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Comparing Metal Toxicity Among *Daphnia magna* Clones: An Approach Using Concentration-Time-Response Surfaces

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Abstract. This study investigates the use of concentration-time-response surfaces as a tool to predict potential long-term effects of metals to *Daphnia magna*, using mortality as an endpoint. Specifically, concentration-time-response surfaces were determined for four *D. magna* clones exposed to four metals (Cd, Zn, Cu or U) in moderate-hard or hard synthetic freshwater for 24–96 h. Mortality data were $\log_e$ transformed into probits and then regressed against the reciprocal of exposure time and concentration. The results obtained clearly showed that mortality was markedly affected at exposure periods longer than 48 h. Thus, an optimal exposure period of 72 h was selected to compare predicted lethality effects (LC) of different intensities (*i.e.*, 10–50%) derived from concentration-time-response surfaces with measured chronic lethal levels obtained from the literature. Only the results of Cd and Cu show good agreement between predicted and measured chronic lethal concentration levels. The apparent disagreement observed for U could be attributed to differences in water quality, and hence, U bioavailability. The high levels of Zn toxicity observed in relation to the predicted values could indicate that chronic mortality of Zn is mediated by toxic anorexia. Overall, the results obtained show that concentration-time-response surfaces offered the potential to assess the effect of time on toxicity, which is desirable to credibly extrapolate from acute to chronic scenarios. Furthermore, by determining lower mortality thresholds (*i.e.*, LC_{10}) at different times, concentration-time-response surfaces were able to emulate the mode of action of the selected metals on chronic exposures. Therefore, the use of concentration-time-response surfaces has potential application in environmental risk assessment.

A major challenge in aquatic ecotoxicology is to bridge the gap between the use of biological indicators of pollutant exposure/damage derived from measurements on individuals, and the health of natural populations (Forbes and Forbes 1994). Recently, the development of individual-based energetic models have allowed us to link environmental variables, including chemical exposure concentrations, to ecophysiological responses of individuals (Hallam *et al.* 1990; Kooijman 1993; Nogueira 1996). These models allow prediction of the potential ecological effects of long-term exposures to toxic chemicals, as measured on individuals, and allow extrapolation from short-term measures of effects, such as measurements of energy balance within the organism, to life-cycle effects, such as growth, mortality, maturation time, and egg output. However, one area that cannot be addressed by most of these models is the ability to predict potential toxic effects not mediated by individual energy imbalance, such as mortality. As a consequence, it is necessary to use empirical models, which can more easily integrate existing toxicity data and estimate time-dependent effects from acute responses (Kooijman 1981; Hendriks and Enserink 1996).

Here, we use concentration-time-response surfaces to predict mortality as a function of concentration and time. Although some controversy among ecotoxicologists exists with respect to the use of concentration-time-response surfaces, or alternately, times-to-death responses (Newman and Dixon 1996), we selected the former approach because it requires a simpler analysis and can be parameterized with existing acute toxicity data. Most laboratory tests are short-duration acute toxicity tests where the combined effects of dose exposure and duration exposure are tested. However, only a single endpoint, reflecting a given dose at the end of the test period, is routinely reported and used (OECD 1997). In contrast, concentration-time-response surfaces provide more complete use of data obtained in acute exposures (Lee *et al.* 1995; Sun *et al.* 1995). The analysis of concentration-time-response surfaces offers the possibility to assess the progress of mortality over time. In addition, it is also possible to assess levels of response lower than midmortality (LC_{50}). Thus, concentration-time-response surfaces can be used to predict chronic lethal concentration levels at different exposure times (Lee *et al.* 1995).

The aim of the present study was to evaluate the use of concentration-time-response surfaces as a tool to predict potential toxic effects of *Daphnia magna*, using mortality as an endpoint. Specifically, concentration-time-response surfaces were determined for four *D. magna* clones exposed to four metals (Cd, Zn, Cu, or U) in synthetic fresh waters with varying water hardness (*i.e.*, moderate-hard and hard waters) over differing time periods (24–96 h). Although the analysis of

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concentration-time-response surfaces have been performed for different fish species (Lee *et al.* 1995; Sun *et al.* 1995), this approach is novel for *D. magna*. Furthermore, given the nature of the standard risk assessment process, it is quite possible that *Daphnia* tests may yield the most sensitive and cost-effective approach (Kenega 1982). Thus, it is important that mortality data generated from concentration-time-response surfaces are relevant and representative of sensitive organisms exposed to a pollutant (Baird and Barata 1997).

