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3

Alternative Biotest on *Artemia franciscana*

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1. Introduction

Recent toxicology, in accordance with recommendations from the European Council, has demanded decrease in the number of vertebrates used in toxicology testing and their partial replacement with invertebrate animals, plants or even organ, tissue, or cell cultures. During the last 50 years various invertebrate species have been tested for their sensitivity to many chemical or physical agents to prove their possible use for pre-screening tests. To the most valuable organisms available for ecotoxicity testing belong crustaceans of the *Artemia* genus, commonly known as brine shrimps.

2. Characterisation of *Artemia franciscana*

The taxonomic status of the *Artemia* genus is as follows (Martin & Davis, 2001):
Subphylum: Crustacea Brünnich, 1772
Class: Branchiopoda Latreille, 1817
Subclass: Sarsostraca Tasch, 1969
Order: Anostraca Sars, 1867
Family: Artemiidae Grochowski, 1896
Genus: *Artemia* Leach, 1819.
To this genus belong the following species:

A. salina, monica, urmiana, franciscana, persimilis, sinica, tibetiana, sp. Pilla & Beardmore 1994, and parthenogenetic population(s) of Europe, Africa, Asia, and Australia. In this report we use *A. franciscana* Kellogg 1906, distributed in America, Caribbean, and Pacific islands.

Populations of the *Artemia* genus are widely distributed in all continents except for Antarctica. Species of this genus inhabit more than 500 salt lakes, but not seas, of temperate, subtropical, and tropical zones. They are very well adapted to high salinity waters (up to 70 $g.L^{-1}$, but can survive even at 250 $g.L^{-1}$, Ruppert & Barnes, 1994) with fairly low diversity of living organisms and absence of predators or competitive species. In these environments the evolution of *Artemia* species is favoured by the abundance of microorganisms such as bacteria, protozoa and algae that are the basis of the *Artemia* diet (Amat, 1985).

Brine shrimps come in many colours - from white to pink, shadow, or green. The different colours probably result from specific diets and environmental conditions. Females can reach

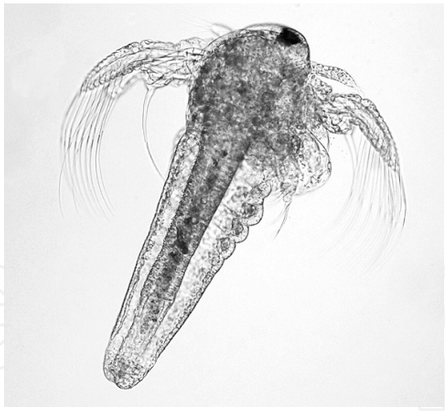

Fig. 1. Nauplius of *A. franciscana.*

the size of 30 mm (most often 12 - 18 mm), males are smaller. Their body is divided into three parts: head, thorax, and abdomen. On the head there is a pair of stalked, lateral, compound eyes and a single, median, unstalked naupliar eye, and two pairs of antennae. The latter are sexually dimorphic. Those of the adult males are modified to form a clasping organ to hold the female during copulation. The female antennae are smaller but much thicker and have a sensorial function. The remainder of the body consists of the segmented thorax, consisted of 11 segments, and posterior abdomen. Each thoracic segment bears a ventral pair of leaf-like appendages, known as phyllopods, used for swimming, respiration, and feeding. Finally, the abdomen consists of 6 segments and a telson – the posterior end of the body. The abdomen of females carries a bunch of spherical-like eggs (cca 200 u in diameter). Their colour varies from creamy to almost blackish brown (Anderson, 1967; Benesch, 1969; Hertel, 1980).

The brine shrimp larva – nauplius is pink or rather auburn, its size about 0.4 mm. Its body consists of a head and short thorax (Fig. 1). On the head there is a dark, red or black, median naupliar eye and two pairs of antennae. The second pair is used for swimming and feeding, while the first pair is a sensory organ.

Artemia franciscana is resistant to high osmotic stress of the hypersaline environments. Its nauplii drink water and secret Na^+ and Cl^- ions. The adults ingest water by mouth and anus. The capability to ingest water, containing bacteria and debris as well, is developed in the 2nd instar stage (Kikuchi, 1971). The larvae hatch usually after 20 - 48 hrs of embryonic development and reach their terminal size after 8 - 10 days, during which they undergo about 17 molts (Gilchrist, 1960).

The life span of *A. franciscana* varies from 2 to 4 months in dependence on salinity and temperature (Browne et al., 1991). Reproduction of the genus *Artemia* may proceed in two modalities: sexual or parthenogenetic. Both, sexual and parthenogenetic populations occur all over the world except for Americas where only sexual populations have been reported (Browne, 1992). Coexistence of both sexual and parthenogenetic strains has been recorded in various habitats; however, partial overlapping in space and/or time may occur. The parthenogenetic reproduction results in unisexual, female populations. While the bisexual specimens have stable number of chromosomes (2n = 42), the parthenogenetic populations may be diploid, triploid, tetraploid, or even pentaploid (Hentschel, 1968).

Females after insemination produce eggs that start to develop immediately after laying. But, under unfavourable conditions dormant stages, known as cysts, are produced. Their metabolism is inhibited and they can survive, even in extreme conditions, for dozens of years. In the cyst there is an embryo in the stage of gastrula, enwrapped with a hard outer membrane. In natural habitats the cysts gather on the water surface, and then drift ashore where they survive the unfavourable season. When the cysts occur in salt water their metabolic activity is again restored and in 20 hrs hatches the umbrella stage that quickly develops to a free swimming nauplius (Browne et al., 1991).

Artemia cysts are produced mainly in the USA, Australia, China, or Saudi Arabia but other countries are also involved. The recent annual yield of the cysts is about 2 000 t. They are produced for the aquaculture or aquarium practice as food for tropical fish. One of the most important sources of the cysts is the Great Salt Lake in Utah, USA. In the northern part (Gunnison Bay) *Artemia salina* cysts are produced in 16 oz. (454 g) cans, while in the southern part (Gilbert Bay) *Artemia franciscana* is produced and packed in 15 oz. (425 g) cans. The latter cysts are more delicate (more than 300 000 specimens per 1 g). The best quality products are sold as the Premium class, and this quality was used in our experiments ("Maxima brine shrimp eggs" produced by the Sanders firm in Utah).

3. Alternative methods

Alternative methods are as follows:

- biotests on plants (e.g. mustard roots, soya, or rice; Horowitz, 1970; Kratky & Warren, 1971) or algae (e.g. *Selenastrum capricornutum, Chlorella kesssleri, or Chlamydomonas reinhardtii*; Fouradzieva et al., 1995);
- biotests on invertebrates (e.g. *Daphnia magna, Tetrahymena piriformis, Asellus aquaticus*, genus *Tubifex*, or flatworms; Balls et al., 1992);
- biotests on vertebrates in early stages of development, before a nervous system is developed and the specimens do not feel any pain (Bagley et al., 1994);
- in vitro methods, i.e., cell, tissue, or organ cultures (Fentem & Balls, 1993), and
- mathematical and computer simulations (Pazourek, 1992).

