**Artemia salina** acute immobilization test: a possible tool for aquatic ecotoxicity assessment

Gabriela Kalčíková, Jana Zagorc-Končan and Andreja Žgajnar Gotvajn

**ABSTRACT**

Despite the fact that the marine crustacean *Artemia salina* is extensively used in ecotoxicology, there is still a lack of information about its sensitivity to commonly used chemicals. In the presented study, acute toxicity of 18 commonly used chemicals – including organic solvents, industrial chemicals, metals and inorganic compounds – to *A. salina* was evaluated. *A. salina* showed a range of sensitivities to tested chemicals. Regarding all of the investigated organics, phenolic compounds expressed the highest toxicity to *A. salina*. Nitrite and mercury were the most toxic inorganic substances applied in the study. On the other hand, dimethyl sulfoxide, nitrate and ammonium were the least toxic. The possibility to use *A. salina* for interspecies correlation was assessed by comparison of sensitivities of different organisms (bacteria, fish, crustacean) to organic compounds. Correlation between various species was observed, especially between *A. salina* and fish. Due to the strong relation between toxicity and the logarithm of the octanol/water partition coefficient log P_{OW}, lipophilicity was found to be the main factor influencing toxicity of the chosen organic compounds. No significant correlation between toxicity to *A. salina* and physico-chemical parameters of metals was observed.

**Key words** | *Artemia salina*, ecotoxicology, interspecies correlation, toxicity test

**INTRODUCTION**

The aquatic environment is continuously endangered by many industrial and domestic activities. Aquatic contamination has been for a long time evaluated only by specific chemical analyses, being a source of information about the quantitative and qualitative composition of contaminants. But many years of experience have shown that such approach is not always sufficient. The use of biological methods for evaluation of the aquatic contamination is an important alternative (Martins et al. 2007). Biological tests integrate the effect of all contaminants including their additive, synergistic and antagonistic effects. They also provide valuable information on the extent of a bioavailable fraction of the contaminants, and they integrate effects of all substances, even those that were not considered or detected by chemical analyses – micropollutants (Pandard et al. 2006; Wolska et al. 2007).

For effective protection of the environment it is necessary to use a battery of biotests containing organisms representing different trophic levels of an ecosystem. *Artemia salina*, an aquatic organism which is sometimes used in toxicity assessment, is popularly known as brine shrimp. *Artemia* have a wide geographical distribution and inhabit saline environments, such as permanent salt lakes and coastal lagoons, which are too extreme for many species which readily prey on *Artemia*, if opportunity offers. They are physiologically able to tolerate large changes in salinity, ionic composition, temperature, and oxygen tension, and they are able to adapt to changeable nutrient resources as they are non-selective filter feeders (Abatyopoulos & Beardmore 2002; Nunes et al. 2006).

In spite of several criticisms against the use of *Artemia* as a test organism in standardized bioassays (absence in most marine ecosystems, low sensitivity to chemical exposure due to the intrinsic resistance to extreme salinity conditions), *A. salina* has recently gained popularity as an organism used in ecotoxicology. The reasons for its popularity are its availability throughout the year, easy culturing (hatching from eggs gives organisms of similar age, genotype and physiological condition), short life cycle, resistance to manipulation, wide geographic
distribution and simplicity and cost-effectiveness of performed tests (Barahona & Sánchez-Fortún 1999; Nunes et al. 2006). Artemia has been employed to assay toxicity of a large number of different contaminants, such as carbamates (Barahona & Sánchez-Fortún 1999), antifouling biocides (Koutsafis & Aoyama 2007), agricultural antibiotics (Migliore et al. 1997), pesticides (Varó et al. 2006) and metals (Hadjispyrou et al. 2001). Artemia was included in a battery of biotests used for assessment of landfill leachate toxicity (Bortolotto et al. 2009) and for evaluation of toxicity of phenolic compounds in industrial effluent (Guerra 2001). Cysts of Artemia were used for evaluation of embryotoxic effects of alcohols (Vismara ef al. 2013) and for evaluation of toxicity of phenolic compounds in industrial effluent (Bortolotto et al. 2009) and for evaluation of toxicity of phenolic compounds in industrial effluent (Guerra 2001). Cysts of Artemia were used for evaluation of embryotoxic effects of alcohols (Vismara et al. 2013), and Brix et al. (2003) demonstrate that a chronic toxicity test with brine shrimp can be successfully performed.