Genetic variability in both acute and chronic tolerance to metals occurs among laboratory *D. magna* clones (Baird *et al.* 1990, 1991). Differences in water quality, including hardness, alkalinity, and/or pH, may also have important effects on the bioavailability and, hence, toxicity of metals to aquatic organisms (Barata *et al.* 1998). Thus, to credibly compare our data with previous studies conducted at different laboratories with different *Daphnia* genotypes and water types, it is necessary to include data from different *D. magna* clones and varying water hardness.

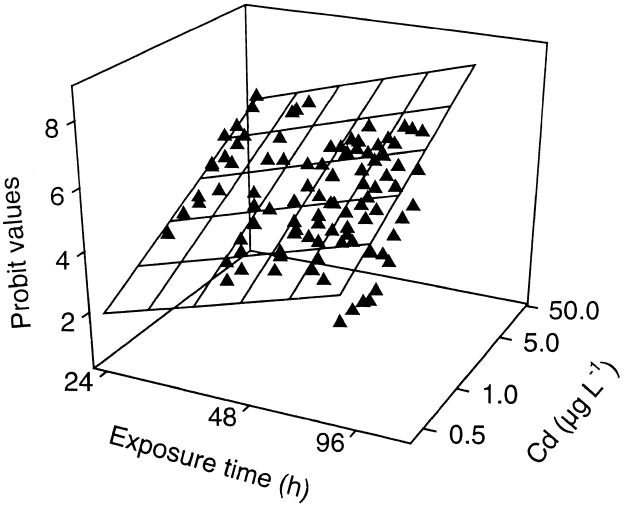

Fig. 1. Concentration-time-response surface of a single *D. magna* clone (European standard, clone A) exposed to Cd in moderate-hard synthetic water. Triangles represent single mortality values transformed into probits

Materials and Methods

Experimental Design

Data from the acute mortality responses of four *D. magna* clones (designated F, C, A, and S-1, *sensu* Baird *et al.* 1991) exposed to Cd, Zn, Cu, or U in moderate-hard or hard reconstituted fresh water (APHA *et al.* 1992) were obtained from a previous study (Barata *et al.* 1998). The clones selected are currently used to conduct toxicity test in Europe and North America (Baird *et al.* 1991) and differ in their tolerance to Cd, Zn, Cu, and U (data from Table 4 in Barata *et al.* 1998). The measured mean pH, alkalinity (in mg L^{-1} $CaCO_3$), and hardness (in mg L^{-1} $CaCO_3$) for moderate-hard and hard water, was 7.7, 62, and 91 and 8.1, 126, and 179, respectively (data from Table 1 in Barata *et al.* [1998]).

Tests were performed in accordance with standard protocols (OECD 1997) using only third to sixth brood neonates. Neonates produced on the same day were pooled in experimental water. Groups of 10 animals were then transferred to the test medium (120 ml). Each test series consisted of one triplicated control and from 8 to 15 triplicated test concentrations, depending on the metal and genotype assayed. The measured toxicological endpoint was mortality, identified as immobility. Mortality was checked at 12-h intervals and tests were terminated at 96 h or when control mortality, probably due to starvation, exceeding 10%. All experiments were conducted at 20 ± 0.5°C.

Data Analysis

Mortality data were $\log_e$ (ln) transformed into probits (P) and then regressed against the reciprocal of exposure time (T) and metal concentration (M). Exposure time and metal concentration were ln transformed to correct for heterogeneity of residuals. The final form of the regression model is

$$\mathrm{P} = \mathrm{a} + \mathrm{b} \ln M + \mathrm{c}\frac{1}{\ln T} \qquad \text{Equation 1}$$

where a, b, and c are constants.

