

# A novel protocol for assessing aquatic pollution, based on the feeding inhibition of *Daphnia magna*

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## ABSTRACT

**Key-words:**  
*Daphnia magna*,  
copper,  
whole effluent,  
feeding activity  
inhibition

In this study, sensitivity of a novel acute bioassay based on the feeding activity of *Daphnia magna* was assessed, using 2 and 4 h of exposure. For calibration purposes, results were compared with those of the standard immobility test as described by the ISO 6341:1996 standard. Using potassium dichromate as the reference chemical, after 4 h of exposure the proposed protocol showed similar sensitivity in comparison with the standard, as the  $EC_{50}$  of the immobility test was  $1.093 \pm 0.098 \text{ mg}\cdot\text{L}^{-1}$ , whereas the  $EC_{50}$  of the feeding inhibition bioassay was  $1.742 \pm 0.133 \text{ mg}\cdot\text{L}^{-1}$ . In order to test the sensitivity of the bioassay, toxicity of two other contaminants, copper and wastewater, was estimated and the results were compared with those of the standard immobility test. For both cases, the feeding inhibition test showed higher sensitivity, as in the case of copper the  $EC_{50}$ s were  $0.0952 \pm 0.0087$  and  $0.0753 \pm 0.0152 \text{ mg}\cdot\text{L}^{-1}$ , whilst the  $EC_{50}$  recorded for the 24-h immobility test was  $0.2407 \pm 0.0159 \text{ mg}\cdot\text{L}^{-1}$ . In the case of the effluent,  $EC_{50}$  values after 2 and 4 h of exposure were  $15.698 \pm 2.681$  and  $12.557 \pm 2.358$  expressed as % of the wastewater, respectively, whereas the  $EC_{50}$  of the immobility test was calculated to be  $36.4688 \pm 5.4887$ .

## RÉSUMÉ

Nouveau protocole pour l'évaluation de la pollution aquatique, basé sur l'inhibition de l'alimentation de *Daphnia magna*

**Mots-clés :**  
*Daphnia magna*,  
cuivre,  
effluent total,  
inhibition de  
l'activité  
d'alimentation

Dans cette étude, la sensibilité d'un nouveau test biologique aiguë basé sur l'activité d'alimentation de *Daphnia magna* a été évaluée, utilisant 2 et 4 h d'exposition. À des fins d'étalonnage, les résultats ont été comparés à ceux de l'essai standard d'inhibition de la mobilité tel que décrit par la norme ISO 6341:1996 standard. En utilisant le potassium dichromate comme produit chimique de référence, après 4 h d'exposition, le protocole proposé a montré une sensibilité similaire en comparaison de la norme, alors que la  $EC_{50}$  du test d'inhibition de la mobilité était  $1,093 \pm 0,098 \text{ mg}\cdot\text{L}^{-1}$ , la  $EC_{50}$  de l'essai biologique d'inhibition d'alimentation a été  $1,742 \pm 0,133 \text{ mg}\cdot\text{L}^{-1}$ . Afin de tester la sensibilité du test biologique, la toxicité de deux autres contaminants, du cuivre et des eaux usées, a été estimée et les résultats ont été comparés à ceux du test de l'inhibition de mobilité standard. Pour les deux cas, le test d'inhibition d'alimentation a montré une sensibilité plus élevée. Dans le cas du cuivre, les  $EC_{50}$  ont été  $0,0952 \pm 0,0087$  et  $0,0753 \pm 0,0152 \text{ mg}\cdot\text{L}^{-1}$ , alors que la

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$EC_{50}$  enregistrée pour le test d'inhibition de la mobilité de 24 h était  $0,2407 \pm 0,0159 \text{ mg}\cdot\text{L}^{-1}$ . Dans le cas de l'effluent, les valeurs  $EC_{50}$  après 2 et 4 h d'exposition étaient  $15.698 \pm 2.681$  et  $12.557 \pm 2.358$  exprimés en % des eaux usées, respectivement, alors que la valeur calculée  $EC_{50}$  du test d'inhibition de la mobilité était  $36.4688 \pm 5.4887$ .