In recent years, the relationship between physico-chemical properties and toxicity of different organic and inorganic compounds, as well as interspecies correlation between several organisms, has been intensively studied. The main goal of such investigations was to apply obtained data for prediction of toxicity of different chemicals to organisms and to reduce excessive testing on vertebrates. Organisms such as fish, protozoans, fresh water crustaceans, bacteria and plants were used for development of quantitative structure-activity relationship (QSAR) (Shultz et al. 1989; Wang et al. 2002; Parvez et al. 2008; Khangarot & Das 2009). However, A. salina has not been used so far, although it offers many advantages. A. salina is one of the few crustaceans that can be stored for a long time in the form of dormant eggs (cysts), and thus the difficult culturing of organisms does not take place. Other stand-by toxicity tests, e.g. luminescent toxicity test using freeze-dried bacteria or phytotoxicity test using seeds of different plants, cannot be always applied, especially if toxicity of chemicals with specific effects on animals (e.g. inhibition of acetylcholinesterase) is investigated. In such cases, Artemia could be an appropriate organism for prediction of toxicity of chemicals and for interspecies relationships.

The aim of our study was to acquire new information about sensitivity of A. salina to different commonly used chemicals, because of the lack of current information in this area. Obtained sensitivities of A. salina and other organisms used for ecotoxicological studies (bacteria, fish, crustacean) were then compared and the relationship between their responses was evaluated. We tried to correlate the relationship between toxicity and physico-chemical parameters of tested chemicals as well.

**METHODS**

**Toxicity test**

The method has been performed according to a modified ARS-test procedure (Persoon et al. 1984). Artificial sea water with a pH of 7.8 ± 0.2 and concentration of dissolved oxygen of 8.5 ± 0.5 mg L⁻¹ was used for hatching as well as a dilution medium for preparing different concentrations of tested chemicals. A measure of 0.1 g of A. salina cysts was incubated in a flask containing 300 mL of artificial seawater, which was heated up to 30 °C and gently aerated and illuminated by a 15 W fluorescent lamp for 24 h. After incubation, the first instar nauplii were transferred with a Pasteur pipette into a beaker containing 250 mL of artificial sea water. Some of the first instar Artemia nauplii were immediately used for evaluation of sensitivity to K₂Cr₂O₇. The remaining nauplii were kept for another 24 h in the beaker. After that time, the second and third instar Artemia nauplii were harvested and also used for evaluation of sensitivity to K₂Cr₂O₇. More sensitive instar Artemia nauplii (the second and the third) were used for toxicity testing. In each test, 10 larvae were placed in small glass Petri dishes (diameter = 9 cm) containing 25 mL of different concentrations of tested chemicals. The test was performed at 25 °C in the dark with 24 h of incubation. Nauplii were considered immobilized if no movement was observed in 10 seconds after gently moving the Petri dishes. Definitive tests were carried out twice, and immobilization of the organisms in the control sample (artificial sea water) with no added chemicals was always 0%.

**Test chemicals**

Chemicals were selected to include as many commonly used compounds as possible: organic solvents (methanol, ethanol, acetone, acetonitrile, dimethylsulfoxide – DMSO), industrial chemicals (phenol, formaldehyde, bisphenol A – BPA), compounds containing inorganic nitrogen (NaNO₃, NaNO₂, NH₄Cl) and metals (HgCl₂, CoCl₂,6H₂O, Cd(NO₃)₂.4H₂O, ZnCl₂, NiCl₂,6H₂O) and compounds used as a reference toxicants (K₂Cr₂O₇, 3,5-dichlorophenol – 3,5-DCF). Tested chemicals were obtained from different producers (Sigma-Aldrich, Merck) in the p.a. purity. All of the tested compounds, except for BPA, were dissolved in artificial sea water. The solvent used to solubilize BPA was ethanol in a concentration non-toxic to A. salina nauplii (0.1 v/v %).
Statistical analysis

Toxicity of tested chemicals to *A. salina* was expressed as 24 h EC$_{50}$ values (with 95% confidence limit) in mg L$^{-1}$. The percentages of immobilized organisms were analyzed by probit analysis (Finney 1972). Linear regression used for interspecies correlation and standard deviation (SD) were calculated by Excel software (Microsoft).

RESULTS AND DISCUSSION

For *Artemia* nauplii tested immediately after hatching (first instar) the mean 24 h EC$_{50}$ of K$_2$Cr$_2$O$_7$ was 39.7 mg L$^{-1}$ ($n$=8) with SD=10.2 mg L$^{-1}$. For *Artemia* nauplii which were tested 24 h after hatching (the second and third instar) the mean 24 h EC$_{50}$ of K$_2$Cr$_2$O$_7$ was lower (27.9 mg L$^{-1}$ ($n$=8)), and SD=5.1 mg L$^{-1}$ showed also the lower variability of the test. The test using the second and third instar of *Artemia* nauplii was assessed as more sensitive with a low variability, and therefore was used for other toxicity testing.