The methods mentioned above have some disadvantages. Most of them are time consuming, require skilled workers, and often are expensive. These disadvantages may be overcome by the alternative microbiotests, in which the effect of dissolved agents on unicellular or small multicellular organisms is evaluated. For such tests are convenient various simple organisms, such as bacteria, fungi, algae, protozoa, or invertebrates (Blaise, 1991).

In the Multicenter Evaluation of In Vitro Toxicity (MEIC) program the special list of chemical agents have been proved in four standardized tests of the acute toxicity realized on the following species: *Artemia franciscana, Streptocephalus proboscideus, Brachionus calyciflorus,* and *Brachionus plicatilis*. The mortality and toxic effects expressed as $LC_{50/24}$ (i.s., the concentration of an agent at which 50 per cent of the tested animals are dead after 24 hrs) were chosen as criteria of the toxicity. Very good correlations of results among the different testing systems have been found. Furthermore, positive correlations between the tests on lower organisms and conventional tests on rats have also been achieved (Calleja & Persoone, 1992).

The "toxkit" tests are based on dormant stages – cysts or ephippia - that hatch up to 24 hrs before the start of a test. Their advantage is particularly the elimination of time and space consuming laboratory maintenance of the test organisms. Commercially available are ROTOXKIT F, ROTOXKIT M, THAMNOTOXKIT F, ARTOXKIT M, etc.

3.1 Alternative biotests on *Artemia* species

Suitability of the *Artemia* species for the toxicity studies was recognized 80 years ago (Boone, 1931). The first bioassay on *Artemia salina* was offered by Michael et al. (1956). Twenty four years later the standardized acute toxicity bioassay - Artoxkit M (Microbiotests, Inc., Mariakerke /Gent/ - Belgium) was developed (Vanhaecke et al., 1980). The reliability and validity of this commercially available test have been confirmed by a large international study (Vanhaecke & Persoone, 1981). Later on, Solis et al. (1993) developed the Artemia Microwell Cytotoxicity Assay.

Various modifications of the biotests on *Artemia* species have been used for the acute or rarely chronic toxicity testing of a large number of inorganic substances: such as cadmium (Hadjispyrou et al., 2001; Sarabia et al., 1998a, 2002, 2006; Brix et al., 2006), mercury (Sarabia et al., 1998b), chromium (Hadjispyrou et al., 2001), stannum (Hadjispyrou et al., 2001), zinc (Brix et al., 2006), copper (Browne, 1980, Brix et al., 2006), arsenic (Brix et al., 2003), potassium permanganate, potassium bichromate, and silver nitrate (see Boone, 1931), phenolic compounds (Guerra, 2001), and trace elements (Petruci et al., 1995). From the organic substances have been tested: organic solvents (Barahona-Gomariz et al., 1994), acrylonitrile (Tong et al., 1996), antifouling agents (Okamura et al., 2000), oil dispersants (Zillioux, et al., 1973), phorbol esters (Kinghorn et al., 1977), phthalates (Van Wezel, et al., 2000), carbamates (Barahona & Sánchez-Fortún, 1998), atropine (Barahona & Sánchez-Fortún, 1998), anesthetics (Robinson et al., 1965), antihelmintics (Oliveira-Filho & Paumgartten, 2000), herbicides, insecticides, pesticides (Varó et al., 1997, 2002), mycotoxins (Schmidt, 1985; Panigrahi, 1993; Hlywka et al., 1997), pharmaceuticals (Touraki et al., 1999, Parra et al., 2001), pollutants (Knulst & Sodergren, 1994), opiates (Richter & Goldstein, 1970), various plant extracts (Cáceres et al., 1998), or toxins (Granade et al. 1976; Vezie et al., 1996; Beattie et al., 2003). The tests on the genus *Artemia* have also been used to specify biological effects of some physical factors, such as ionizing radiation (Grosch & Erdman, 1955; Easter & Hutchinson, 1961), radionuclides (Boroughs et al., 1958), or UV (Dattilo et al., 2005). In the project Biostack carried out on Apollo 16 the effects of cosmic irradiation on cysts of *Artemia franciscana* were studied (Ruther et al., 1974; Graul et al., 1975; Heinrich, 1977).

3.2 Advantages and disadvantages of the *Artemia* species for toxicity test

The *Artemia* species have been found convenient for various short- or long-term toxicity testing. In spite of this fact, several criticisms against their suitability for such purpose have been published (Persoone & Wells, 1987; Nunes et al., 2006). The most important of them is the lower sensitivity of the *Artemia* species to several chemical or physical agents in comparison to the other invertebrate test organisms (Sorgeloos et al., 1978; Song and Brown, 1998, Okamura et al., 2000; Guerra, 2001; Nalecz-Jawecki et al., 2003; George-Ares, et al., 2003; Mayorga et al., 2010). The second disadvantage is a decreased solubility of some

chemical substances in saline or sea medium (e.g. Mayorga et al., 2010), however, this problem may be overcome by using convenient co-solvents (see bellow).

In general, the reliability of results of the *Artemia* tests may be affected by various conditions of a test, such as temperature, pH, chemical composition of the medium, oxygen, photoperiod, nutrients, some population effects, type of sexual reproduction, etc. (Soares, et al., 1992). For example, George-Ares et al. (2003) reported dependence of the *Artemia* test on the concentration and composition of medium, or on the age of nauplii. Some populations of *Artemia* species may consist of strains with different reproduction strategies (sexual versus parthenogenetic reproduction) and different sensitivity to tested agents. But, Triantaphyllidis et al. (1994) suggested a simple method to separate nauplii of bisexual and parthenogenetic strains by hatching at different temperatures, moreover specific strains may be distinguished by DNA-analysis (Abatzopoulos et al., 1997). Of course, the effects of almost all test factors mentioned above may be easily overcome by the strict standardization of test conditions. An important disadvantage of all alternative tests is their unsuitability to test the chemical agents that require metabolic activation in mammals.

On the other hand, the *Artemia* alternative tests offer many advantages to favour them as convenient for the standardized testing in ecotoxicology: high adaptability to variety of testing conditions, high fecundity, bisexual versus parthenogenetic reproduction strategies, small body size, varied nutrient resources, high hatchability, simple availability, and low cost of the tests. High sensitivity of the *Artemia* specimens to some chemical agents has also been reported. For example, Oliveira-Filho and Paumgartten (2000) found the higher sensitivity of *Artemia* to niclosamide in comparison with *Daphnia similis* or *Ceriodaphnia dubia*. Hlywka et al. (1997) have shown similar sensitivities to the mycotoxin fumonisin B1 in the *Artemia* test and the chicken embryo screening test. And Parra et al. (2001) compared the sensitivity of the acute toxicity *Artemia* test of several plant extracts with corresponding tests on mice and found significant correlations. Undoubtedly, the *Artemia* test is a suitable, sufficiently accurate, simple, and inexpensive alternative to pre-screening chemical toxicity with mammals. Of course, all results derived from the *Artemia* or any other invertebrate test have to be validated by bioassays on mammals.

