamination with other heavy metals (Cd, Mn, Cu) (SERBAN et. al., 2001; PALICI et. al., 2001). The same parameters (ventilation movements) undergo diametrically opposite changes and become accelerated in the case of methanol and biguanide contamination. Mobility, however, is not affected as it is in the case of Pb contamination.

Table 3. Responses (TL50, TL100) obtained by category of size (stages) during the analysis period.
Tabel 3. Răspunsurile obţinute (TL50, TL100) pe categorii de mărime (stadii) în perioada de analiză.

Stages	**Weight Wet average (g)**	**Concentrations**	**LT50**	**LT100**
Adult (10-13 mm)	3.14	C1	>8	>8
	2.94	C2	6	7
	3.14	C3	6	8
Pre-adult (7-8 mm)	1.27	C1	>8	>8
	1.05	C2	5	6
	1.08	C3	5	8
Juvenile (4-6 mm)	0.199	C1	4	6
	0.299	C2	4	5
	0.262	C3	4	5

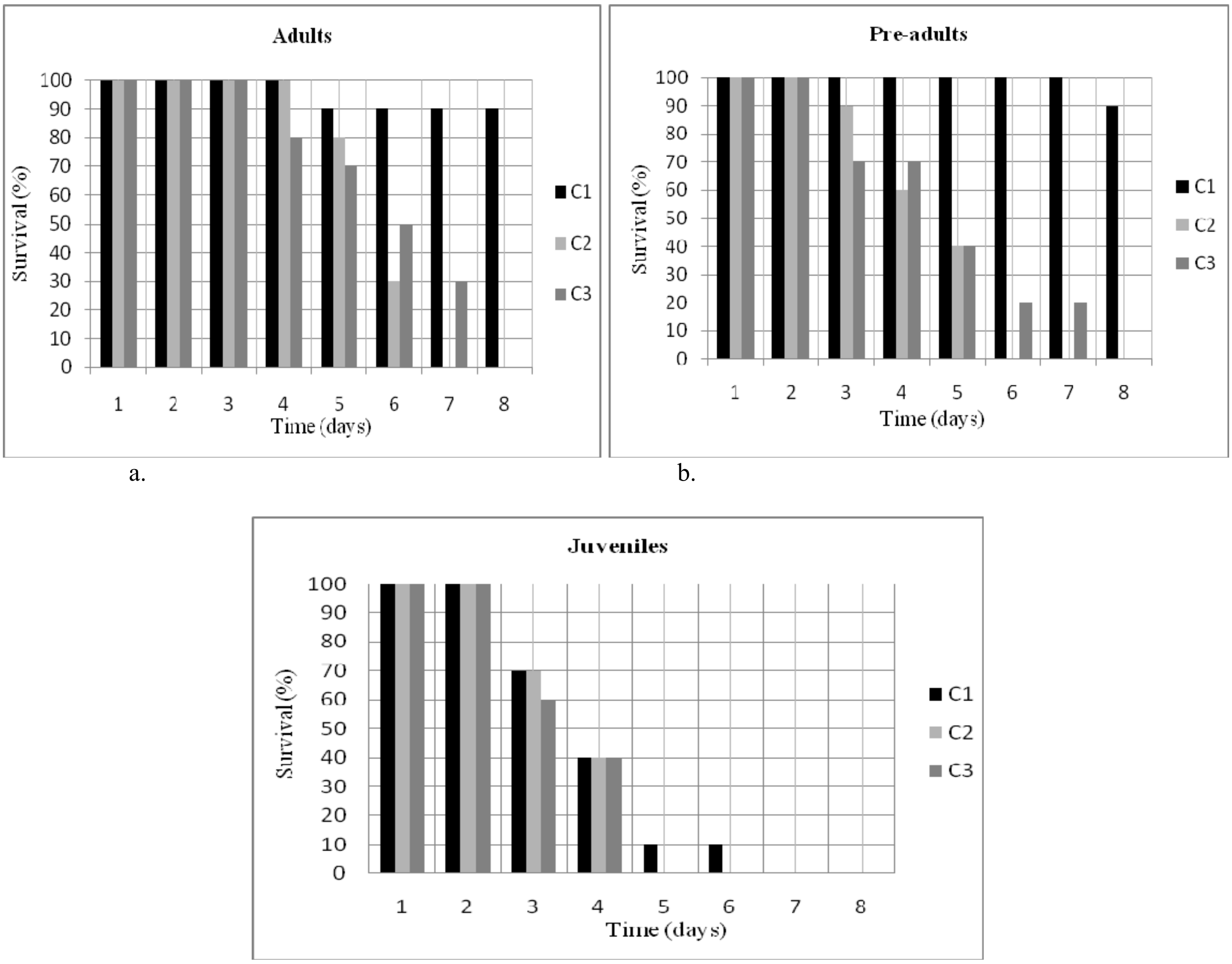
Thus, the most vulnerable forms are those of small dimensions, whose survival rate plummeted after 48-96 hours, reaching LT50 4 days into the experiment (Table 3, Fig. 5c). In the following 24 hour interval there is clear evidence of the same toxic shock in the case of this category so that C2 and C3 concentrations correspond to LT100.

The second category (7-8 mm) appears to be more resistant than the very small forms (Fig. 5b) to reduced concentrations of 50 ppm. At 100 ppm and 200 ppm respectively, there is evidence of an acute toxicity, which results in an LT50 toxicity index within 5 days (Table 3).

In the case of the third category of organisms, those ranging from 10 to 13 mm, sublethal effects appear within 24-96 hours. Lethality occurred 96 hours after contamination (Fig. 5a), LT50 being recorded on the 6th day for the second and third concentrations used (Table 3). Notably, 90-100 % of organisms survived at a reduced concentration of 50 ppm, for eight days (Figs. 5a, b).

