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The application of the *Lymnaea stagnalis* embryo-test in the toxicity bioindication of surfactants in fresh waters

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ABSTRACT

The aim of this study was to assess the efficiency of the new acute toxicity bioassay with the application of embryological criteria, using aquatic invertebrates such as *Lymnaea stagnalis* L. We were looking for optimal methods of water bio-monitoring, comparing the sensitivity of different biotests. Different forms of snails (embryonic and juvenile) were tested and the tests compared to each other and to the daphnia (EN ISO 6341) test as well. The tested substances were surfactants, which are now regarded global threat to surface waters. The main source of detergent pollution is municipal and industrial wastewater. The tested groups were exposed to various concentrations of diluted surfactant agents known as Brij 32, Brij 58, Bri 72, Brij 76, Brij 78 and the detergent known under the trade name of Ludwik. The results proved that juvenile forms showed higher sensitivity to the tested toxicants. Therefore, they could be used as a potential tool to monitor the acute toxicity of surfactants, which could be presence in aquatic ecosystems.

1. Introduction

According to the International Organization for Standardization (ISO) – water monitoring is a planned process of collecting samples and data, followed by measuring and subsequently recording and signalling different degrees of water quality (Bartram and Ballance, 1996). Chemical and physical methods of surface water monitoring are not sufficient for the comprehensive assessment of actual quality of aquatic ecosystems because they do not take into account the accumulation of toxins in food chains, changing their chemical structure and nature as a result of metabolic processes and synergistic effects of some xenobiotics (Dominik et al., 1999, 2000). Thus biological methods are needed to supplement physical and chemical methods of biomonitoring.

Organisms, to be classified as bioindicators, have to meet some basic criteria, known as 5R criteria: relevant, reliable, robust, responsive and reproducible (Walker et al., 2002). Apart from the above criteria, the organisms should be in good health condition, with no parasites or disease symptoms. They should be characterized by a wide spectrum of sensitivity to different substances (Rand, 1995). The same authors also recommended narrow genetic diversity, like in case of any laboratory animals. APHA require that the breeding of laboratory organisms and preparation of bioassays is not very expensive, so that bioassays are more available and can be widely applied in industry (APHA, 1995; Laskowski, 2010). Organisms meeting the above requirements can also be used as bioindicators for a specific group of toxic compounds as indicators attributed to one particular type of factors (Mazur, 2008).

The aim of the study was to find a new sensitive biotest for the toxicity of detergents (surfactants) and compare it with a standard biotest. The proposed new biotest used early developmental stages of the great pond snail *Lymnaea stagnalis* L. The standard test used for comparison was the test on *Daphnia magna* Strauss. The reason for focussing on detergents is the fact that they make serious environmental problem by deteriorating the oxygen balance in waters, and, consequently disturbing self-purification processes (Chełmicki, 2001, p. 108; Dojlido, 1995, Yamane et al., 1984; Chawla et al., 1987; Warne and Schifko, 1999a,b; Pettersson et al., 2000; Aizdaicher and Markina, 2006; Markina and Aizdaicher, 2007; Sanchez-Fortun et al., 2008; Markina, 2010; Azizullah et al., 2011). Surfactants also prolong the time when heavy metals stay in waters, increase their solubility (Miller, 1995; Desai and Banat, 1997) and thus can increase their bioaccumulation. In higher concentrations detergents directly influence water organisms, e.g. the gills of fish (Lal et al., 1984; Sandbacka et al., 2000). The products of detergent decomposition often contribute to the eutrophica-

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tion of surface waters. Even though the inflow of surfactants with wastewater is limited by constructing more modern wastewater treatment stations, leachates from landfills still make a great problem. In the rivers located near the landfills, the concentration of detergents reached 7.7 mg/l, (Dojlido, 1995) and in ground waters near these objects their concentration was 0.25 mg/l (Prygiel and Haury, 2006). Thus, it is important to study the toxic effects of these agents and to monitor their risk to humans and the environment.

The use of early developmental stages in our studies is based on the fact that embryonic and juvenile forms clearly show greater sensitivity of early developmental stages to low doses of toxins present in freshwater (Dobrowolski, 1976; Vaughan and van Egmond, 2010). This phenomenon can be explained based on the information theory, which explains the impact of information noise (coming from the external environment) to changes in the genetic material (Dobrowoslki and Tadeusiewicz, 1986). Finally, the effects of these changes on the phenotypic expression in developing organisms are observed. The most critical developmental stages are not the earliest ones, because at early stages there is an excess of genetic material in the relation to the current needs of the developing embryo. Embryological and juvenile criteria, first introduced to Polish literature by Dobrowolski (Dobrowolski, 1976), start playing more and more important role in the bioindication (Mazur and Lewicki, 2008).