Our test is based on the cysts produced for purposes of the aquaculture and aquarium practice. The test was established in 1992 (Dvorak, 1995), especially for the extensive dynamic studies including simultaneous treatments with two agents, or possibly of a radiation and a chemical agent. The total expenses of our tests are lower than those of the commercial toxkits.

4. Methods

4.1 Hatching of cysts

Cysts are hatched in water of the following composition (Table 1). The total concentration of salts is 4.7%. In the following text this water is specified as the "sea water".

In water of this quality the brine shrimps mature in 37 days and start their reproduction, providing they are fed with a convenient diet, and the sea water is changed every third day. The hatching proceeds at temperature of 25 ^{o}C and the first larvae occur after 24 hrs. During the hatching process the sea water is aerated by a membrane compressor.

salt (per analysis)	$g.L^{-1}$
NaCl	23.900
$MgCl_2 . 6H_2O$	10.830
$CaCl_2 . 6H_2O$	2.250
KCl	0.680
$Na_2SO_4 . 10 H_2O$	9.060
$NaHCO_3$	0.200
$SrCl_2 . 6H_2O$	0.040
KBr	0.099
H_3BO_3	0.027

Table 1. Composition of hatching water.

4.2 Solubility of chemical agents

Solubility of various chemical agents in 4.7% sea water is often decreased. Consequently, in our pharmacological studies the concentration of sea water is reduced to 0.9% (i.e. the concentration of the blood salts). But, in this low concentration of salt water *A. franciscana* does not consume glucose; consequently, in such low concentration of medium this sugar cannot be used to prolong the test.

The solubility of some low soluble chemical agents may be improved by specific co-solvents. For example, increased solubility of some insoluble purine inhibitors of cyclin-dependent kinases may be achieved by the non-ionic tensides: polysorbate 80 and poloxamer Pluronic F68.

The co-solvent is a non-water solvent that can be mixed with water and the resulting mixture has increased capability to solve some chemical compounds. For example, dimethylsulfoxide (DMSO) is used as solvent and co-solvent for its ability to solve the most of organic or inorganic chemical compounds, as well as an enhancer of some medicines into skin. It is a colourless liquid, hygroscopic and miscible with water and many other organic solvents (Rowe et al., 2006). DMSO is included in some veterinary medicines. In medicaments used in human medicine it is used only rarely for some undesirable effects. The analgetic, antiflogistic, vasodilatative, myorelaxative, and antiviral effects of DMSO have been reported (Jacob & Herschler, 1986; Rowe et al., 2006). The systemic toxicity of this drug in vivo is rather low (Rowe et al., 2006).

The tweens are used in the pharmaceutical, cosmetics or food industries. Polysorbate 80 (Tween 80) is the tenside widely used in pharmaceuticals as helping agents for the preparation of oral or parenteral suspensions. In parenteral medicines the content of this agent must not exceed the concentration in which it causes a hemolysis of erythrocytes (Rowe et al., 2006). It is an oil-like, yellowish but clear liquid with a specific odour and rather bitter taste. This substance is miscible with water or ethylacetate, but insoluble in fatty oils or a paraffine liquid. The pH value of a 5% water solution varies from 6.0 to 8.0. Chemically it is a polyoxyethylensorbitanmonooleate and it consists of the partial esters of sorbitol and its anhydrides, copolymerized with cca 20 moles of ethylenoxide per mol of sorbitol and sorbitol anhydrides with various fatty acids, mainly the oleic acid. The toxicity of polysorbate 80 is rather low – the acute toxicity LD_{50} in mice treated perorally is 25 $g.kg^{-1}$

(Rowe et al., 2006). We used polysorbate 80 produced by the Jan Kulich firm (Hradec Kralove, Czech Republic) applied in mixture with DMSO. Concentration of the stock solution of polysorbate 80 was 200 $g.L^{-1}$. In our experiments we combined the concentrations 10.0 $g.L^{-1}$ of polysorbate 80 and 5.0 $g.L^{-1}$ of DMSO.

Poloxamers are non-ionic agents used in pharmaceuticals as emulgators, sufractants, wetting agents, or solubilisers. In pharmaceuticals, they are widely used as helping agents for various medications. These agents are non-toxic, non-irritable and are not metabolised in the living organisms. For their inability to cause a hemolysis (Rowe et al., 2006) they are used in various parenteral medicaments. Poloxamers are commercially produced under the names of Lutrol® or Pluronic® (e.g. commercial name of the Poloxamer 188 used in this study is Pluronic F68). The acute toxicity of these agents applied in mice is 15 $g.kg^{-1}$, if applied perorally, or 1 $g.kg^{-1}$, when applied parenteraly (Rowe et al., 2006). In our experiments we used a concentration of 10.0 $g.L^{-1}$ applied with DMSO.

4.3 Treatment with polychlorinated biphenyls and ionizing radiation

Delor 103 is one of the polychlorinated biphenyls (PCB's). These compounds produce colloidal solutions. The initial delor 103 colloidal solution in salt water was obtained by three-day agitation. The excessive undissolved delor 103 was then removed. For the treatment we used the concentration of 4.5 $ng.L^{-1}$. This final concentration was detected by the capillary gas column chromatography analysis (41 peaks identified; from dichloride to pentachloride derivatives). The Kovats index was used as a method of evaluation.

As source of ionizing radiation we used the beta emitter strontium 89 in solution with the volume activity of 33 $kBq.L^{-1}$. The isotope was supplied by the Amersham Comp. as strontium chloride dissolved in water. The manufacturer guarantees specific strontium activity of 1.85 - 7.40 $GBq.g^{-1}$ and purity < 0.5% ^{85}Sr < 0.1% ^{90}Sr, with pH within the 5.0 - 9.0 range. The final activity used in experiments was obtained by re-calculation of the original activity to the reference date. Potassium dichromate $K_2Cr_2O_7$ of analytical grade was used in the concentration of 5 $mg.L^{-1}$ and cadmium chloride $CdCl_2 . 2.5H_2O$ in the final concentration of 20 $mg.L^{-1}$.

4.4 Subacute toxicity

Ten freshly hatched nauplii are placed into a polystyrene Petri dish (diameter 60 mm) by a thin plastic Pasteur pipette. The total volume of salt water is adjusted to 9 ml per dish. All the agents tested are dissolved in sea water, always in a concentration ten times higher than that finally required for a treatment. Consequently, to a dish with 9 ml of the standard sea water 1 ml of the tenfold concentrated stock solution is supplemented (in case of the control dishes 1 ml of the standard salt water). If the simultaneous treatment of two agents is tested, the original volume of sea water with specimens per dish is 8 ml and then each agent is supplemented in 1 ml of its stock solution.

