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Assessment of acrylamide toxicity using a battery of standardised bioassays

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Acrylamide is a monomer widely used as an intermediate in the production of organic chemicals, e.g. polyacrylamides (PAMs). Since PAMs are low cost chemicals with applications in various industries and waste- and drinking water treatment, a certain amount of non-polymerised acrylamide is expected to end up in waterways. PAMs are non-toxic but acrylamide induces neurotoxic effects in humans and genotoxic, reproductive, and carcinogenic effects in laboratory animals. In order to evaluate the effect of acrylamide on freshwater organisms, bioassays were conducted on four species: algae *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata*, duckweed *Lemna minor* and water flea *Daphnia magna* according to ISO (International Organization for Standardisation) standardised methods. This approach ensures the evaluation of acrylamide toxicity on organisms with different levels of organisation and the comparability of results, and it examines the value of using a battery of low-cost standardised bioassays in the monitoring of pollution and contamination of aquatic ecosystems. These results showed that EC_{50} values were lower for *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata* than for *Daphnia magna* and *Lemna minor*, which suggests an increased sensitivity of algae to acrylamide. According to the toxic unit approach, the values estimated by the *Lemna minor* and *Daphnia magna* bioassays, classify acrylamide as slightly toxic (TU=0-1; Class 1). The results obtained from algal bioassays (*Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata*) revealed the toxic effect of acrylamide (TU=1-10; Class 2) on these organisms.

KEY WORDS: *battery of bioassays;* Daphnia magna*;* Desmodesmus subspicatus*; ISO standards;* Lemna minor*; polyacrylamide;* Pseudokirchneriella subcapitata

Acrylamide has been identified as a toxic substance with the ability to induce carcinogenic, genotoxic, and reproductive effects in mammalian cells. In the last few decades, a substantial amount of efforts have been devoted to the development of reliable methods for detecting the presence of acrylamide in environmental and drinking water. According to the EU Report (1), it is estimated that the total acrylamide production capacity within the EU is at 80,000-100,000 tons per year and the total amount of acrylamide from all known sources released into waterways is 280 kg day^{-1}.

Acrylamide is the main ingredient of polyacrylamides (PAMs), non-toxic polymers, which have a wide range of applications - in drinking water and wastewater treatment, crude oil production processes, paper and textile industries, ore, concrete and mineral processing, soil and sand treatment for stabilising soil erosion, manufacture of dyes and cosmetic additives, and other miscellaneous uses - photographic emulsion, adhesives and coatings (1, 2). Also, PAMs are blended with pesticides as a thickening agent (3).

Although PAM has been seen as a non-toxic chemical, the toxicity of its non-polymerised residual monomer content – acrylamide, which is a known neurotoxin (4, 5) classified as a "probable human carcinogen" by IARC, is of concern (6). It is the main source of drinking water contamination and since PAM is used for drinking water treatment, acrylamide has been included in the monitoring of the quality of water intended for human consumption (7). The results of the analysis of some samples from public drinking water supply wells showed acrylamide concentrations ranging from 0.04 μg L^{-1} to 5 μg L^{-1} (8, 9). Also, the discharged concentrations of acrylamide into natural water were monitored and the measured concentrations were from 0.082 μg L^{-1} to 9.700 μg L^{-1} (10, 11).

In accordance with the new European Union legislation on chemicals (12, 13), acrylamide is classified as a carcinogen substance in the Category 1B, as a mutagen substance in the Category 1B, and as a reproductive toxic substance in the Category 2. For acrylamide, the WHO/FAO's estimated cancer risk is at 3.3×10^{-4}, i.e. 33 additional cases per 100,000 people (14). Animal studies have showed

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that acrylamide can induce an increased incidence of brain, central nervous system, thyroid and other endocrine gland cancer, as well as the reproductive organ cancer in mice and rats (1, 6, 14, 15). The biological monitoring of exposure to acrylamide in laboratory animals and humans shows that a significant fraction of ingested acrylamide is converted metabolically to the chemically reactive and genotoxic epoxide glycinamide, which has an important role in the reproductive and genotoxic/carcinogenic effects of acrylamide (16, 17).

