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Temperature-dose relationships with aflatoxin M_1 in milk on the brine shrimp (*Artemia salina*) larvae

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Summary

Temperature-dose relationships with aflatoxin M_1 (AFM_1) were studied using the brine shrimp *Artemia salina* larvae as an biological indicator in the temperature range from 20 °C to 40 °C. Increase in the incubation temperature resulted in sensitivity increase by the brine shrimp to AFM_1. Optimum sensitivity occured at 30 °C. Positive results were obtained at 0.18 μg AFM_1 x L^{-1} of whole pasteurized milk with a mortality of over 15%. Greater than 90 % mortality occurred at dose levels of 0.9 μg AFM_1 x L^{-1} and above. The test can be conducted during 30-60 hours.

Key words: aflatoxin M_1, bioassay, *Artemia salina*, LC_{50} and T_{50} doses

Introduction

It is known that some moulds produce various toxic metabolites under appropriate temperature and moisture conditions. These metabolites, called mycotoxins, may be hazardous for human health (van Egmond, 1989, 1991; Wood, 1991; Duraković and Duraković, 2003).

The discovery of aflatoxin in 1960 dramatically stimulated interest in mycotoxins and mycotoxicoses and clearly revealed the real and potential danger of toxic fungal metabolites to men and animals (van Egmond, 1989; Wood, 1991; IARC, 1993a; Bhat and Vasanthy, 1999).

Exposure to mycotoxins through food is widely recognized as human health hazard (Bhat and Vasanthy, 1999; Duraković and Duraković, 1999, 2003; Duraković et al., 2008). Of all mycotoxins, aflatoxin B_1 (AFB_1) is considered to be the most toxic/carcinogenic compound (Škrinjar et al., 1992; IARC, 1993b; Škrinjar et al., 1995; Duraković et al., 2008). It is biotransformed by hepatic microsomal cytochrome P_{450} to AFM_1.

Milk is usually contaminated with small amounts of AFM_1 as a consequence of the AFB_1 metabolism by animals that are fed with AFB_1 contaminated feed (van Egmond, 1991; Škrinjar et al., 1992, 1995; Taveira and Midio, 2001; Tratnik et al., 2001). The forming of AFM_1 occurs in liver and is secreted into milk in mammary gland (Stoloff, 1980; van Egmond, 1989). Like AFB_1, AFM_1 is toxic and carcinogenic, although toxicity of AFM_1 is slightly lower than of AFB_1 (Trucksess, 1999). The toxicological concern with AFM_1 arises in principle from its close structure similarity to AFB_1 (Figure 1), which has been shown to be of the most potent carcinogens (van Egmond and Wagstaffe, 1987; Duraković, 2007).

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Table 1. Current and proposed legal limits for aflatoxin M_1 in milk and milk products in some European countries* (van Egmond and Wagstaffe, 1987; Creppy, 2002)

Country	Milk ($\mu g \times L^{-1}$)	Other milk products ($\mu g \times L^{-1}/kg^{-1}$)
Belgium	0.05 (V)	0.10 (P) milk for infant foods
Bulgaria	0.50 (V)	0.10 (P) milk powder for infant foods
Croatia	0.05 (V)	0.025 (P) milk for infant foods
Czech Republic	0.50 (V)	0.10 (P) milk powder for infant foods
France	0.05 (P)	0.03 (P) milk powder for infant foods
Germany	0.05 (P)	0.10 (P) milk powder for infant foods
The Netherlands	0.05 (P)	0.04 (P) milk powder for infant foods 0.20 (P) cheese
Russia	0.50 (V)	0.10 (P) milk powder for infant foods
Sweden	0.05 (P)	0.10 (P) milk powder for infant foods
Switzerland	0.05 (C)	0.01 (C) milk powder for infant foods

* (C) = current limit ; (P) = proposed limit ; (V) = voluntary limit

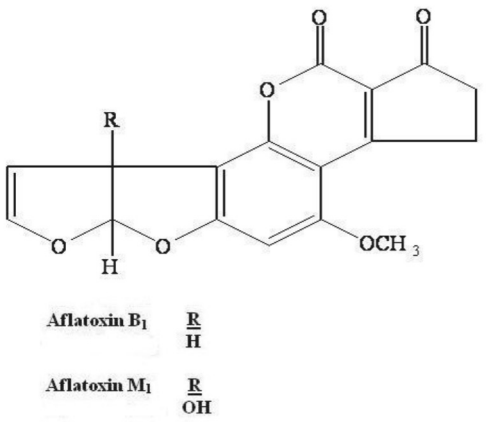

Figure 1. Chemical structures of aflatoxins B_1 and M_1 (van Egmond and Wagstaffe, 1987; Duraković, 2007)