The biotest proceeds in darkness, at temperature of 20 ± 0.5 ^oC, in covered dishes, and without aeration. The specimens are not fed, and consequently, under these conditions they start to die of starvation after 120 hrs. When the temperature is increased to 24 ^oC the lifespan of unfed nauplii is shortened to about 96 hrs.

For every experimental combination five Petri dishes (50 specimens in total) are used. Alive specimens are counted after 24, 48, 72, 96, or 120 hrs (in case of prolonged tests). The dead nauplii are not removed but their presence has not any deleterious effect on the final results of the biotest. If more than 10% of the experimental specimens died in the control group, the exposure was discarded. The evaluation of results of an experimental series should be always performed by one person.

As dead we consider the specimens that do not move even after the stimulation by movement of water caused by rotation of the dish. The evaluation is carried out under a microscope or magnifying glass. To express results of such a test, two terms are, generally used: mortality and lethality. The first means the percentage of dead specimens from the total number of originally healthy specimens. On the other hand, lethality means the percentage of dead specimens from all ill specimens. We use the latter term because, in fact, all specimens exposed to the agents tested at first become ill and only then some of them die.

4.5 Long term toxicity

The prolongation of life span of nauplii in biotests may be achieved by feeding. However, utilisation of various artificial diets, algae or yeast is always complicated with the contamination with some undesired chemical substances or metabolites. Hence, we prolong the *Artemia* test by the treatment with 3% solution of glucose that is used by the specimens as a source of energy. The other parameters of prolonged tests correspond to those of the standard test. The evaluation of results is carried out in steps of 24 hrs for 10 days (Dvorak et al., 2005).

4.6 Expression of concentrations

Concentrations of agents may be expressed in toxicology in $mmol.L^{-1}$ or $mg.L^{-1}$ (in some papers is used ppm unit, but it is not included in the International System of Units). The first method is used to compare different chemical compounds which toxicity depends on only part of the molecule, such as cations of metals, or molecules accompanied with bound molecules of water. In these cases the concentration is expressed only for the active part of the molecule (e.g. mmol $Cd^{2+}.L^{-1}$). This expression accentuates rather more the chemical properties of the molecule than its biological effects. Actually, a biological effect often depends on physical properties of the agent, particularly on its solubility and absorption. Consequently, we prefer the expression of concentrations in units of weight, providing that the precise chemical composition of the substance is included. For this reason we express the drug concentrations in our experiments in $mg.L^{-1}$.

4.7 Evaluation of results

All 50 specimens (i.e., 5 Petri dishes with 10 specimens each) of one experimental combination are evaluated as a whole. Each specimen is judged as alive or dead (criterion see above). Significance of results is calculated according to Hayes (1991). The lethality is expressed in percentage of all specimens used. Results are displayed in the form of 3D charts – concentrations versus time of treatment.

4.8 Validity of results

The principle validity criterion of results is the mortality in control dishes lower than 10%. This criterion is generally used in the *Artemia*, as well as, in *Dafnia* toxicity tests. Because the

specimens in our experimental system are not fed and starve, it is sometimes difficult to keep this criterion at the exposures higher than 72 hrs. In this case, we terminate the experiments at this exposure, in spite of the fact that in the exposure of 96 hrs the nauplii are the most sensitive to some toxic agents and difference between the treated and control group is the highest. In the prolonged experiments (120 hrs or more) in medium supplemented with glucose (see above) we use the higher criterion of mortality (20%). The glucose is probably not sufficient to secure the optimal development of the experimental specimens. Consequently, we generally finish experiments when the mortality of the control group exceeds 20%.

5. Results and discussion

5.1 Simultaneous treatment with ^{89}Sr and low concentrations of cadmium, chromium, and delor 103

For principal information about the treatment with polychlorinated biphenyls and ionizing radiation see 4.3. Results are summarized in Fig. 2. Neither the treatment with delor 103 or ^{89}Sr per se, nor the simultaneous treatment with the both agents did not affect survival of the experimental specimens (results of the separate treatments are not included in figure, simultaneous treatment see 'PCB + Strontium 89' in Fig. 2). On the contrary, potassium dichromate increased the lethality up to 27% (at 72 hrs). The simultaneous treatments with two agents (cadmium + potassium dichromate) or with all four agents altogether (cadmium + potassium dichromate + delor 130 + ^{89}Sr) resulted in the expressive increase in lethality of the experimental specimens.

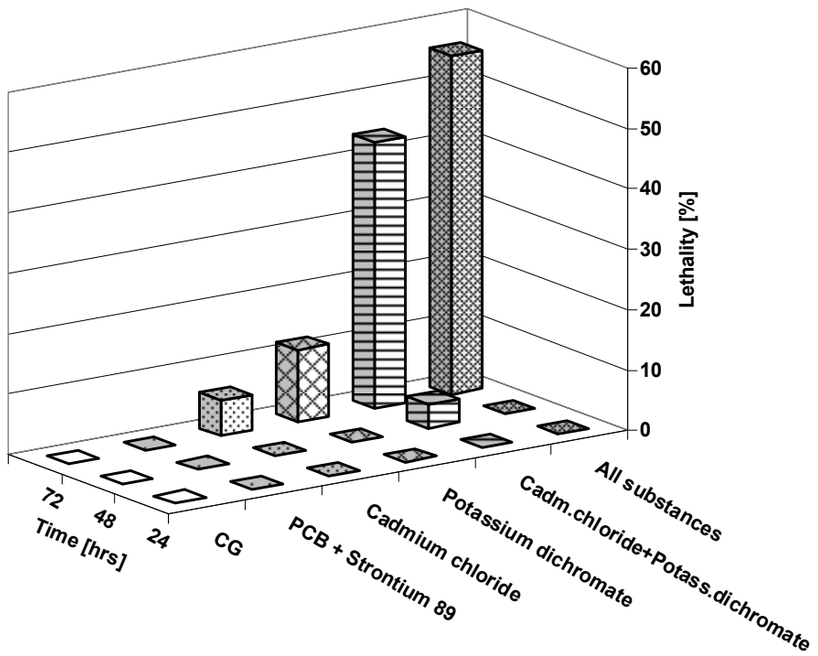

Fig. 2. Effect of simultaneous treatment with strontium 89 [33 kBq.L^{-1}], PCB - delor 103 [4.5 ng.L^{-1}], cadmium chloride ($CdCl_2$. $2H_2O$) [Cadm.chloride; 20 mg.L^{-1}], and potassium dichromate [Potass.dichromate; 5 mg.L^{-1}] on lethality of *A. franciscana* ; CG – control group (no agents).

Isotope ^{89}Sr is a beta emitter; consequently, it is impossible to satisfactorily convert its activity to a dose the experimental specimens actually receive during the exposure. For this reason, the strontium effects are given in the volume activity units of $kBq.L^{-1}$. The hexavalent chromium can damage DNA, decrease its synthesis and enhance the oxidative processes in cells. The analogous effects are induced by the ionizing radiation, as well.