Since acrylamide could be released into the environment, there is a need to understand and evaluate its effect. It is widely known that a test battery composed of bioassays of different species can reduce the uncertainty of the detection, control and monitoring of the quality of the environment (18, 19). The aquatic organisms such as plants and algae are important components of aquatic ecosystems due to their role as primary producers but animal organisms are also constituents of food webs. Most of them are very sensitive to a wide range of pollutants and therefore have already been used as test organisms in toxicity assessments of pollutants. For our study, freshwater green algae *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*, plant *Lemna minor* and water flea *Daphnia magna* were chosen as bioassay representatives of different taxonomic groups of organisms in water ecosystems.

The ecotoxicological data on acrylamide toxicity from previous studies showed that algae are the most sensitive organisms while water fleas and fish are much more tolerant (6, 20-23). There is no literature evidence that the toxicity evaluation of acrylamide has ever been performed on *Desmodesmus subspicatus* and higher plants.

The results of the previous studies conducted on freshwater organisms are not always comparable because the toxicity of acrylamide was not usually estimated by using the same method. In order to ensure the comparability of these results and to follow the proposal of the new European Union legislation relating to the preference of tests (e.g. on algae or water flea), this work will focus on determining the sensitivity of the selected freshwater organisms and examine the value of using the battery of ISO standardised bioassays in the monitoring of acrylamide pollution and contamination of aquatic ecosystems. With respect to everything mentioned above, *Desmodesmus subspicatus*, *Pseudokirchneriella subcapitata*, *Lemna minor,* and *Daphnia magna* have been chosen for toxicity evaluation of acrylamide, primary due to their high sensitivity and ease of culture and manipulation in laboratory conditions (24-29).

MATERIALS AND METHODS

High purity acrylamide (Standard Acrylamide, ≥99 %, Sigma Aldrich, CAS 79-06-1) was used as the test substance. A stock solution (1,000 mg L^{-1}) was prepared as an aqueous solution and added to the algal nutrient medium and the solution for *Daphnia magna* cultivation in such volumes to achieve final concentrations of acrylamide of 10, 25, 50, 75, and 100 mg L^{-1}. In the nutrient medium for *Lemna minor*, the same stock solution was added in order to achieve the following concentrations of acrylamide: 10, 25, 50, 75, 100, and 150 mg L^{-1}.

Algae *Pseudokirchneriella subcapitata* (Korshikov) Hindak, CCAP 278/4, formerly called *Selenastrum capricornutum* (30), and *Desmodesmus subspicatus* (Chod.) Hegew. & Schmidt, CCAP 276/22, formerly called *Scenedesmus subspicatus* (31), were obtained from the Culture Centre of Algae and Protozoa (Ambleside, Cumbria, UK). Algae used as stock cultures were cultivated in chamber conditions (23±2 °C) under the 138 μE m^{-2} s^{-1} light intensity; 16/8 h light/dark cycle, provided by cool-white fluorescent bulbs. The experiment was conducted according to the ISO 8692:2012 standard (27). Six replicates per treatment and six replicates for controls were prepared and incubated for three days (72 h) at 23±2 °C under a shaking procedure of 110 rad min^{-1} (Innova 4340, New Brunswick Scientific, New Jersey, USA), exposed to an overhead light of an intensity of 138 μM m^{-2} s^{-1} to ensure exponential algal growth. Every 24 h, the algal density was quantified using spectrophotometric measurements at 750 nm (Lambda 14P, Perkin Elmer, Norwalk, Connecticut, USA). The pH value in all the samples was measured at the beginning and end of the experiment using a glass electrode (pH 526 WTW, Weilheim, Germany).

Growth inhibition (I) was determined using the following equation:

$$I = \frac{B_c - B_n}{B_c - B_0} \times 100$$

where B_0 is the biomass of the control at the beginning of the test, B_c is the biomass of the control at the end of the test, and B_n is the biomass in the treated samples at the end of the test.