The direct evidence for the carcinogenicity of AFM_1 is slender, primarily because insufficient quantities of the pure compound are available for toxicological studies, and is based largely on limited experiments with rainbow trout (Abedi and Scott, 1969; Hsieh and Ruebner, 1984) and Fisher rats (Abbas et al., 1984). The toxicological risks, however, are sufficiently high to justify legal controls. Current and proposed limits for AFM_1 in European countries are given in Table 1 (van Egmond and Wagstaffe, 1987; Creppy, 2002).

Evidence of hazardous human exposure to AFM_1 through milk and dairy products has been shown by several investigators (Lukač et al., 1991; Galvano et al., 1998; Trucksess, 1999; Samaržija et al., 2003; Galvano et al., 2009). AFM_1 is of great concern because of high human, namely children consumption of milk, dairy products and protein concentrates based on milk such as whey protein concentrates (Tratnik, 1998; Brnčić et al., 2008a; Brnčić et al., 2008b; Brnčić et al., 2009). There are also some innovative techniques for milk processing like ultrasound, pulsed electric fields and cold plasma that could potentially solve problems like this one (Bosiljkov et al., 2011).

Regulatory limits throughout the world are influenced by economic consideration and vary from one country to another (Stoloff et al., 1991). The European Community and Codex Alimentarius prescribe that the maximum level of the AFM_1 in liquid milk and dried or processed milk products should not exceed 50 ng x kg/L^{-1} (Škrinjar et al., 1995; Trucksess, 1999; Codex Alimentarius Commission, 2001; Kamkar, 2006). However, according to US regulation by FDA, the level of AFM_1 in milk should not be higher than 500 ng x kg/L^{-1} (Stoloff et al., 1991; Kamkar, 2006).

Therefore, contamination of milk and dairy products with AFM_1 has been recognized as a significant human health hazard.

The aim of this study was to determine the contents of AFM_1 in milk and to estimate LC_{50} and T_{50} values to *Artemia salina* larvae in experiments with different temperatures of incubation.

There are several studies on the effects of aflatoxins on the brine shrimp *Artemia salina* eggs and larvae (Harwing and Scott, 1971; Duraković et al., 1986, 1987, 1989; Schmidt, 1989; Logrieco et al., 1996; Hartl and Humpf, 2000; Duraković et al., 2002; Moretti et al., 2007).

Thus, Duraković and co-workers (1989, 2002) reported that concentration resulting in 50 % mortality of AFB_1 exposed to temperature of 20 °C for 24 hours was 3.0 μg x L^{-1}. Investigations by the same authors at temperature of 30 °C during the 24 hours resulted in 50 % mortality of investigated larvae by 0.9 μg x L^{-1} of this toxin. Research by Schmidt (1989) and Logrieco et al. (1996) on *Fusarium* and *Aspergillus* mycotoxins shows that *Artemia salina* are especially attractive for use in short-term bioassay for these toxins.

According to previous findings (Brown, 1969; Hartl and Humpf, 2000; Favilla et al., 2006) the *Artemia salina* larvae appear to be as susceptible as biological indicator of toxicity of some mycotoxins in foods and feeds.

Materials and methods

AFM_1 standard

AFM_1 standard was obtained from Immunolab Gmbh, Kassel (Germany). Toxin was dissolved in methanol to obtain concentrations 5.0, 10.0 and 50.0 μg x mL^{-1}.

Milk

Whole pasteurized milk, from retail shop was used. The milk showed no detectable levels of AFM_1 (results not presented) (Antunac et al., 2002) and the toxin was added in concentration that varied from 0.1 to 5.0 μg x L^{-1} of milk. The samples were stored frozen in the dark until used.