Both agents - strontium 89 or delor 103 - attack the membrane integrity, the result of which may be the increased penetration of potassium dichromate or cadmium chloride into cells. Cadmium induces production of metallothioneins (e.g. Huska et al., 2008) that express, among others, the radioprotective effects (Benova et al., 2006).

This study demonstrated the suitability of the *Artemia* test to evaluate effects of rather complex experiments with simultaneous treatments of several chemical and/or physical agents.

5.2 Changes in morphology of *Artemia* specimens during treatment with gamma rays or inhibitors of cyclin-dependent kinases

In our previous paper the lethality of *A. franciscana* after irradiation with the ionizing radiation was studied. The LD_{50} value was estimated 96 hrs after the irradiation with 600 - 700 Gy (Dvorak & Benova, 2002). This result is in accord with the phylogenetic position of this species.

In the following experimental series the freshly hatched nauplii were irradiated by the gamma rays (^{60}Co) with 100, 250, 500, or 1000 Gy (dose rate of 2.7 $kGy.h^{-1}$). At the time of 72 or 96 hrs after the irradiation the specimens were killed and fixed with formaldehyde. The morphological changes were determined and documented by microphotography.

During the standard development of the control group we observed segmentation of the thoracal part and formation of appendages. The development of those structures in the specimens irradiated with 100 Gy (96 hrs after the irradiation) was comparable with control specimens. But after the irradiation with 250 Gy the segmentation was less apparent, and at the highest doses (500 or 1000 Gy) it was not observed at all.

Another morphological change observed after the irradiation was an atypical development of the intestine epithelium. In control specimens the ratio of the height of epithelium to the diameter of intestine lumen was 1 : 1.2. After irradiation the ratio changed: with 100 Gy to 1 : 1.3, with 250 Gy to 1 : 1.5, and even with 500 Gy to 1 : 1.8. When the doses increased to 1000 Gy the intestine epithelium was undistinguishable. The high sensitivity of intestine epithelium to irradiation corresponds with high sensitivity of this tissue in vertebrates caused by the high mitotic activity of intestine cells. Consequently, the effects of irradiation on the intestine epithelium in the *Artemia* species were consistent with the analogous response in vertebrates.

We also looked for some morphological changes caused by the inhibitors of the cyclin-dependent kinases – olomoucin or roscovitin to compare them with effects of the ionizing radiation. Both agents, in the concentration of 100 $mg.L^{-1}$, caused only slight changes in the intestine epithelium of nauplii stages of *A. franciscana*, but they did not affect the segmentation of thorax and further development of appendages. Also the length or morphology of the body were not affected by these drugs. In the concentration of 50 $mg.L^{-1}$ no effects on the intestine epithelium were apparent.

5.3 Toxicity of newly synthesized inhibitors of cyclin-dependent kinases

The synthetic inhibitors of cyclin-dependent kinases (CDKI) represent possible anticancer drugs. Their anticancer activities have been confirmed in clinical pilot studies. The first synthetic purine inhibitor of cyclin-dependent kinases was olomoucin (Vesely et al., 1994). Since that time a lot of other derivatives were synthesized (Hajduch et al., 1999; Sklenar, 2006). One of the most promising derivatives is the lipophilic roscovitin. In the *Artemia* experimental system we tested the toxicity of some newly synthesized CDKI, and the results compared with the toxicity of olomoucin.

The aim of this study was to test the acute toxicity of roscovitin and three other CDKI, namely TSP 1, TSP 2, and TSP 3 in sea water (salinity 0.9%). As co-solvents were used dimethylsulfoxide (DMSO), polysorbate 80 (TW), or Pluronic F68 (PL). Their toxicities were compared with the control groups (in charts labelled as CG).

The mixture of DMSO and PL occurred inconvenient because of the precipitation of roscovitin that started 24 hrs after dissolving. On the other hand, the mixture of polysorbate 80 and DMSO proved useful and the agents did not precipitate, at all.

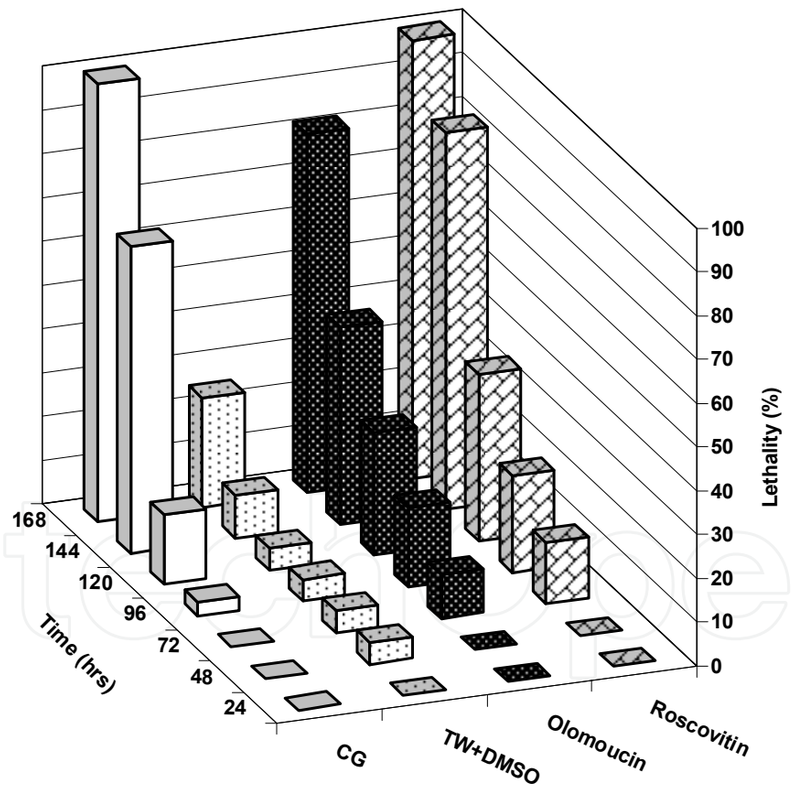

Fig. 3. Effect of olomoucin or roscovitin (100 mg.L^{-1}) dissolved in mixture of 10.0 g.L^{-1} of polysorbate 80 (TW) plus 10.0 g.L^{-1} of dimethylsulfoxide (DMSO) on lethality of *A. franciscana;* CG – control group without any TW or DMSO.

In Fig. 3 we compare the toxicity of olomoucin or roscovitin, both dissolved in mixture of 10.0 g.L^{-1} of polysorbate 80 plus 10.0 g.L^{-1} of DMSO. The final concentration of the tested

agents was 100 mg.L^{-1}. The toxicity of these agents was evaluated over 168 hrs. The values of lethality of both agents were significantly different from the toxicity of the control group (TW+DMSO) at the exposures higher than 96 hrs. Both agents, i.e., olomoucin and roscovitin gave the comparable results except for the exposure of 144 hrs, at which the lethality of roscovitin was significantly higher than that of olomoucin.