## INTRODUCTION

*D. magna* is one of the most widely used test organisms in ecotoxicology (Persoone *et al.*, 2009; Jeon *et al.*, 2010). The ISO 6341:1996 standard uses *Daphnia magna* Straus in an acute toxicity test, where the exposure is 24 and/or 48 h with an endpoint of inhibition of motility, practically mortality. To minimise the differences and uncertainties emerging from the application of various genotypes and to avoid the expensive maintenance of stock cultures, toxkits have been developed such as the Daphtoxkit F<sup>TM</sup> which contains dormant eggs, ephippia, as a source of neonates whenever they are required (Persoone, 1996).

However, while mortality is an ultimate and non-specific endpoint, its relevance to forecasting ecological threat can be questioned. More attempts have been made to specify other ecological effects which can be used as potential test endpoints. Baylor used *Daphnia* heart beat rate tests (Baylor, 1942) as early as 1942, which is still considered an applicable endpoint (Villegas-Navarro *et al.*, 2003). Some authors recorded morphological abnormalities (e.g. Leoni *et al.*, 2008). Wu *et al.* (2008) suggested the phototaxis index (response to the light) of *D. carinata* as an appropriate test endpoint for toxicity testing of environmental contaminants. Recording movement patterns such as average speed, turns and circling movements is relatively widely used (e.g. Untersteiner *et al.*, 2003; Jeon *et al.*, 2008). The IQ Toxicity Test<sup>TM</sup> kit developed by Aqua Survey, Inc. is based on fluorescent tagging of metabolic processes of the test organisms. In this assay daphnids are fed a fluorescent sugar reagent. In the presence of toxins, the metabolism of *Daphnia magna* is reduced, blocking their normally visible light emission. Some authors suggest a close relation between decreased grazing activity and damage of the digestive tract of daphnids caused by some toxicants (Nogueira *et al.*, 2006; Soares *et al.*, 2009). Life-history traits such as somatic (individual) growth (Burks *et al.*, 2000), time to first reproduction, development of parthenogenetic eggs (Sobral *et al.*, 2001; Khangarot and Das, 2009) and number of newborns (Lürling and van der Grinten, 2000) can also be studied. Lürling (2003) used population growth as the measure of toxic effect. These assays provide an alternative approach to studying the ecological response of daphnids other than mortality, yet some of them are difficult to quantify. An assay based on feeding activity of *D. magna* was developed by our team. The test gives quantifiable results and can be applied in routine testing.

Our aim was to assess the sensitivity of this new method, in comparison with the standard immobility test using potassium dichromate as the reference toxicant. Reference values are presented in the ISO 6341 standard, where the immobility test can be considered valid if the 24-h  $EC_{50}$  falls in the  $0.6\text{--}2.1 \text{ mg}\cdot\text{L}^{-1}$  range. In order to test the sensitivity of the novel assay, toxicity of two other contaminants, copper sulphate and wastewater, was estimated and results were compared with those of the standard immobility test.

Copper is a predominant pollutant in aquatic habitats as copper-based compounds have been used as herbicides, algicides and molluscicides. Its environmental levels range from  $0.00004\text{--}0.294 \text{ mg}\cdot\text{L}^{-1}$  (An and Kampbell, 2003), reaching  $20 \text{ mg}\cdot\text{L}^{-1}$  in critical situations (Goodyear and McNeill, 1999). Copper is one of the metals included in the EU Water Framework Directive (WFD) list of priority substances (Banas *et al.*, 2010). The effects of copper have been demonstrated with studies on the inhibition of motility of daphnids (De Schamphelaere *et al.*, 2002; Jonczyk and Gilron, 2005; Pernet-Coudrier *et al.*, 2008; Kungolos *et al.*, 2009; Park *et al.*, 2009; Jeon *et al.*, 2010).

Municipal wastewater is one of the major sources of pollutants introduced into the aquatic environment (Gros *et al.*, 2010). The discharge of municipal wastewater effluents can have

negative effects on the community and population structure of the receiving water body. The whole-effluent toxicity of municipal wastewater treatment plants can be greatly variable because the effluents are a mixture of various chemicals (Mitteregger *et al.*, 2007). To assess whole-effluent toxicity, the *Daphnia* immobility test according to ISO 6341:1996 is generally prescribed by national regulations such as, for example, the 27/2005 Decree of the (Hungarian) Minister for Environmental Affairs and Water Management on the control of discharging used and wastewaters.