Larvae

Brine shrimp dry eggs were obtained from Hans Brustman Co., Düsseldorf (Germany). Hatching procedure followed the one described in ARC test, standardized short term toxicity test with *Artemia nauplii* (Vanhaecke and Persoone, 1981a; Vanhaecke et al., 1981b). The hatching medium was artificial seawater of normal seawater salinity (35 g x L^{-1}). For each experiment, 100-200 mg of brine shrimp was placed in each 100 mL of hatching medium contained in a 500 mL Erlenmeyer flasks, and these were shaken as described by Favilla et al., (2006). Hatching can occur in less than 24 hours at 27 °C. Throughout hatching period the same conditions of light sensitivity and temperature were maintained.

Assay method

Disk screening method

Blank paper discs (8 mm diameter) were saturated with a selected solution of AFM_1 (about 20 μL/disc), and each disc was placed directly in the well (Duraković et al., 1987, 1989). Toxicity of each solution was evaluated in triplicate. Two drops (about 0.1 mL) of a larvae suspension (containing 20 to 40 larvae) were added to each well. Trays were incubated at 20 °C, 25 °C, 30 °C, 35 °C and 40 °C, for about 48-96 hours. Mortality was determined by counting the immobile (dead) larvae under a stereoscopic microscope, after killing the living larvae with heat or formalin and then counting the total number. Mortality in controls was determined simultaneously with each screening test. Natural mortality associated with discs saturated in noninoculated media or water average 3 and 1 % respectively (Duraković et al., 1987, 1989; Ben Naceur et al., 2009; Tsolaki et al., 2010).

Results and discussion

Tests were conducted at 20 °C, 25 °C, 30 °C, 35 °C, and 40 °C. The sensitivity of the brine shrimp increased with increase of incubation temperature to about 40 °C; optimum sensitivity to AFM_1 occurred at 30 °C. Tests at 35 °C and 40 °C produced significant mortality among controls. The mortality

Table 2. Percent mortality of *A. salina* larvae at 30 °C after 48 hours at various dose levels of aflatoxin M_1

Aflatoxin M_1 ($\mu g \times L^{-1}$)	Average number of brine shrimp/Test	% Mortality	Number of tests
Control	46	11	10
0.15	42	25	8
0.30	56	50	12
0.40	35	65	10
0.50	38	75	9
0.90	48	95	8

response of the larvae varied according to dosage and it was possible to develop a standard curves in an observation period of 48 and 96 hours, respectively (Figures 2a, 2b, 3a and 3b).

In experiments at 20 °C the larvae started to die in 30 hours and were all killed within 96 hours at a highest dose level (4.8 $\mu g \times L^{-1}$ of AFM_1). Data derived from conducting tests at this temperature are presented in Figures 2a and 2b. At least eight tests were conducted at all dose levels. The tests give positive results at 0.5 $\mu g \times L^{-1}$ of AFM_1 with a mortality of over 5 %. The dose of 2.5 $\mu g \times L^{-1}$ of this toxin in 60 hours produced mortality of 50 % of investigated larvae. Greater than 90 % mortality was achieved after 96 hours at dose levels of 4.5 $\mu g \times L^{-1}$ and above; 0.5 $\mu g \times L^{-1}$ was the lowest level of AFM_1 tested.