The simultaneous treatment with DMSO and polysorbate 80 in the concentrations used in our experiments did not cause any deleterious effects on the tested specimens (TW + DMSO). On the contrary, this treatment actually prolonged duration of the test as result of the polysorbate 80 used as energy source by the experimental specimens. Hence, results of the toxicity tests were compared with the control group treated with mixture of DMSO and polysorbate 80 (TW + DMSO).

In the following bar chart (Fig. 4) we compare toxicity of the newly synthesized CDKI, namely TSP 1 (=6-benzylamino-2-(2-aminoethylamino)-9-isopropyl-purine), TSP 2 (=6-benzylamino-2-[(E)-(4-aminocyclohexylamino)]-9-isopropylpurine), and TSP 3 (= 6-benzylamino-2-(3-aminopropylamino)-9-isopropylpurine) with the toxicity of olomoucin. All substances were dissolved in DMSO and used in the concentrations of 100 mg.L^{-1}. While the toxicities of TSP 1 or TSP 3 were comparable with the toxicity of olomoucin, TSP 2 occurred significantly more toxic than olomoucin. The toxicity of TSP 2 was even higher than that of cadmium; hence, this agent was discarded from the further pharmaco-toxicological studies. In general, chromium, cadmium, or zinc are more toxic than olomoucin, roscovitin, TSP 1, or TSP 3 (Sklenar, 2006).

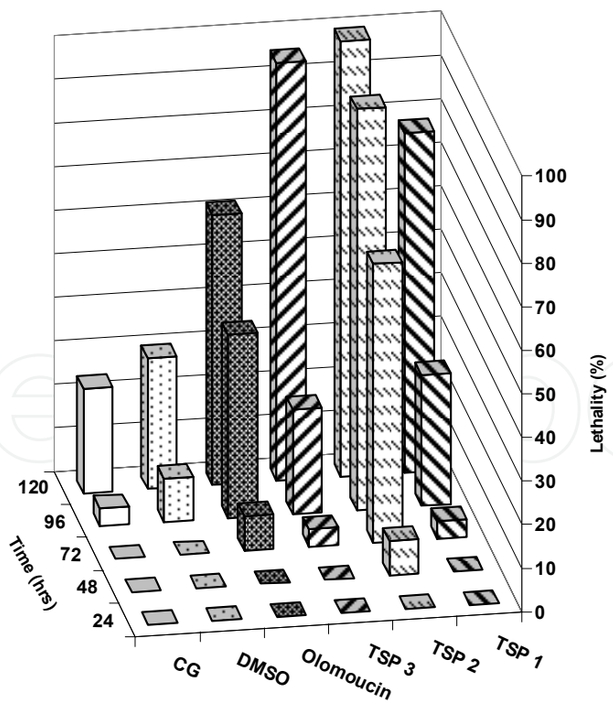

Fig. 4. Comparison of effects of newly synthesised CDKI (TSP 1, TSP 2, and TSP 3) with effects of olomoucin (100 mg.L^{-1}) on lethality of *A. franciscana.* All substances were dissolved in dimethylsulfoxide (DMSO, 15 g.L^{-1}); CG – control group without DMSO.

5.4 Anti-oxidative and pro-oxidative effects of ascorbic acid

The action of ascorbic acid on the oxidative effects of hydrogen peroxide (0.4 $g.L^{-1}$) was studied. Both, the pro-oxidative and anti-oxidative effects of ascorbic acid were evaluated. The first occurred at the concentration of ascorbic acid 0.3 $g.L^{-1}$, while the latter at 0.1 $g.L^{-1}$. The highest anti-oxidative effect was evaluated at the exposure of 96 hrs when the lethality decreased by 34% in comparison with that of hydrogen peroxide per se (Fig. 5).

The search for the antagonists of the oxidative effects of reactive forms of oxygen (free radicals) undoubtedly has been one of the priorities of recent pharmacology. Such antagonistic effects are generally dependant on the concentration of the drug used. In our experiments the ascorbic acid in a concentration of 0.1 $g.L^{-1}$ partially decreased the oxidative effect of hydrogen peroxide. This finding is in accord with the well-known anti-oxidative efficiency of the ascorbic acid (Young et al., 1992). On the other hand, the higher concentration of ascorbic acid (0.3 $g.L^{-1}$) operated contrary and increased the final oxidative effect in the simultaneous treatment with 0.4 $g.L^{-1}$ of hydrogen peroxide. These results are in agreement with the clinical studies showing that the treatments with ascorbic acid at concentrations higher than 1000 mg per day have the pro-oxidative effects, while the effects of the lower concentrations are contrary (Soska et al., 1994). Consequently, the alternative *Artemia* test proved competent for the extensive studies of simultaneous treatments with two agents.

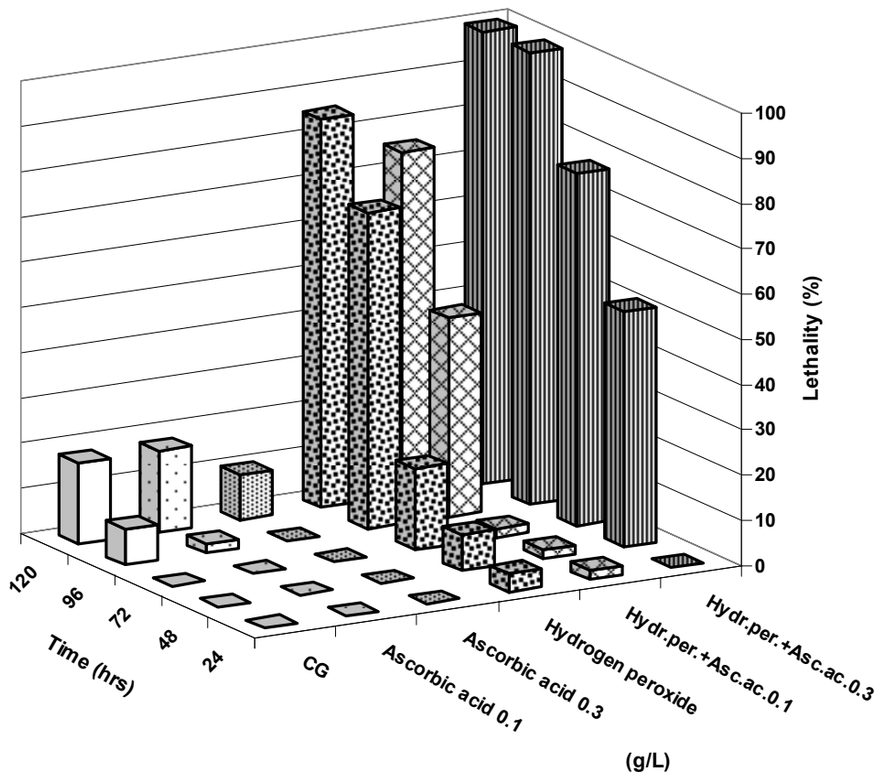

Fig. 5. Effects of simultaneous treatments with ascorbic acid (Asc.ac.; 0.1 or 0.3 $g.L^{-1}$) and hydrogen peroxide (Hydr.per.; 0.4 $g.L^{-1}$) on lethality of *A. franciscana;* CG – control group.