Lemna minor L. was taken from a laboratory stock culture originally collected in the Botanical Garden of the Faculty of Science, University of Zagreb and sterilised according to Krajnčič and Devidé (32). For the long term cultivation of duckweed, Pirson-Seidel's nutrient solution was used (33). The stock cultures were grown in 300 ml Erlenmeyer flasks in chamber conditions (temperature 24±2 ºC, 16/8 light/dark cycle and fluorescent light of 90 μ$Es^{-1}m^{-2}$ (TEŽ, Zagreb). The experiment was conducted after two weeks of precultivation of plants on Steinberg nutrient medium (in 300 mL Erlenmeyer flasks), according to ISO 20079:2005 standard (28). Individual colonies with two to three fronds were taken and transferred to Steinberg medium supplemented with acrylamide at the above mentioned concentrations. Each treatment culture and control was prepared in eight replicas. The test was carried out for seven days (168 h) in the climatic test exposure

cabinet, calibrated at a temperature of 24±2 °C with a neutral light intensity of 85 μE s^{-1} m^{-2}.

The duckweed growth rate (r) was calculated on the basis of the fronds number according to the equation:

$$r = \frac{\ln(FN_{t2}) - \ln(FN_{t1})}{t_2 - t_1}$$

where r is the growth rate per day, FN_{t1} is the number of fronds on day t_1, FN_{t2} is the number of fronds on day t_2, $t_2 - t_1$ is the time period between FN_{t2} and FN_{t1}, expressed in days.

The percent inhibition of growth rate was determined using the following equation:

$$ir = \frac{rc - rt}{rc} \times 100$$

where ir is the inhibition of the average growth rate (%), rc is the average growth rate of the control (%), and rt is the average growth rate of the treatment groups (%).

Experimental water flea *Daphnia magna* Straus were obtained from the "Ruđer Bošković" Institute, Zagreb and cultured in the laboratory for more than three generations. The Daphnia tests were conducted following the ISO 6341:2012 standard (29) using water fleas younger than 24 h. The concentrations of acrylamide solution were 10, 25, 50, 75, and 100 mg L^{-1}. The test was run at temperatures of 20±2 °C in the dark. Immobility was estimated after 48 h.

Concentrations causing 50 % growth inhibition (EC_{50}) were determined and used as toxic end points (Table 1). EC_{50} values with 95 % confidence limits were estimated by the linear regression of the probit of percentage inhibition on the log dose of acrylamide. Triplicate measurements were made and all the results are presented as mean ± standard deviation (S.D.). After data processing, EC_{50} values were translated into toxic units (TU) according to the formula: $TU=[1/EC_{50}]\times100$ (34). Due to the lack of a standard classification system to express the degree of toxic hazard, the arbitrary (log) toxicity scale was used, which ranks toxicity in five classes: Class 0 (nontoxic) TU=0; Class 1 (slightly toxic) TU<1; Class 2 (toxic) TU=1-10; Class 3 (very toxic) TU=11-100 and Class 4 (extremely toxic) TU>100 (34).

RESULTS

EC_{50} values

The growth inhibition curves for *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata* at different concentrations of acrylamide showed that acrylamide was more toxic for *Desmodesmus subspicatus* than for *Pseudokirchneriella subcapitata* (Figure 1a and b). The dose-response curve of immobilisation of *Daphnia magna* at different concentrations of acrylamide showed that acrylamide was more toxic for *Daphnia magna* than for *Lemna minor* (Figure 1c and d).