Previous studies have shown that the model given in Equation 1 provides a good fit to toxicity data for several fish species (Lee *et al.* 1995). Furthermore, concentration-time-response surface parameters provide a biologically meaningful explanation of toxicity data, in the sense that the intercept, a, and the coefficients, b and c, are measurements of the initial strength of toxic action, the shape of concentration response and the shape of the time response, respectively (see Sun *et al.* [1995] for further explanation). Parameters a, b, and c were estimated by multiple linear regression analysis using the least square method (Zar 1996). To satisfy the assumptions of the least square method, regression models were restricted to mortality data between the LC_{10} and LC_{90} (Sun *et al.* 1995).

To evaluate the use of concentration-time-response surfaces as a useful tool to predict the potential toxic effects of metals on *D. magna*, predicted mortality concentration (LC) levels were compared with observed chronic mortality levels. By holding P constant, the metal concentration (M) inducing a given level of response (*i.e.*, LC_x) as a function of exposure time (T) can be obtained by rearranging Equation 1, as follows:

$$\mathrm{Ln}\, M = \frac{\mathrm{P} - \mathrm{a} - \dfrac{\mathrm{c}}{\ln T}}{\mathrm{b}} \qquad \text{Equation 2}$$

Using Equation 2, it is possible to predict different mortality levels (LC_x). Consequently, LC_{10}, LC_{20}, LC_{30}, LC_{40}, and LC_{50} values were predicted by making P equal to 3.72, 4.16, 4.48, 4.75, and 5, respectively (Lee *et al.* 1995). In addition, by examining the effect of exposure time on mortality in unexposed and exposed animals, an optimal time of exposure (T) was chosen. These results were compared with observed results in chronic exposures. It should be noted that in *D. magna* chronic tests, reproductive output, rather than mortality, is typically used as an endpoint (OECD 1997). Thus, chronic mortality results have been reported in only a few studies. The observed chronic mortality data was reviewed for three cladoceran species, *D. magna*, *D. pulex*, and *Ceriodaphnia dubia*. Observed chronic mortality for *Daphnia* sp. and *C. dubia* was assessed at 15 and 7 days, respectively. At 20°C, 15-day-old *Daphnia* sp. and 7-day-old *C. dubia* had already laid their first three broods, which generally determined all population growth rates (Porter *et al.* 1983; Mount and Norberg 1984). Furthermore, the metal sensitivity of these cladoceran species is very similar

Table 1. Equations and coefficients of determination (r^2) of the concentration-time-response surfaces obtained for four *D. magna* clones exposed to Cd, Zn, U, and Cu in moderate-hard (MHW) and hard (HW) synthetic waters

		Regression Parameters				
Clone	Water Hardness	a (intercept)	b	c	r^2	n
Cadmium						
Clone A	MHW	8.68	0.45	−17.77	0.40*	74
	HW	9.40	0.51	−22.57	0.58*	17
Clone F	MHW	4.34	0.88	−11.18	0.63*	67
	HW	6.69	0.75	−17.35	0.73*	33
Clone C	MHW	5.98	0.60	−11.03	0.45*	106
	HW	7.23	0.55	−18.30	0.68*	33
Clone S-1	MHW	5.69	1.23	−25.32	0.55*	25
	HW	5.51	0.79	−18.61	0.33*	35
Zinc						
Clone A	MHW	−1.68 ns	1.94	−20.21	0.76*	41
	HW	0.52 ns	1.70	−25.80	0.60*	20
Clone F	MHW	3.93	0.86	−15.74 ns	0.41*	26
	HW	5.70	0.93	−25.63	0.57*	23
Clone C	MHW	0.72 ns	1.52	−23.33	0.73*	39
	HW	4.84	1.57	−39.83	0.49*	28
Clone S-1	MHW	1.15 ns	1.09	−13.64	0.60*	27
	HW	0.88	1.29	−16.44	0.85*	27
Uranium						
Clone A	MHW	−4.72 ns	1.72	−19.27	0.46*	20
	HW	−4.49	1.64	−13.54	0.61*	30
Clone F	MHW	2.40 ns	0.66	−11.72	0.31*	24
	HW	1.75 ns	0.72	−13.42	0.23*	40
Clone C	MHW	−12.56	3.52	−56.80	0.78*	15
	HW	−0.96 ns	1.20	−23.46	0.60*	41
Clone S-1	MHW	−5.22	1.73	−21.33	0.68*	18
	HW	−0.35 ns	0.94	−14.63	0.40*	44
Copper						
Clone A	MHW	6.81	0.63	−11.39	0.79*	42
	HW	5.19	1.46	−18.49	0.74*	20
Clone F	MHW	6.63	1.32	−12.15	0.85*	22
	HW	3.96	1.14	−10.27	0.76*	24
Clone C	MHW	4.93	1.16	−12.13	0.57*	29
	HW	4.55	0.84	−12.44	0.57*	27
Clone S-1	MHW	5.37	1.10	−8.67	0.79*	52
	HW	3.63	1.19	−10.24	0.56*	20