5.5 Anti-oxidative effect of *Orobanche flava* plant extract

To the *Orobanche* genus belong annual or perennial parasitic plants that lack chlorophyll, and consequently, fail to perform photosynthesis. They get the nutrients from their host dicotyledonous plants. In our experiments we used *Orobanche flava* (order Scrophulariales, family Orobanchaceae). The pharmacodynamic studies of this plant have shown the positive effects on fatigue, and supporting effects on the immunity system or male potency. From tissues of this plant various chemical substances have been extracted, such as phenylpropanoid glycosides, verbascoside, orobanchoside, tropones, tocochromanoles, fatty acids, phytosterols, carotenoids, or D-mannitol (Erickson, 1969). Some of these agents are supposed to produce anti-oxidative effects. Consequently, effects of the alcoholic extract of this plant on the treatment with hydrogen peroxide were evaluated by the *Artemia* alternative test.

The oxidative effects were induced by hydrogen peroxide in concentrations of 0.4 $g.L^{-1}$ or 0.2 $g.L^{-1}$. Use of such low concentrations of hydrogen peroxide is on principle in concordance with the real concentrations causing the oxidative effects in vivo. The co-solvent DMSO in concentration of 12.5 $g.L^{-1}$ was used to improve solubility of the plant extract in sea water. In the other experiments, where the effect of the plant extract on the toxicity of DMSO was studied, the latter was used in the concentrations of 50 $g.L^{-1}$ or 100 $g.L^{-1}$. The alcoholic plant extract was dissolved in DMSO and applied in the concentrations of 0.05, 0.10, 0.25 or 0.50 $g.L^{-1}$. All concentrations of the plant extract proved non-toxic, or they even decreased the lethality in comparison with the control dishes. At the concentration of 0.5 $g.L^{-1}$ no dead specimens were found. This effect was probably caused by some carbohydrates present in the extract that served as sources of energy for the experimental specimens.

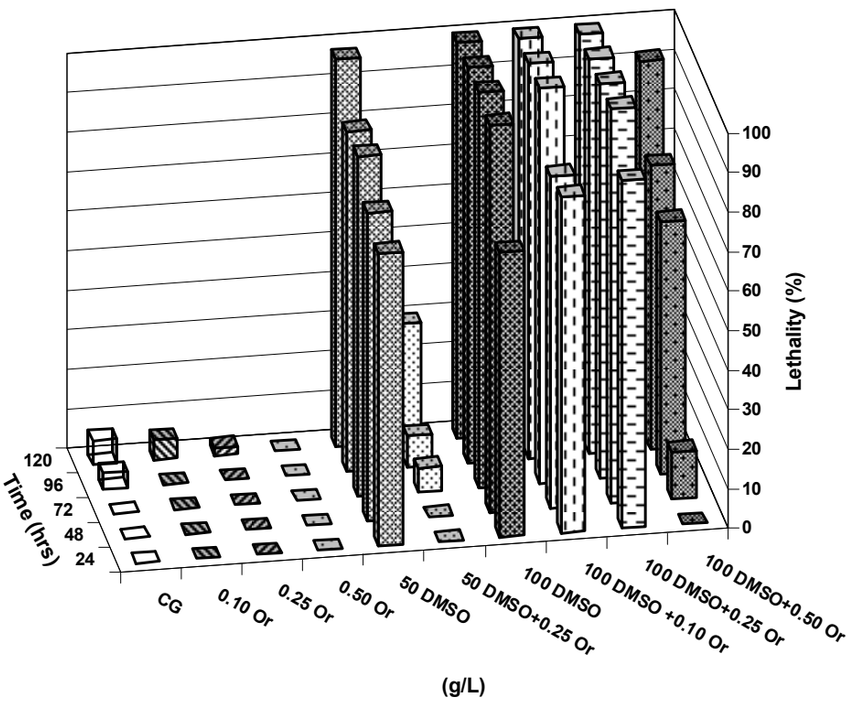

Fig. 6. Decrease in toxic effect of dimethylsulfoxide (DMSO) induced by the plant extract from *Orobanche flava* (Or); CG - control group (no agents); according to Hrbasova (2006).

In the first model experiment we studied the effect of the plant extract on toxicity of the co-solvent per se (Fig. 6). In two experimental combinations (100 g.L^{-1} DMSO + 0.50 g.L^{-1} of plant extract, or 50 g.L^{-1} DMSO + 0.25 g.L^{-1} of plant extract) the toxicity of DMSO was significantly reduced. The other experimental combinations proved rather ineffective.

In the second model experiment the anti-oxidative effects of the plant extract on the oxidative effects of hydrogen peroxide were evaluated. In all simultaneous treatments with the plant extract (0.05 g.L^{-1} or 0.10 g.L^{-1}) plus hydrogen peroxide (0.2 g.L^{-1} or 0.4 g.L^{-1}) the anti-oxidative effects of the plant extract were detected (Fig. 7). While in the combinations with 0.10 g.L^{-1} of the plant extract the anti-oxidative effects were significant, after 96 or 120 hrs, the effect of 0.05 g.L^{-1} occurred positive but insignificant. Thus, we came to the conclusion that the *Artemia* test had proved fully competent for the extensive studies of simultaneous treatments with combinations of drugs.