By developing new methods of toxicity studies, such as new sensitive bioassays we can contribute to a comprehensive evaluation of the quality of aquatic environment (Łebkowska et al., 2004; Dach et al., 2012). A special opportunity in this field is provided by the development of experimental embryology and its use in environmental biotechnology for the development and protection of human health (Mazur, 2004; Mazur, 2006). It is possible to predict long term ecological effects of toxic chemicals (i.e. demographic responses) by the application of short-term sublethal and acute toxicity assays (Allen et al., 1995; Barata and Baird, 2000; Mc William and Baird, 2002; Barata et al., 2007, 2008).

2. Methods and materials

The Probit Method was used as a parametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Finney, 1978). The main requirements: to calculate the LC. The STATISTICA software package was applied to analyze the data. The applied test was ANOVA50, two or more of the observed proportion mortalities must be between zero and one.

2.1. The characteristicsof L. stagnalis (L.)

Juvenile organisms and embryos in cocoon of *L. stagnalis* were studied to investigate the impact of selected surfactants. The great pond snail *L. stagnalis* (L.) fulfils the criteria required for good bioindicators. It is a widespread species with no major species-specific threats. It is easy to collect and breed. This species inhabits slow or stagnant waters, such as the edges of ponds, streams, lakes. They prefer muddy sand or crushed stone bottom, and feed on algae, aquatic macrophytes and dead organic matter such as the remains of other gastropods. The studies on the specimens from different locations (A. Wagner, unpublished) showed no significant difference in the reaction to toxicants.

Selected species meets the criteria necessary for choosing it as the proper bioindicator for the development of bioassay using juvenile and embryological criteria. The designed biotest can be categorized as an embryo assay. The degree of sensitivity the tested organisms for impacts used toxicants was compared with the bioassay on *Daphnia magna*.

Toxicity tests were carried out for six detergents: *Brij* 32, *Brij* 58, *Brij* 72, *Brij* 76, *Brij* 78, Ludwik (washing-up liquid).

2.2. Culture techniques and experimental procedures

The laboratory livestock of *L. stagnalis* were kept in the ecotoxicology laboratory facility at the Laboratory of Environmental Biotechnology end Ecology, AGH University of Science and Technology in Krakow. Adult's great pond snails were maintained in covered rectangular 21-l aquariums. Water temperature ranged from 22 to 18 °C. Each tank was supplied with continuously aerated de-chlorinated city water filtered through active carbon beds. L. The water in tanks was adjusted to a pH of 7.0. All the parameters such as pH, temperature and light period were controlled.

Feeding: the snails were fed with green lettuce leafs every 3 days. Livestock tanks were cleaned by siphoning every week to remove faeces.

All the snails kept in aquariums were characterized by high reproductive potential. Mature individuals laid they cocoons with eggs, attached to the walls of the aquarium, so that it was necessary to provide additional rectangular glass to facilitate obtaining the biological material for testing.

The average number of eggs deposited per female in cocoon was 70 ± 30.

All the embryos in cocoons selected for tests were in good life condition, the choice was preceded by microscopic evaluation of kinetic parameters. The unfertilized eggs or those with immobilized embryos were removed.

2.3. Toxicity tests on L. stagnalis

Embryo-toxicity test: pond snail egg mass were collected and cocoons envelopes were initially damaged due to improve the solution migration inside, but no egg inside was destroyed.

Juvenile-toxicity test (after hatching): juveniles were collected and immersed in the prepared test solutions in plastic containers 100 ml volumes, 32 individuals to each container.

The toxicity test algorithm was similar as in case of *Daphnia magna*.

Tests with the egg mass show that the mortality effect of embryonic forms increases after longer time than in the biotest with *Daphnia magna*, and the duration of development is much longer than in the control. For this reason the cocoon envelopes were initially damaged to increase the speed detergent molecules penetration into egg with embryos. Tests were conducted in 5 replications for each toxicant.

2.4. Toxicity tests on Daphniamagna

The *Daphnia* test was performed to compare the results of the tests on *L. stagnalis* with the results of a standard test, according to OECD standard protocol for *Daphnia magna* Straus, no. – ISO 341: 1996 Water quality, EN ISO 6341 (acute toxicity tests). Daphnia were cultured in glass aquariums containing 2l of aerated, reconstituted, moderately hard water. The colony was maintained in a 21–23 °C room with a photoperiod of 12 h light/10 h dark. Each day the adult Daphnia were fed with 10 ml of *Scenedesmus* sp. algae. The neonates were separated from adults according to their size (<1.5 mm) by sieving.

The tests were conducted in 120 ml polystyrene containers, with 100 ml liquid in each. Thirty two neonates were transferred to containers containing different concentrations of the test detergents, and the containers were closed with a cap. There was no feeding and no aeration during the tests. Immobilization was determined visually after 24 h. For each toxicant, controls and ten concentrations were used for the determination of EC_{50} and the experiments were