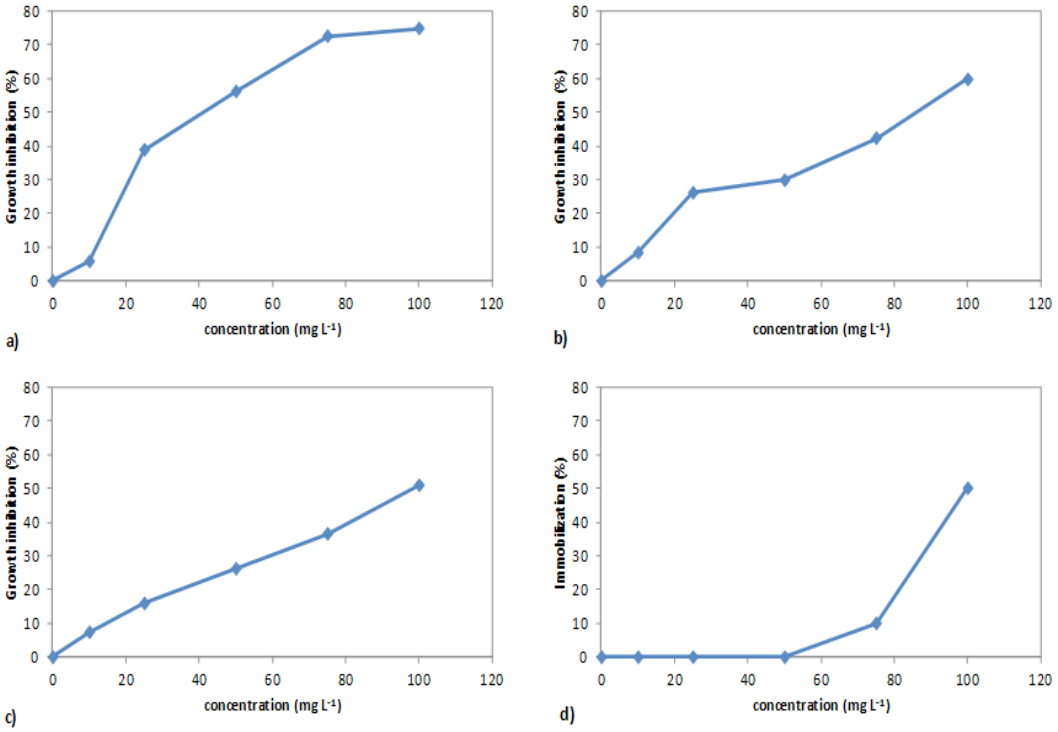

Figure 1 *Growth inhibition curves for: a) freshwater green algae* Desmodesmus subspicatus, *b) freshwater green algae* Pseudokirchneriella subcapitata, *c) duckweed* Lemna minor, *and d) dose-response curve of immobilisation of water flea* Daphnia magna *treated with different concentrations of acrylamide*

The EC_{50} values (±S.D.) ranged from 39.8±0.261 mg L^{-1} to 142±2.944 mg L^{-1} and this indicates that *Desmodesmus subspicatus* is the most sensitive (39.8±0.216 mg L^{-1}), while *Lemna minor* (142±2.944 mg L^{-1}) is the most tolerant species to acrylamide (Table 1).

Toxicity assessment of acrylamide using the TU approach

Using the TU approach, the degree of acrylamide toxicity for all four test organisms was determined (Table 1). For *Lemna minor,* the estimated value was 0.704, and for *Daphnia magna* 1.000, which showed slight acrylamide toxicity (TU=0-1; Class 1). Acrylamide toxicity for the algae was ranked Class 2 (TU=1-10, toxic), due to the values determined for *Desmodesmus subspicatus* (2.513) and *Pseudokirchneriella subcapitata* (1.225).

DISCUSSION

The harmful effects of monomer acrylamide on freshwater organisms has already been confirmed but the results were not always comparable because toxicity was not estimated using the same method. This work focuses on the tests conducted on freshwater green algae *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata*, duckweed *Lemna minor* and water flea *Daphnia magna* according to the standardised methods ISO 8692:2012, ISO 20079:2005, and ISO 6341:2012 in order to ensure the comparability of results and to evaluate the toxicity of acrylamide in organisms with different levels of organisation. The fact that ISO developed standards specifically for environmental samples has been the decisive factor for selecting these tests. Also, the inhibition parameters and effective concentration values (EC_{50}) in ISO standards are now based on growth/inhibition rates (35) allowing a reliable comparison of toxicity between the tests with different species from diverse taxa (36-38). Therefore, the emphasis is placed on the evaluation of a battery of ISO standardised biological tests in the monitoring of the pollution and contamination of aquatic ecosystems caused by acrylamide.