Results from the toxicity testing of different chemicals to *A. salina* are presented in Table 1. *A. salina* showed a range of sensitivities to tested chemicals. Acetonitrile was the most toxic of the organic solvents, almost 10 times more toxic than the least toxic solvent DMSO (8,463 compared with 76,450 mg L$^{-1}$). Due to the low toxicity, DMSO may be an appropriate carrier solvent for tests with *A. salina*. Phenolic compounds, phenol, 3,5-DCF and BPA, showed the highest toxicity from all organic compounds. Very similar toxicity of phenol to *A. salina* (24h LC$_{50}$=17.35 mg L$^{-1}$) was presented in another study (Guerra 2001). *A. salina* seems to be very tolerant to inorganic substances, especially to nitrate and ammonium. The mechanism of ammonium toxicity includes a number of physiological effects (e.g. disruption of enzyme systems and membrane stability, elevation of blood pH); however, toxic effect is usually expressed by greater susceptibility to disease and reduction in growth rather than mortality (Taylor 1996). Low sensitivity of *A. salina* to ammonium contents (ammonium concentration 800 mg L$^{-1}$ gave only 20% immobility of *A. salina*) has been already reported by Svensson et al. (2005), as well as its low sensitivity to metals. On the other hand, higher toxicity of nitrite was noticed. This can be explained by the fact that nitrite, in comparison with ammonium and nitrate, has a different physiological effect on crustaceans. The toxic action of nitrite is a consequence of its oxidation of the respiratory pigment hemocyanin to methemocyanin, which cannot carry oxygen (Taylor 1996). Mercury (inorganic as well as organic form) is one of the metals that are the most toxic to marine organisms (Neff 2004); therefore the highest toxicity of mercury from all tested metals was expected. Zinc, an essential micronutrient, and non-essential cadmium showed similar toxicity to *A. salina*, probably due to several physico-chemical similarities of both metals. Essential zinc is only moderately toxic to some marine organisms, but cadmium is one of the more toxic metals. However, bioavailability and toxicity of cadmium decreased as salinity increased, due to the formation of non-bioavailable complexes; therefore its toxicity to marine organisms can be significantly lower (Elinder & Piscator 1978; Neff 2004). Nickel and cobalt seem to be relatively non-toxic when compared with other metals. However, both have already shown a potential for chronic toxicity to invertebrates in very low concentrations (Wright & Welbourn 2002).

With enormous production of new chemicals it is unfeasible to test toxicity of chemicals to all species. Correlation between the toxicities to different organisms allows us to predict toxicity of a chemical to one species based on the toxicity of the same chemical to other species (Khangarot & Das 2009). Rapid, simple, cost-effective toxicity tests used for interspecies correlation could save a lot of time.

Table 1 | Toxicity of different chemicals to Artemia salina expressed as 24 h EC$_{50}$ (95% confidence limit) in mg L$^{-1}$

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>24 h EC$_{50}$ (mg L$^{-1}$)</th>
<th>95% CL (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>31.039</td>
<td>27.555-34.522</td>
</tr>
<tr>
<td>DMSO</td>
<td>76.450</td>
<td>73.700-79.200</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>8,463</td>
<td>7,878-9,048</td>
</tr>
<tr>
<td>Phenol</td>
<td>25.2</td>
<td>23.7-26.6</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>2,671</td>
<td>1,744-3,597</td>
</tr>
<tr>
<td>BPA</td>
<td>56.1</td>
<td>55.8-56.3</td>
</tr>
<tr>
<td>3,5-DCF</td>
<td>2.02</td>
<td>1.99-2.04</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>33.9</td>
<td>32.6-35.1</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>n.t.</td>
<td>/</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>n.t.</td>
<td>/</td>
</tr>
<tr>
<td>Co</td>
<td>2,815</td>
<td>2,450-3,180</td>
</tr>
<tr>
<td>Cd</td>
<td>457</td>
<td>369-544</td>
</tr>
<tr>
<td>Zn</td>
<td>556</td>
<td>483-628</td>
</tr>
<tr>
<td>Hg</td>
<td>41.7</td>
<td>30.9-52.4</td>
</tr>
<tr>
<td>Ni</td>
<td>1,209</td>
<td>1,102-1,315</td>
</tr>
</tbody>
</table>

n.t. - no toxic effect observed at concentrations higher than 3 g L$^{-1}$.
/  - not determined.
costs and primarily could avoid additional toxicity testing (e.g. fish). The possibility of using A. salina for interspecies correlation was evaluated by comparison of the experimental acute toxicity data of selected organics (methanol, ethanol, acetone, DMSO, 3,5-DCF, phenol) with toxicity data (LC50/EC50) obtained from literature for fish (96 h) (Zagorc-Končan et al. 2002; Martins et al. 2007), luminescent bacteria Vibrio fischeri (5 min) (Jenning et al. 2001) and water flea Daphnia magna (24 h) (Guilhermino et al. 2000; Barbosa et al. 2003; Martins et al. 2007) (Figure 1).