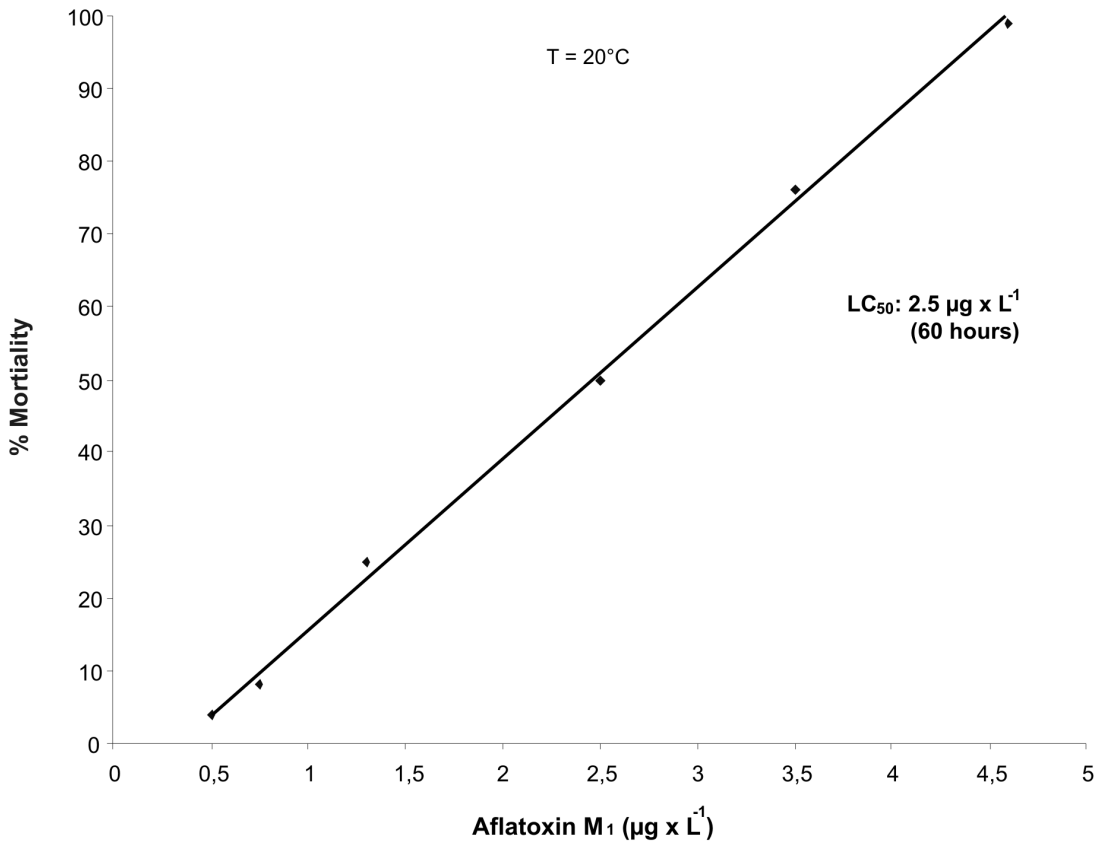

Figure 2a. Dosage-mortality response of *A. salina* larvae to AFM_1 with observation period of 96 hours at 20 °C