To express the degree of toxic hazard caused by acrylamide, the degree of toxicity (expressed by TUs) for all four test organisms was determined (34). The toxic effect of acrylamide was confirmed in all of the test organisms included in this work but their sensitivities differed depending on the degree of organization. For *Desmodesmus subspicatus,* the TU value was 2.513, and for *Pseudokirchneriella subcapitata* it was 1.225. Due to these results, acrylamide can be classified as Class 2. On the other hand, acrylamide showed slight toxicity (Class 1) for *Daphnia magna,* where a TU value of 1.000 was determined, and for *Lemna minor*, which proved to be the most tolerant species (TU=0.704). The results indicate that acrylamide is differentially toxic for the tested species. *Lemna minor* and *Daphnia magna* (Class 1) appear to be relatively tolerant to acrylamide exposure compared to both green algae.

Comparing the EC_{50} values of the test organisms, the algae proved to be the most sensitive and plant *Lemna minor* the most tolerant species. Algae are commonly used to assess the harmful effects of industrial chemicals on the environment (39). In this study, the EC_{50} value for *Pseudokirchneriella subcapitata* was 81.6 mg L^{-1}, which is comparable with the literature data on the toxicity tests conducted on marine algae *Sceletonema costatum* (90 mg L^{-1}) reported by Weideborg et al. (10). The results of other studies (20, 40) were not comparable because the determined EC_{50} value was 33.8 mg L^{-1} and the test was performed according to the OECD 201 Guidelines (41) and EEC Directive 92/69 Method C.3 (42). Furthermore, in this work EC_{50} for algae *Desmodesmus subspicatus* was determined for the first time. Comparing the EC_{50} data obtained from the two algal bioassays included in this work, remarkable differences were observed (81.6 mg L^{-1} for *Pseudokirchneriella subcapitata* and 39.8 mg L^{-1} for *Desmodesmus subspicatus*) leading to the conclusion that these two algal species vary in their sensitivity to acrylamide.

Based on the inhibition of the plant growth rate, the results of this work revealed that the EC_{50} value for plant *Lemna minor* was the highest (142 mg L^{-1}). According to the EC_{50} value, *Lemna minor* proved to be a much more tolerant species than the algae. The lower susceptibility to acrylamide could be explained by the differences in morphology and physiology between plants and algae. It is supposed that part of the acrylamide monomer is retained

Table 1 EC_{50} values (±S.D.) and TU values for the effect of acrylamide on algae *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata*, duckweed *Lemna minor* and water flea *Daphnia magna*

Test organism	EC_{50}±S.D. (mg L^{-1})	TU
Desmodesmus subspicatus	39.8±0.216	2.513 (Class 2)
Pseudokirchneriella subcapitata	81.6±0.249	1.225 (Class 2)
Lemna minor	142±2.944	0.704 (Class 1)
Daphnia magna	100±2.494	1.000 (Class 1)

Class 0 (nontoxic) TU=0; Class 1 (slightly toxic) TU=0-1; Class 2 (toxic) TU=1-10; Class 3 (very toxic) TU=11-100 and Class 4 (extremely toxic) TU>100

on the lower epidermis of *Lemna minor* fronds in the area of the cell wall or it can also accumulate in the cell vacuoles. There are reports suggesting that duckweed plants can tolerate some environmental pollutants and have a capacity for adaptation to a local enrichment of anthropogenic chemicals (43-46).

For water flea *Daphnia magna*, the estimated EC_{50} value was 100 mg L^{-1}, which is comparable with the previously published research of acrylamide toxicity, where EC_{50} was 98 mg L^{-1} (47). Some other acrylamide aquatic toxicity studies reported higher LC_{50} values of up to 160 mg L^{-1} (23).