The model used is given in Equation 1
n = number of observations; ns, coefficients not significantly different ($p \geq 0.05$) from 0
* $p \leq 0.05$

(Koivisto *et al.* 1992; Lilius *et al.* 1995; Koivisto and Ketola 1997; Versteeg *et al.* 1997).

Results

Since the concentration-time-response regressions were ln transformed to linearize experimental data and attain model adequacy, they could be defined as planes (see Figure 1 for a typical example). All linear regression equations obtained were significant ($p \leq 0.05$, Table 1), and the residuals were normally distributed (Shapiro-Wilk test, $p > 0.05$). In 23 of the 32 treatments, the regression models explained at least 50% of variability (*i.e.*, $r^2 > 0.50$–0.85) in mortality (Table 1).

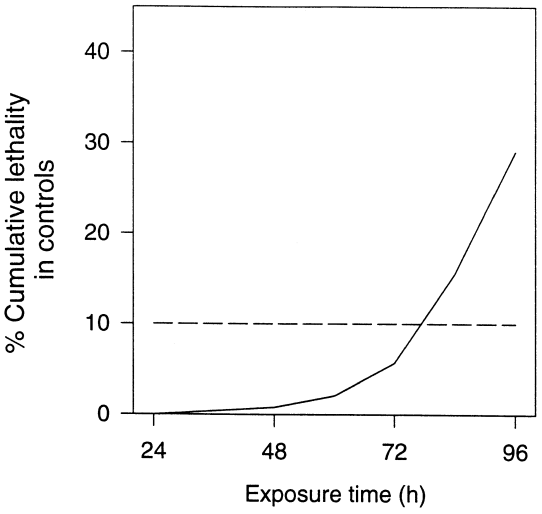

Fig. 2. Cumulative mortality of *D. magna* neonates under control conditions (*i.e.*, starvation time). The results are based on data pooled from 22 trials, 4 clones, and 2,000 individuals. In addition the 10% mortality threshold (dashed line) is depicted

Except for clone F exposed to Zn in moderate-hard water, exposure time significantly affected percentage mortality (the coefficient, c, was significantly [$p \leq 0.05$] different from zero) (Table 1). Thus, to credibly extrapolate from acute to chronic mortality, it is necessary to choose the longest exposure time. Nevertheless, since acute *D. magna* tests are performed with newly born individuals (neonates) in the absence of food (OECD 1997), it is important to know the length of time that *Daphnia* neonates can survive under starvation. Based on data pooled from 22 trials and 2,000 individuals, cumulative mortality in control treatments showed that exposure times greater than 72 h were not recommended, since mortality, probably due to starvation, exceeds 10% (Figure 2).