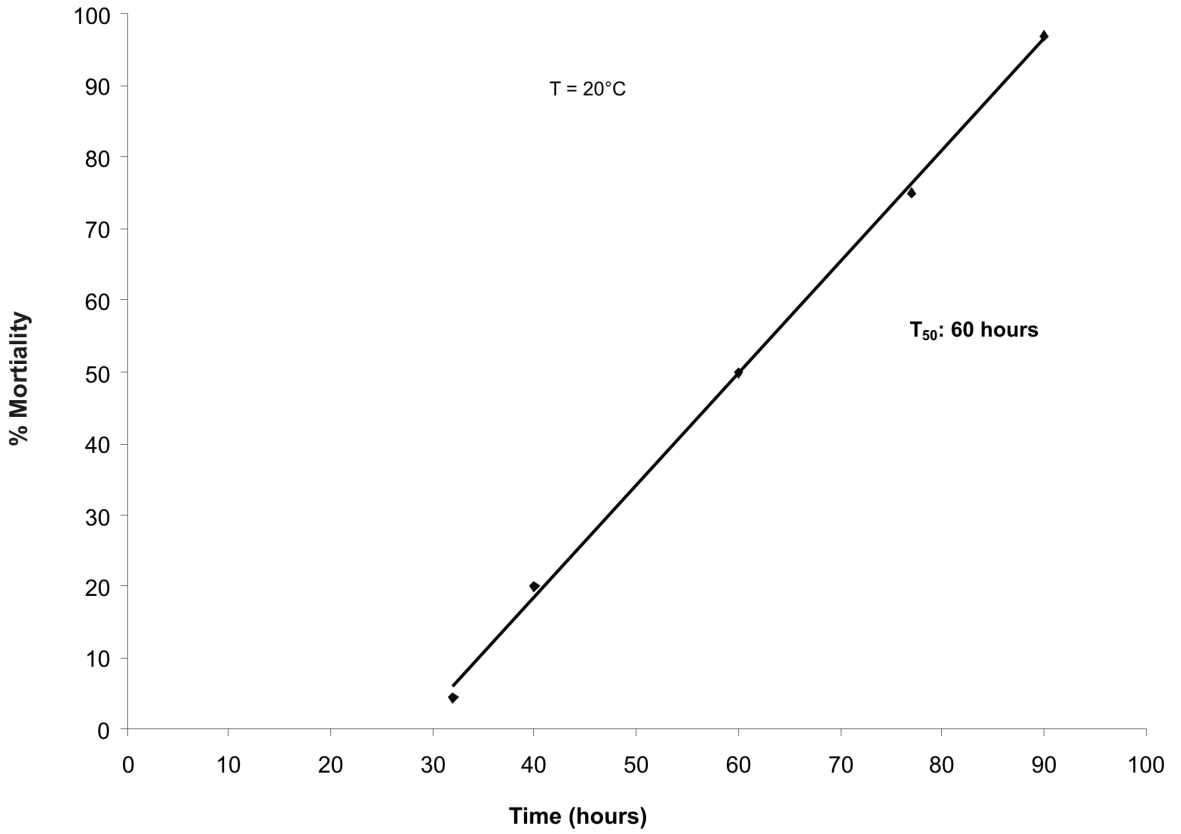

Figure 2b. Time-mortality response of *A. salina* larvae to lethal concentration of AFM_1 at 20 °C

Total mortality (100 %) was reached with 4.8 μg x L^{-1} of this toxin (Figures 2a and 2b). The values are in good accordance with the findings of Duraković and co-workers (1987, 1989), Schmidt (1989), Duraković and co-workers (2002) and Duraković (2007) who have stated 0.5 and 1.0 ng x mL^{-1} as the minimal concentrations of aflatoxins that produce LC_{50} value in experiments with *A. salina* larvae.

Figures 3a and 3b and Table 2 represent data from the tests conducted at 30 °C. AFM_1 at dose levels of 0.4 μg x L^{-1} and above produced greater than 60 % mortality after 48 hours (Table 2). The larvae started to die in 10 hours and were all killed within 55 hours; 30 hours was the time for a 50 % kill. Greater than 90 % mortality was achieved after 48 hours at dose levels of 0.9 μg x L^{-1} and above. At this incubation temperature and toxin concentration of 1.2 μg x L^{-1} all the larvae were killed within 55 hours. At a lower temperature of incubation and lower concentrations of AFM_1 this period was prolonged by a few hours up to several days (Figures 2a, 2b, 3a and 3b).

Dosage mortality (LC_{50} - 48/96 hours) and time mortality (T_{50} - 48/96 hours) were calculated by graphical interpolation.

The percentage mortality between 5 % and 95 % were calculated from the average number of dead larvae per concentration, and plotted on log-probate paper. A straight line is drawn at sight through the points. The intersection of this line with the 50 % mortality horizontal line determines LC_{50}.

The test aims at the determination of the LC_{50} and T_{50} in the 48/96 hours on the basis of the critical range concentrations obtained in the preliminary test. Concentrations and dilutions are chosen from a logarithmic scale (Duraković et al., 1986, 1987). In principle five concentrations should be sufficient. For satisfactory LC_{50}, however, at least three data must be situated in the mortality range 5-95 %. If this not the case, the test should be repeated with additional concentrations from dilution scale. For each concentration, including the control, three replications should be used.