The toxicity evaluation on other freshwater organisms, for example bluegill sunfish (*Lepomis macrochirus*) and rainbow trout (*Oncorhynchus mykiss*), determined the LC_{50} value of 100 mg L^{-1} and 96 mg L^{-1}, respectively (22, 23). Aquatic toxicity tests were conducted on marine organisms as well. The toxicity tests with mysid shrimp *Mysidopsis bahia* showed a very similar sensitivity to the test with *Daphnia magna*, and the toxicity test determined the LC_{50} lowest value of 78 mg L^{-1} (21). For crustacean *Acartia tonsa*, the LC_{50} value of 72 mg L^{-1} was reported (10). Due to the fact that some chemicals are highly toxic to both paramecia and microalgae, some protozoa have already been used for the evaluation of environmental contaminations. Takahashi (48) proposed green paramecium *Paramecium bursaria* as a convenient and sensitive bioindicator for the evaluation of environmental acrylamide toxicity. It should be noted that one cell paramecium possesses several hundred endosymbiotic green algae which are morphologically very similar to the algae of genus *Chlorella*. To determine the effect of acrylamide, the test has been conducted for one, three, five, and seven days with various concentrations (0 - 15.000 mg L^{-1}). It has been reported that the IC_{50} values were 7.8 mg L^{-1} for endosymbiotic algae and 120 mg L^{-1} for *Paramecium bursaria* (48).

CONCLUSION

Data from this work indicate differences in the sensitivities to acrylamide between species since it was slightly toxic to aquatic plant *Lemna minor* and water flea *Daphnia mangna* (TU=0-1; Class 1) and toxic to planktonic green algae *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata* (TU=1-10; Class 2). This work confirms that freshwater organisms are suitable bioindicators that represent an irreplaceable tool for ecological research. The new EU regulation on chemicals (13) promotes the methods for the hazard assessment of substances in order to reduce the number of tests on animals (e.g. aquatic toxicity on *Daphnia* and algae). Due to this recommendation, the results of this work could be a basis for the proposal of a standardised routine and a convenient bioassay system for monitoring acrylamide toxicity in the environment. The results obtained in this research could also contribute to the improvement of data required by certain environmental policies, monitoring systems, and environmental information services (database), which are designed to monitor the global effect of industrial activities on health (toxicity) and the environment.

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Procjena toksičnosti akrilamida pomoću standardiziranih biotestova

Akrilamid je monomer koji se koristi kao intermedijer u proizvodnji organskih kemikalija, npr. poliakrilamida (PAM-a). PAM se primjenjuje u različitim industrijama te u obradi otpadnih voda i tretmanu vode za piće, a prihvatljiv je i zbog niske cijene. Široka upotreba PAM-a u spomenute svrhe predstavlja rizik od toga da određena količina nepolimeriziranog akrilamida dospije u vodene ekosustave. PAM je netoksičan, ali akrilamid ima neurotoksične učinke u ljudi te je također dokazana njegova genotoksičnost, kancerogenost i štetan utjecaj na reprodukcijski sustav u laboratorijskih životinja. U cilju procjene toksičnosti akrilamida provedeni su biotestovi na četirima vrstama slatkovodnih organizama: na zelenim algama *Desmodesmus subspicatus* i *Pseudokirchneriella subcapitata*, vodenoj leći *Lemna minor* i vodenbuhi *Daphnia magna*. Biotestovi su provedeni prema standardiziranim ISO metodama, što osigurava procjenu toksičnosti akrilamida na organizme različitog stupnja organizacije, usporedivost rezultata i procjenu pogodnosti korištenja niza standardiziranih biotestova prihvatljive cijene za praćenje zagađenja i onečišćenja vodnih ekosustava. Rezultati su pokazali da su EC_{50} vrijednosti niže nakon izlaganja zelenih algi *Desmodesmus subspicatus* i *Pseudokirchneriella subcapitata* akrilamidu u odnosu na vrijednosti dobivene za vodenu leću *Lemna minor* i vodenbuhu *Daphnia magna*, što pokazuje veću osjetljivost algi. S obzirom na klasifikacijski sustav za izražavanje stupnja toksičnosti, a na temelju vrijednosti utvrđenih za vodenu leću *Lemna minor* i vodenbuhu *Daphnia magna*, akrilamid se može klasificirati kao slabo toksičan (TU=0-1; razred 1). Rezultati dobiveni testovima na algama (*Desmodesmus subspicatus* i *Pseudokirchneriella subspicata*) dokazuju toksičan učinak akrilamida na tim organizmima (TU=1-10; razred 2).

KLJUČNE RIJEČI: *"baterija" bioloških testova;* Daphnia magna; Desmodesmus subspicatus; *ISO standardi;* Lemna minor; *poliakrilamid;* Pseudokirchneriella subcapitata