## MATERIALS AND METHODS

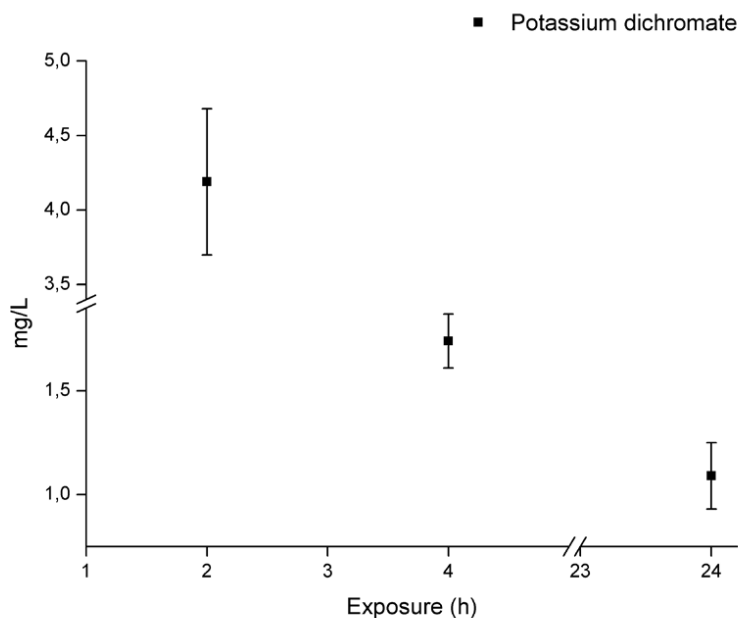
Potassium dichromate (Renal, Rt., Hungary) stock solution ( $1.982 \text{ mg}\cdot\text{L}^{-1}$ ) was used to prepare 5 serial dilutions (0.124, 0.248, 0.495, 0.991 and  $1.982 \text{ mg}\cdot\text{L}^{-1}$ ). Copper sulphate ( $\text{CuSO}_4$ ; anhydrous, Renal, Rt., Hungary) was tested at five concentrations (0.024, 0.049, 0.059, 0.079 and  $0.099 \text{ mg}\cdot\text{L}^{-1}$ , values are presented as Cu). Untreated municipal wastewater was collected from a municipal wastewater treatment plant, and stored at  $+2 \text{ }^\circ\text{C}$  until testing the day following the collection. Five serial dilutions (v:v), using synthetic freshwater (6.25, 12.5, 25, 50 and 100%) were used. The synthetic freshwater (supplied by Microbiotest Inc., Belgium) was used for preparing serial dilutions of the tested pollutants and for the control. For both the immobility test and the feeding inhibition test, Daphtoxkit F<sup>TM</sup> kits (Microbiotest Inc., Belgium) were used as the source of the test organisms. Neonates were hatched from ephippia after 3 days of incubation at  $20 \text{ }^\circ\text{C}$  in synthetic freshwater, under continuous illumination (light source 10 000 lux). The immobility test was carried out according to the protocol described by the ISO 6341:1996 standard. Briefly, daphnids were exposed in the sample at  $20 \pm 1 \text{ }^\circ\text{C}$  in the dark. The number of immobile organisms was recorded after 24 h of exposure. Four replicates were used.

The *D. magna* feeding inhibition toxicity assays were carried out as described by Ács *et al.* (2009). Neonates were exposed to five serial dilutions in addition to the control and incubated at  $20 \text{ }^\circ\text{C}$  in darkness for periods of 2 h and 4 h. Immediately after incubation, 0.2 ml of red polystyrene microsphere suspension with a particle size of  $5 \mu\text{m}$  (MicroBiotest Inc., Ghent, Belgium) were added to each cell. Daphnids were allowed to feed for 30 min at  $20 \text{ }^\circ\text{C}$  in darkness. After incubation, a fixative was added, and the number of daphnids with red microspheres in their digestive tracts was recorded using a stereomicroscope. Again, four replicates were used. The mean percentage of feeding inhibition (*FI*) of particle uptake for each replicate was calculated using the following formula:  $FI = 100 (A-B)/A$ , where *A* is the mean percentage particle uptake in the control, and *B* is the mean percentage particle uptake in the test solution.

The  $EC_{50}$  values were calculated using probit analysis according to Finney (1971). All data ( $EC_{50}$ ) are expressed as mean  $\pm$  SE. For statistical analysis, Welch's ANOVA was used, to discover significant differences between pairwise groups.