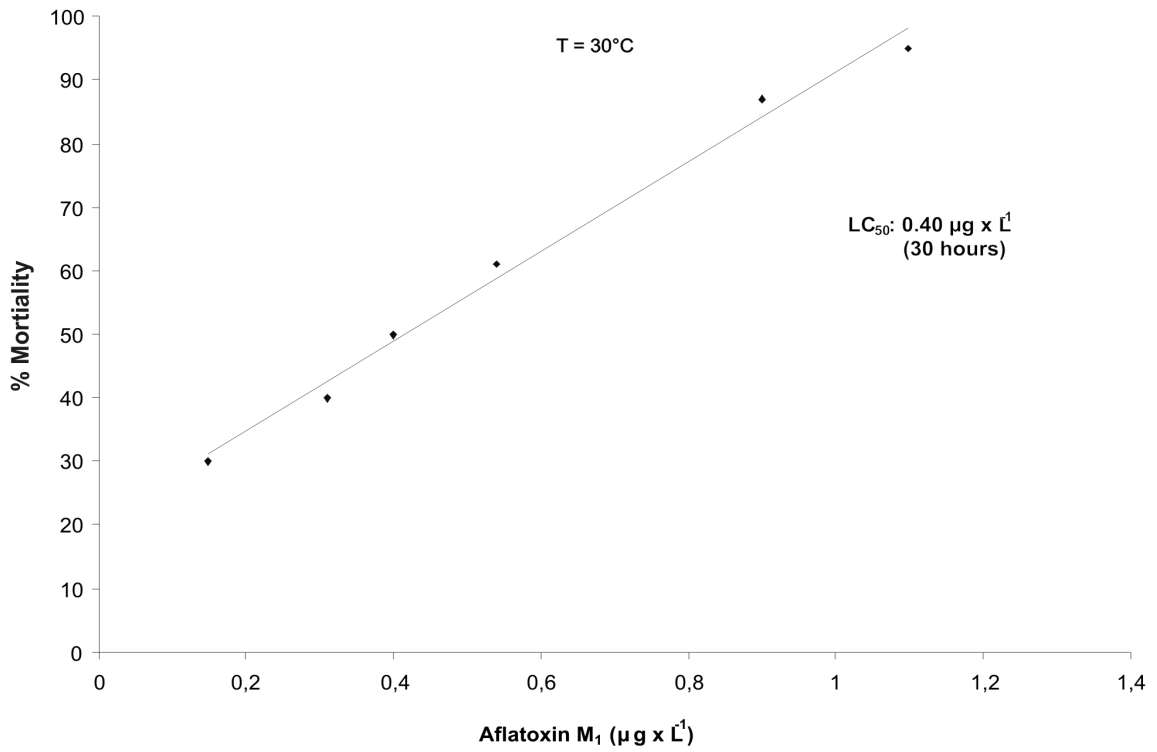

Figure 3a. Dosage-mortality response of *A. salina* larvae to AFM_1 with observation period of 48 hours at 30 °C

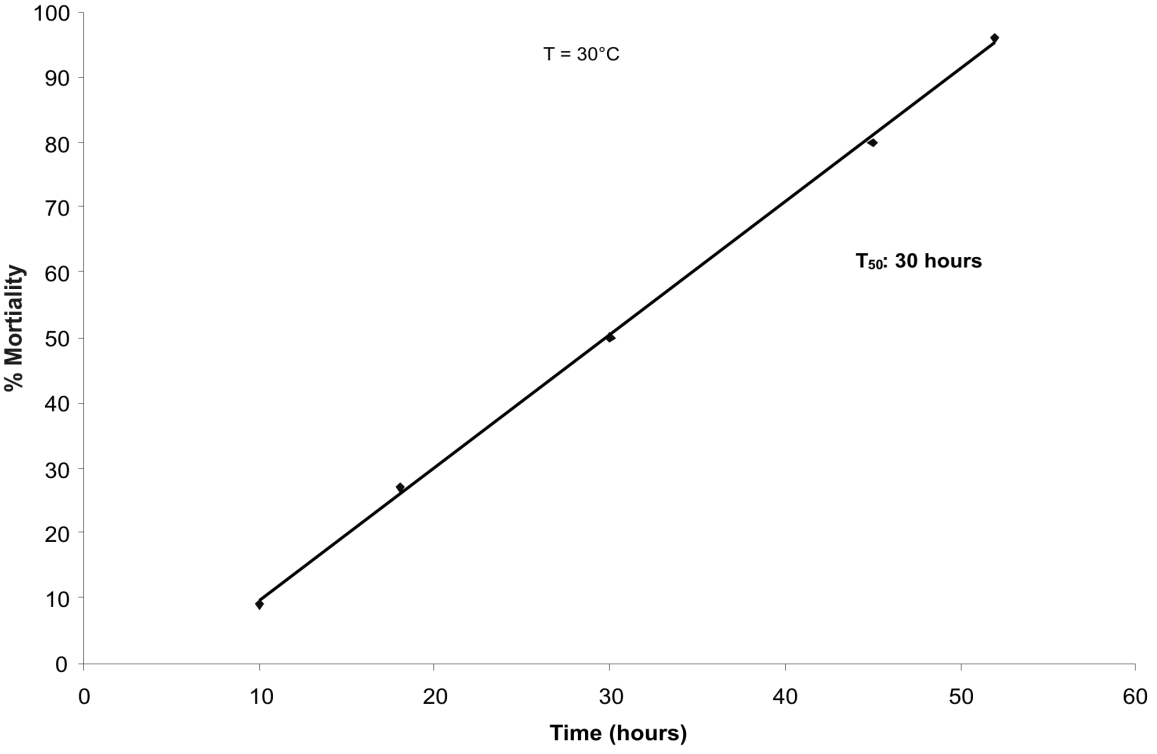

Figure 3b. Time-mortality response of *A. salina* larvae to lethal concentration of AFM_1 at 30 °C