General observations include the high toxicity of the contaminant at concentrations of 100 ppm and 200 ppm, a fact also noted in the case of the isopod species *Sphaeroma pulchellum* (unpublished data), whereas *Idotea baltica* is characterized by reduced sensitivity at this level. This suggests that these species have different reactions to the same concentrations, and it is possible that these responses are due, to a large extent, to the direct influence of the organism's physiological condition and its capacity for bioaccumulation.

Isopod species have a completely different behaviour in terms of this metal accumulation. Thus *Idothea baltica* accumulates this metallic ion, whereas *Sphaeroma* does not display these tendencies. Tests indicate level 0 in the case of the latter species, which explains the different reaction to induced toxicity (ŞERBAN, 2004).



a.

b.

c.

Figure 5. Survival of organisms (a-adults, b-pre-adults, c-juveniles) in conditions of Pb^{2+} ion contamination, C1=50 ppm, C2=100 ppm, C3=200 ppm.

Figura 5. Supraviețuirea organismelor (a-adult, b-preadult, c-juvenili), în condițiile de contaminare cu ionul Pb^{2+}, C1= 50 ppm, C2=100 ppm, C3= 200 ppm.

There is documentary evidence that metallic ion toxicity changes according to the pH (SCHABAUER-BERIGAN, 1993; ALBERTS, 1993). Below the pH 7, the toxicity of the lead ion on *Hyalella azteca* (Amphipoda, Crustacea) appears to decrease. How such effects might affect *Euxinia maeotica* is unknown, as experiments on this species have been conducted at a pH of 8.32±0.177 (Table 1), taking into account the natural environment values for the species under analysis. The differences recorded between the analyzed size categories highlight the fact that there is a higher sensitivity in the case of juvenile forms, as demonstrated by an approximately 30% lower survival rate of these individuals in the first 72 hours, the first mortality case occurring in the first 48 hours.

This sensitivity is probably due to the faster effect of lead at an enzymatic level, these individuals being characterized by extremely rapid molting and cell growth rhythm (SERBAN, 2004). There is, at temperatures similar to the ones at which the experiment was conducted (Table 1) and 17% salinity, a 5-7 day interval between moltings, which coincides with the period in which mortalities were recorded.

The way other categories responded can also be connected to the molting rhythm (the moment of ecdysis and post-moult being regarded as critical). In the case of small concentrations (10-20μg g^{-1}), there are no short-term effects on adult species. Some amphipods are actually capable of accumulating lead (RAINBOW, 1998). *Euxinia maeotica* demonstrates an average accumulation level, compared with other Romanian littoral species (ŞERBAN, 2004).

Statistical interpretation has consisted in drawing comparisons between samples by means of the ANOVA test (repeated ANOVA measurements) (Table 4).

Table 4. Survival Rate (SR) statistical analysis by means of the ANOVA repeated measurements method (Medcalc statistical programs) on duplicate samples.
Tabel 4. Analiza statistică a Rs (ratei de supravieţuire) prin metoda Anova (Medcalc statistical programs) a probelor duplicat.

Stages	**Concentration**	**With subjects factors (for samples duplicates)**		**Trend analysis**
		Mean	**SE**	
Adults	C1 C2 C3	9.62 6.37 6.50	0.18 1.62 1.29	T = -2.63 DF=7 P=0.034
Pre-adults	C1 C2 C3	9.87 4.75 5.50	0.125 1.544 1.224	T= -3.64 DF= 7 P=0.008
Juveniles	C1 C2 C3	4.12 3.37 3.75	1.52 1.67 1.57	T= -2.049 DF=7 P=0.079

The analyzed data reveals small differences between the duplicates used, ensuring the accuracy level accepted for each of the experimental versions, a fact highlighted by both SE values and the mean difference between the pairs of the analyzed samples. The P significance index ranges between relevant values (0,008-0,034) for adults and pre-adults, P values are less than 0.05 (Table 4).

CONCLUSIONS

The study represents a significant insight into the causes of mortality in *Euxinia maeotica* in conditions of heavy metal contamination. It has revealed important information regarding the organism's level of response (survival or mortality, behaviour) by age categories (adult, pre-adult and juvenile) and demonstrated clear differences in juveniles due to increased sensitivity deriving from their faster growth rhythm, which leads to critical levels of toxicity in a shorter timeframe.

The higher mortality witnessed in juveniles can also be correlated to the accentuated respiratory rhythm of juveniles, which demonstrates an increased rate in conditions of contamination at higher concentrations. This permits faster absorption and higher dosage levels of the contaminant tested.

It is clear that adult organisms exposed to rising concentrations of the contaminant greatly reduce their respiration rate, thus demonstrating a defensive strategy, which we may consider to be an “acquired” adaptation or one which is hard for juveniles to employ.

Pb is a possible contaminant in port and estuarine zones, therefore, knowledge of mortality factors in these marine species may represent an important point of reference in assessing pollution and, correspondingly, the ongoing condition of the ecosystem in both the short and long term.

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Schröder Verginica
Department of Cellular and Molecular Biology
Faculty of Pharmacy
"Ovidius" University of Constantza
E-mail: virgischroder@yahoo.com

Sîrbu Rodica
Department of Physics and Chemistry
Faculty of Pharmacy,
"Ovidius" University of Constantza
E-mail: sîrbu_27@yahoo.com

Negreanu-Pîrjol Ticuța & Negreanu-Pîrjol Bogdan
Department of Chemistry Faculty of Pharmacy,
"Ovidius" University of Constantza
E-mail: ticuta_np@yahoo.com

Received: April 30, 2010
Accepted: July 21, 2010