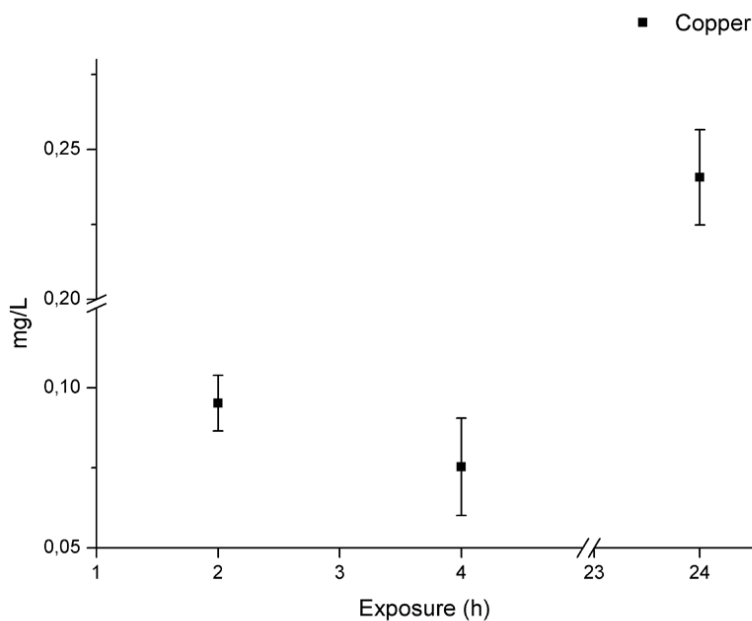
## RESULTS

The data presented in Figure 1 show the potassium dichromate  $EC_{50s}$  for both the immobility and feeding inhibition tests. For the standard immobility test, the 24-h  $EC_{50}$  was  $1.093 \pm 0.098 \text{ mg}\cdot\text{L}^{-1}$ . For the feeding inhibition test, the 2-h  $EC_{50}$  was  $4.190 \pm 0.491$ , which falls outside the range of validity as defined by the ISO 6341 standard. In the case of 4 h of exposure, the  $EC_{50}$  ( $1.742 \pm 0.133$ ) fell within the same range as the standard immobility test. Toxicity of copper determined by the feeding inhibition test was as follows: the 2-h  $EC_{50}$  was  $0.0952 \pm 0.0087 \text{ mg}\cdot\text{L}^{-1}$  and the 4-h  $EC_{50}$  was  $0.0753 \pm 0.0152 \text{ mg}\cdot\text{L}^{-1}$  (Figure 2). In comparison with the  $EC_{50}$  of the standard immobility test,  $0.2407 \pm 0.0159 \text{ mg}\cdot\text{L}^{-1}$ , these results show higher sensitivity. The  $EC_{50s}$  of the 2- or 4-h feeding tests may reflect actual



**Figure 1**

Acute toxicity,  $EC_{50}$  of potassium dichromate ( $mg \cdot L^{-1}$ ) to *Daphnia magna*, using novel feeding inhibition assay test compared with normal mortality test (24 h  $EC_{50}$ ).

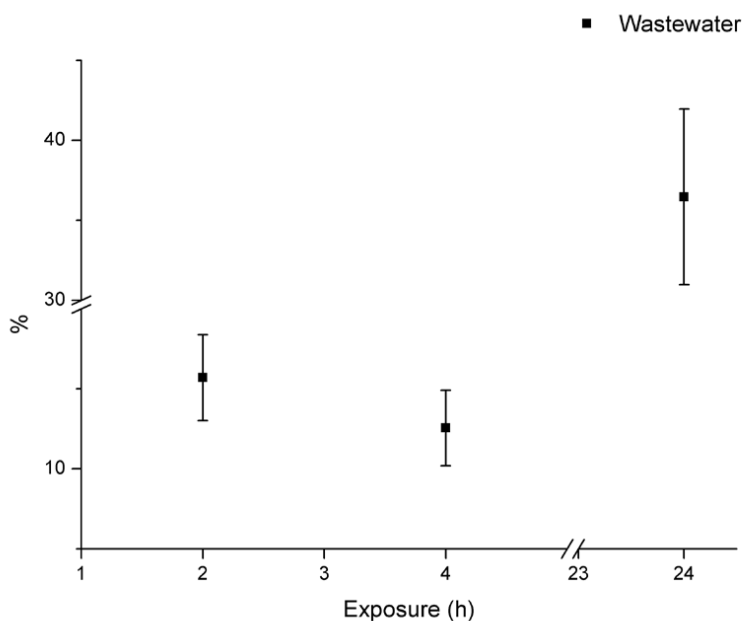


**Figure 2**

Acute toxicity,  $EC_{50}$  of copper ( $mg \cdot L^{-1}$ ) to *Daphnia magna*, using novel feeding inhibition assay test compared with normal mortality test (24 h  $EC_{50}$ ).