Since AFM_1 is soluble in artificial seawater to a concentration of about 20 μg x mL^{-1}, the sensitivity of this test is below the upper solubility limits. The test volume, 1 mL can maintain up to 50 brine shrimp larvae without affecting the results. Living culture of test organism needs to be maintained; enough larvae to conduct a test can be made available by placing eggs in artificial seawater at 27 °C from 18-24 hours before they are needed (Duraković et al., 1989; Dabrowski, 1991; Coldwell et al., 2003; Moretti et al., 2007; Munoz et al., 2008). The brine shrimp test was also used effectively with partially purified other mycotoxins and chloroform extracts of a toxigenous moulds cultures (Brown, 1969; Duraković et al., 1987; Logrieco et al., 1996; Sarabia et al., 2002; Favilla et al., 2006).

For various reasons the brine shrimp *Artemia salina* is a uniquely suitable test species for laboratory experiments. In cysts they can be stored for years under dry conditions without loosing their viability. The advantages of *A. salina* as the best "first choice" for toxicity studies in milk can be summarized as follows:

- the cysts are commercially and readily available so that the tests can be carried out worldwide with the same original material and without any problem of provisioning; moreover, the quantity of cysts required per test is very small so the price of the biological material is negligible,
- the necessity of year-round maintenance of stock cultures, with all the biological and technical difficulties and the considerable economic repercussions, is completely eliminated,
- large number of test organisms of exactly the same age and physiological condition can be easily obtained to start the tests.

Conclusions

Over the past several years, the toxicity of mycotoxins to the brine shrimp *Artemia salina* has been studied. Most investigations were conducted over 16-24 hours period and showed LC_{50} values as low as the 500 ng x kg/L^{-1} level (Stoloff, 1980; van Egmond and Paulsch, 1986; Moretti et al., 2007; Galvano et al., 2009).

Although these studies promise in using *A. salina* as a bioassay for mycotoxins, several inadequacies existed in the data. The conditions for the studies were not the same; therefore, comparisons of data from different researchers are difficult. Also, in consideration of current concern over the possible use of mycotoxins as biochemical warfare agents, the detection limit needed to be extended to the low μg x L^{-1} level (van Egmond and Wagstaffe, 1987; van Egmond, 1989; Creppy, 2002).

A. salina are especially attractive for the use in a short-term bioassay for aflatoxins. The dry eggs can withstand adverse environmental conditions and still hatch out in 24-48 hours in the presence of artificial seawater. Continuous maintenance of stock culture is not necessary. Test populations can be as large as desired, and the fresh larvae can live for 48 hours, during which time most bioassay can be conducted. A continuous system of hatching can be used to provide fresh larvae daily.

Compared to other organisms, the larvae are free from the problem of sterilization, culture media, techniques of toxic administration, etc., and are economical, time saving and readily available.

Utjecaj inkubacijske temperature i koncentracije aflatoksina M_1 u mlijeku na larve račića Artemia salina

Sažetak

Proučavani su utjecaji inkubacijske temperature i koncentracije aflatoksina M_1 (AFM_1) na larve račića *Artemia salina* kao biologijskog indikatora u temperaturnom rasponu od 20 °C do 40 °C. Povišenje temperature inkubacije uzrokovalo je povećanu osjetljivost račića na AFM_1. Optimalna osjetljivost očitovala se pri 30 °C. Pozitivni rezultati dobiveni su pri koncentraciji AFM_1 0,18 μg x L^{-1} pasteriziranog mlijeka uz izraženu smrtnost višu od 15 %. Smrtnost viša od 90 % očitovala se pri koncentracijama AFM_1 0,9 μg x L^{-1} i višim. Test se može provesti u tijeku 30-60 sati.

Ključne riječi: aflatoksin M_1, biotest, *Artemia salina*, vrijednosti LC_{50} i T_{50}

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