Based on the regression model given in Equation 2, the predicted concentrations of Cd, Zn, U, and Cu causing 10, 20, 30, 40, and 50% mortality for the four clones in moderate-hard and hard waters at 72 h of exposure were compared with observed results in chronic exposures (Figure 3). Since clonal differences in metal tolerance have been previously shown to be important (Barata *et al.* 1998), it was considered more appropriate to express the results in terms of clonal boundaries. In particular, for each water hardness and metal, the survival response from the least and most sensitive clone was chosen as the upper and lower clonal boundaries, respectively (Figure 3). Using this method, the predicted genetic variability among *D. magna* clones could be illustrated. It is important to note that the clonal and water hardness boundaries shown in Figure 3 represent mean predicted values. Therefore, some of the observed variability may have been due to the inherent variability of the tests. Nevertheless, Barata *et al.* (1998) showed that the amount of variability inherent to the tests (*i.e.*, experimental error) was small compared to the genetic and water chemistry variability observed for the acute tolerance of *D. magna* to metals. Except for Zn and possibly U (Figures 3B and 3C), predicted lethal effect concentration boundaries were consistent with observed values from chronic tests (Figures 3A, 3C, and 3D). For Cu and U, however, the effects of water hardness on predicted and observed values differed.

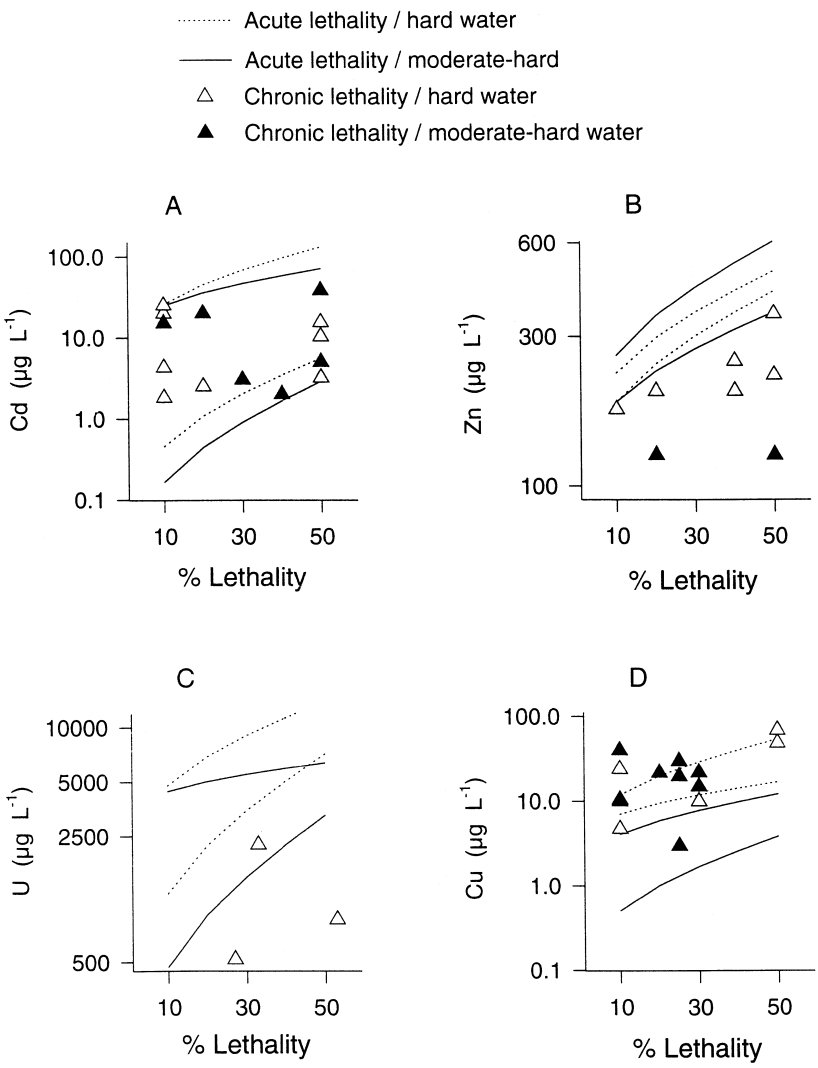

Fig. 3. Predicted mortality at 72 h of exposure for Cd (A), Zn (B), U (C), and Cu (D) in moderate-hard and hard water. For each metal, delimited areas between straight and dashed lines indicate the range of response among the clones studied in moderate-hard and hard waters, respectively. Triangles represent measured chronic mortality data reported in previous studies for *C. dubia* (Carlson *et al.* 1986; Winner 1988; Belanger *et al.* 1989; Cerda and Olive 1993), *D. pulex* (Winner 1984, 1985, 1986; Roux *et al.* 1993; Koivisto and Ketala 1995), and *D. magna* (Biesinger and Christensen 1972; Flickinger *et al.* 1982; Poston *et al.* 1984; Blaylock *et al.* 1985; van Leeuwen *et al.* 1985, 1988; Elnabarawy *et al.* 1986; Knowles and McKee 1987; Bodar *et al.* 1988; Paulauskis and Winner 1988; Santojanni *et al.* 1995)

Discussion

The results reported in Table 1 indicate that the concentration-time-response surfaces obtained in this study were useful and were able to extract much of the information provided in acute *Daphnia* toxicity tests. Furthermore, by using probits instead of percentage mortality and by restricting the regression models to values between 10 and 90% mortality (*i.e.*, LC_{10}–LC_{90}), model adequacy was obtained. Thus, the parameters of the models could be easily determined by the least squares method using a multiple linear regression approach (Sun *et al.* 1995; Zar 1996).