environmental concentrations, as Neal *et al.* (1996) reported copper levels of  $0.034 \text{ mg} \cdot \text{L}^{-1}$  in the Rhine River, whilst the environmental quality standard (EQS) is  $0.006\text{--}0.01 \text{ mg} \cdot \text{L}^{-1}$ .

For the whole-effluent sample,  $EC_{50}$  values of the feeding inhibition test after 2 and 4 h of exposure were  $15.698 \pm 2.681$  and  $12.557 \pm 2.358$  expressed as % of the wastewater, respectively (Figure 3). The  $EC_{50}$  of the immobility test was  $36.4688 \pm 5.4887$ , also expressed as % concentration of the effluent.



**Figure 3**

Acute toxicity,  $EC_{50}$  of wastewater (% effluent) to *Daphnia magna*, using novel feeding inhibition assay test compared with normal mortality test (24 h  $EC_{50}$ ).

For statistical analysis, Welch's ANOVA was used. For  $K_2Cr_2O_7$  and for copper, the  $EC_{50}$  was significantly affected by exposure time ( $F_{df1,df2} = 95.531$ ,  $P < 0.0001$ ;  $F_{df1,df2} = 149.433$ ,  $P < 0.0001$ , respectively), and all conditions differed significantly from each other (XX *post-hoc* test,  $\alpha = 0.05$ ). For the wastewater effluent, the effect of exposure time was also significant ( $F_{df1, df2} = 42.244$ ,  $P < 0.0001$ ), yet the difference was significant only between  $EC_{50}$ -24 h on the one hand, and 2h and 4 h, on the other hand.

## DISCUSSION

Feeding behaviour of zooplankton could be considered as an important endpoint in ecotoxicological studies (Kiss *et al.*, 2003; Palanivelu *et al.*, 2005). The inhibition of feeding has been applied by several authors to assess the possible ecotoxicological effects of different contaminants, such as metals (Flickinger *et al.*, 1982; Ferrando and Andreu, 1993) and pesticides (Fernandez-Cassalderrey *et al.*, 1994). Hartgers *et al.* (1999) and Lüring *et al.* (2011) found that the clearance rate of *Daphnia magna* using artificial beads is a more sensitive endpoint than an acute lethal test. Alterations in the feeding and digestive behaviour of daphnids as endpoints were compared by De Coen and Janssen (1998) and De Coen *et al.* (1998), where ingestion rate was assessed using fluorescent microbeads and measuring fluorescence. In these studies, a clear inhibition of the ingestion activity was experienced, while the digestive enzyme activity proved to be less affected.

From an ecological point of view, feeding inhibition can be considered as a relevant endpoint. Also, considering reliability, the proposed protocol shows equal or higher sensitivity than the standard immobility test. In the case of potassium dichromate, the 2-h  $EC_{50}$  showed lower sensitivity than either the 4-h or 24-h mortality  $EC_{50}$ s. However, after longer (4 h) exposure, the feeding inhibition test proved to have similar sensitivity in comparison with the standard immobility test and also gave valid results according to the ISO 6341 standard. It is important to note that, in the case of the other chemicals tested, the novel protocol showed higher sensitivity than the standard immobility test: 2- and 4-h copper as well as 2- and 4-h whole effluent.

In conclusion, this novel acute bioassay provides more accurate and earlier responses. This is a necessary requirement for toxicity assessment in aquatic habitats and provides more protection to aquatic organisms. In addition, using a sublethal endpoint, feeding inhibition, is regarded as ecologically more relevant. Using shorter exposure, it gives earlier information on the toxicity of the sample. Finally, the protocol fulfils quality assurance components such as repeatability which means it can be applied in routine testing.

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