Methanol, ethanol, acetone, DMSO, acetonitrile phenol and 3,5-DCF were selected as model organic compounds due to their widespread applications and availability of toxicity data for all compared organisms. Surprisingly, A. salina showed good correlation with the toxicities of all chosen organisms. Despite the fact that A. salina showed good correlation with the toxicities of all chosen organisms. Despite the fact that A. salina and Danio rerio are very different species, very good correlation between them was observed (R²=0.999). A similar situation has occurred in another study (Silva et al. 2004), which focused on toxicity of landfill leachate evaluated by A. salina, Daphnia similis, V. fischeri and D. rerio. Among all tested organisms only the correlation between A. salina and D. rerio was appreciable (R² = 0.900).

The relationship between physico-chemical properties of tested organics and toxicity to A. salina was also determined. Toxicity of chemicals is a combination of penetration into the organism and interaction between toxicants and target sites. The possibility of penetration into the organism can be assessed by the octanol/water partition coefficient (P_{OW}), and the dissociation of weak acid groups, expressed as pKₐ, can affect the toxicity of chemicals. Mostly due to the presence of nitrogen and other heterogeneous atoms, hydrogen bond interaction can occur (Shultz 1987; Wang et al. 2002). Poor correlation of toxicity with the negative logarithm of the acidity dissociation constant pKₐ (R² = 0.608) and good correlation (R² = 0.904) (Figure 2) with the logarithm of the octanol/water partition coefficient logP_{OW} are evidence that lipophilicity is the main factor influencing toxicity of chosen organic compounds (ethanol, methanol, acetone, DMSO, acetonitrile, phenol, formaldehyde, BPA, 3,5-DCF). Parameter pKₐ has a major effect on solubility and permeability. Ionized molecules are less permeable than neutral molecules, which are much more lipophilic (Kerns & Di 2008). However, organic compounds with properties of weak acids do not dissociate completely in water, and it could be a reason for poor correlation of pKₐ to toxicity, as was observed by another author (Wang et al. 2002). The highest variation of compared results is caused by phenol-based compounds (Figure 2), probably due to their specific mechanism of toxic action.

Different physico-chemical properties of six metal ions tested in this study (Co, Cd, Cr, Ni, Hg, Zn) were also related to results of toxicity tests (Figure 3). Electrochemical potential (ΔE₀), ionization potential (IP), electronegativity (Χₘᵣ), atomic radius (AR), ionic radius (IR) and atomic weight (AW) were chosen as representative parameters of tested metals (Wolterbeek & Verburg 2000). The correlation coefficient’s values ranged from R² = 0.027 to R² = 0.430. No significant correlations between chosen parameters for metals have been observed, and toxicity to A. salina cannot be correlated to properties of metals, indicating poor sensitivity of Artemia to metals.

The presented results showed sensitivity as well as tolerance of A. salina to different chemicals. Very good interspecies correlation between A. salina and other important aquatic organisms used for ecotoxicological testing has been observed. Therefore A. salina could be used as an
appreciable tool for prediction of toxicity to other organisms, especially to fish. The relationship between toxicity and lipophilicity \( \log K_{\text{OW}} \) was also significant; however, for development of QSAR with \( A. \text{salina} \) it is essential to compare more data and to divide the tested chemicals to special groups, as can be seen from the increased variation of phenol-based compounds. \( A. \text{salina} \) does not seem to be a good organism for correlation of toxicity and physicochemical parameters of metals, probably due to its low sensitivity to metals.

**CONCLUSION**

The aim of our study was to assess sensitivity of \( A. \text{salina} \) to commonly used chemicals and to compare obtained results to responses of other organisms used in ecotoxicological studies. \( A. \text{salina} \) showed higher tolerance to many chemicals; however the acute toxicity test using the second and third instar \( Artemia \) nauplii is easy to perform, fast, and is low cost, and comparison of sensitivities of different species showed significant interspecies correlations. This approach could help us to avoid excessive toxicity testing from both ethical and economical perspectives. For particular types of substances a lot of time, work and testing organisms (e.g. fish) could be saved using interspecies correlation.

**ACKNOWLEDGEMENT**

Ms Petra Spurná is acknowledged for her help with English language editing.

**REFERENCES**


bioassays. Environmental Toxicology and Pharmacology 28 (2), 288–293.


Guerra, R. 2001 Ecotoxicological and chemical evaluation of phenolic compounds in industrial effluent. Chemosphere 44 (8), 1737–1747.


First received 14 January 2012; accepted in revised form 12 April 2012