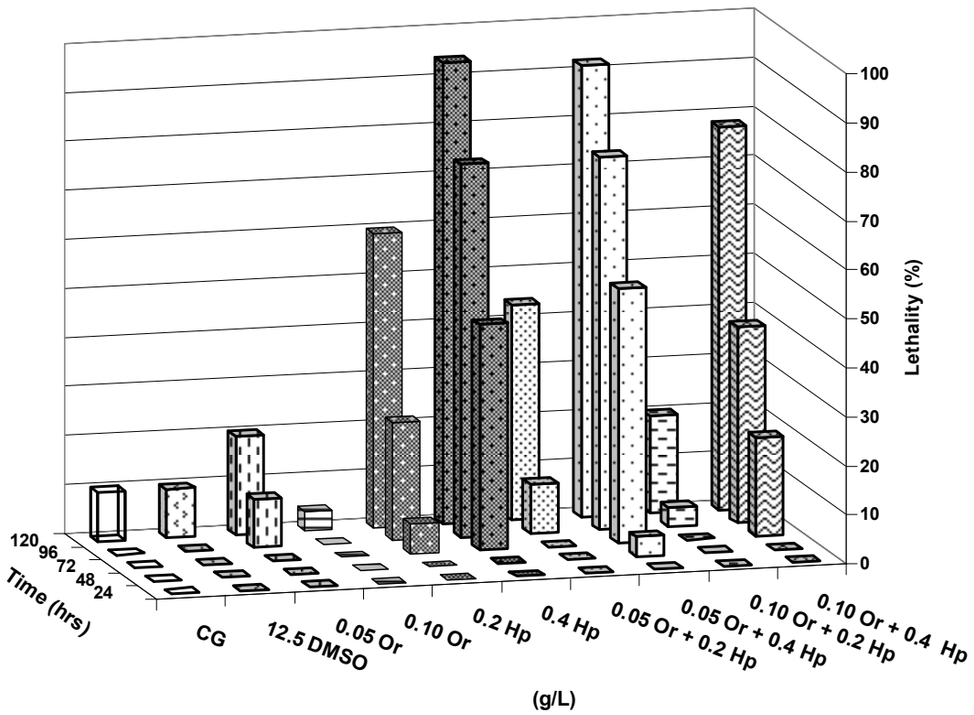

Fig. 7. Decrease in oxidative effect of hydrogen peroxide (Hp; 0.2 g.L^{-1} or 0.4 g.L^{-1}) induced by the plant extract from *Orobanche flava* (Or; 0.05 or 0.10 g.L^{-1}) dissolved in dimethylsulfoxide (DMSO); CG – control group treated with 3% glucose; according to Hrbasova (2006).

5.6 Prolonged treatment with cadmium

Cadmium, as well as zinc or copper, damage SH groups of proteins, resulting in effects analogous to ionizing radiation. These metals bind in tissues to metalothioneins so abundantly, that they may represent up to 11 per cent of the molecular weight of the metalothioneins. If the synthesis of metalothionein is insufficient the toxic effects of cadmium become apparent (Jaywickreme & Chatt, 1990). Lethal treatment of cells, such as

lymphocytes, with cadmium may result in the apoptosis (El Azouzi et al., 1994). On the other hand, production of the metalothioneins induced by cadmium may lead to the radioprotective effects (Benova et al., 2006). Genes that control production of the metalothioneins affect also the repair of DNA (Privezencev et al., 1996).

In this test survival of nauplii is prolonged by the treatment with 3% glucose. The aim was to study the effect of cadmium on *Artemia* in the prolonged test system (Fig. 8). The life span of nauplii was prolonged even up to 240 hrs. The mortality of nauplii in the control dishes treated with glucose decreased to only 4% after 216 hrs of exposure. Hence, 3% glucose proved useful for the prolongation of the *Artemia* test up to 10 days. As expected, the toxicity of cadmium increased with increasing concentration and exposure time.

The supplement of glucose to the *Artemia* test system even reduced the toxic effects of cadmium (Dvorak et al., 2005). This finding was significant for the exposure from 24 to 72 hrs and concentrations higher than 50 $mg.L^{-1}$. The same relationship was valid also for the LC_{50} values expressed in $mg.L^{-1}$ (value without glucose versus value with glucose): at 24 hrs – value 238 versus 482, at 48 hrs – value 250 versus 482, and at 72 hrs – value 195 versus 263. In the same paper we also compared LC_{50} values for three agents used to standardize the microbiotests, namely $K_2Cr_2O_7$, $CdCl_2$. $2H_2O$, and $ZnSO_4$. $7H_2O$. Surprisingly, while the dependence of the toxicity of zinc sulphate on the exposure time was linear, that of the cadmium chloride was logarithmic, and that of potassium dichromate even quadratic.

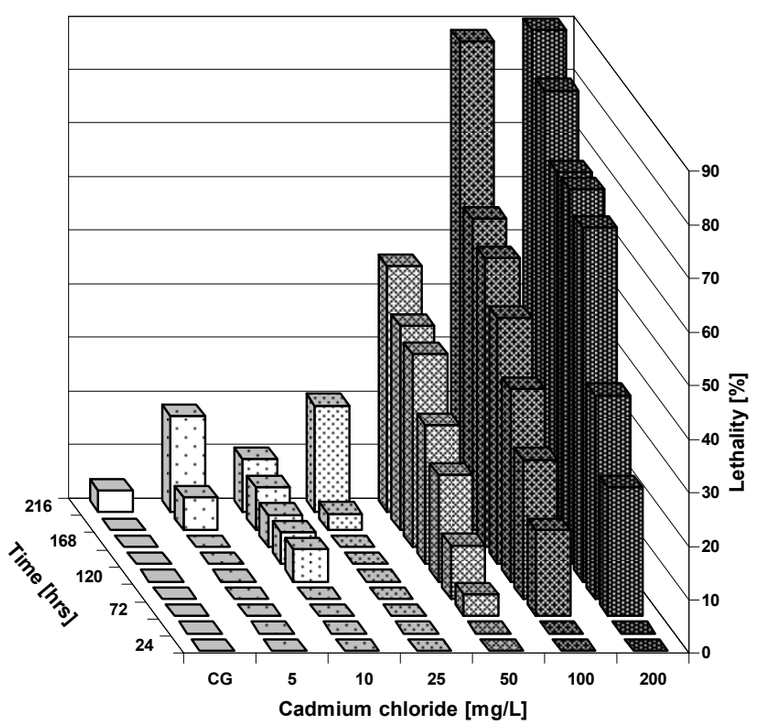

Fig. 8. Effect of cadmium chloride on lethality of *A. franciscana* in test prolonged by glucose (3%), CG - control group supplemented with 3% glucose; according to Dvorak et al. (2005).

6. Conclusion

Eighty years of usage of the *Artemia* species in toxicology testing, and hundreds of papers published all over the world on this theme, have confirmed the exclusivity of this invertebrate genus not only in toxicology or pharmacology, but in biology and medicine as a whole. Our almost twenty years experience with *A. franciscana* have proved that the *Artemia* system is not only convenient for acute toxicity testing, but for extensive dynamic studies, as well, including model experiments with simultaneous treatments with two or more agents of chemical or physical character, as has been demonstrated in this chapter. The *Artemia* tests are fully competent to belong to the group of test systems used for the pre-screening of toxic agents.

7. Acknowledgment

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This is a good book on upcoming areas of Ecotoxicology. The first chapter describes genotoxicity of heavy metals in plants. The second chapter offer views on chromatographic methodologies for the estimation of mycotoxin. Chapter three is on effects of xenobiotics on benthic assemblages in different habitats of Australia. Laboratory findings of genotoxins on small mammals are presented in chapter four. The fifth chapter describes bioindicators of soil quality and assessment of pesticides used in chemical seed treatments. European regulation REACH in marine ecotoxicology is described in chapter six. X-ray spectroscopic analysis for trace metal in invertebrates is presented in chapter seven. The last chapter is on alternative animal model for toxicity testing. In conclusion, this book is an excellent and well organized collection of up dated information on Ecotoxicology. The data presented in it might be a good starting point to develop research in the field of ECOTOXICOLOGY.

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