The analysis of regression coefficients clearly showed that exposure time markedly affected the toxicity of Cd, Zn, U, and Cu (Table 1), whereby toxicity typically increased (lower LC_{50}) as the exposure period increased. Mortality was markedly affected at exposure periods longer than 48 h, which is the recommended standard *Daphnia* endpoint (OECD 1997). Therefore, to credibly extrapolate from acute to chronic scenarios, longer exposures should be selected. In the present study, an optimal exposure period of 72 h was shown to minimize starvation effects at lower mortality thresholds (*e.g.*, LC_{10}) (Figure 2).

To evaluate the use of the concentration-time-response surface as a tool to predict potential toxic effects of Cd, Zn, Cu, or U on the mortality of *D. magna*, predicted lethal 72-h $LC_{10–50}$ values were compared with measured chronic lethal concentration levels obtained from the literature. Only the results for Cd and Cu show good agreement between predicted and measured chronic lethal concentration levels (Figure 3). The apparent disagreement observed for U in Figure 3C should be interpreted with caution, since the differences in measured chronic values derive from replicated trials of a single study (Poston *et al.* 1984). In addition, it is evident that the effects of water hardness on the measured chronic toxicity data reported for Cu and U do not agree with the predicted values. This discrepancy could be mediated by differences in water quality, including pH, alkalinity, hardness, and food levels between this study and those from which measured chronic data were obtained.

Several studies used natural waters with a poorly or undefined chemistry (Poston *et al.* 1984; Belanger *et al.* 1989; Koivisto and Ketala 1995). Consequently, complexation of Cu and U species with inorganic ligands and/or competition with hardness ions (*e.g.*, Ca^{2+} and/or Mg^{2+}) may have affected the free hydrated metal ion concentration (*i.e.*, Cu^{2+} or UO_2^{2+}), and thus, the observed toxicity of Cu and U (Barata *et al.* 1998). In addition, it is known that the presence of food can ameliorate Cu toxicity to daphnids (Biesinger and Christensen 1972; Belanger *et al.* 1989; Koivisto *et al.* 1992).

Koivisto *et al.* (1992) reported that the tolerance of *D. magna* to Cu in spring water (pH 8.4; alkalinity 70 mg L^{-1} $CaCO_3$; hardness 33.8 mg L^{-1} $CaCO_3$) decreased fourfold when 2×10^5 cells ml^{-1} of the unicellular alga *Scenedesmus* sp. was added to the test medium. Therefore, the presence of food in the test medium could contribute to explaining the higher tolerance of *D. magna* to Cu previously reported in chronic studies with varying water hardness. In this study, *D. magna* were not fed during the acute metal exposures. Thus, the toxicity observed in these acute tests could have been lower if organisms were fed.

For Cd, clonal differences in toxicity, rather than differences in Cd speciation, were observed and are consistent with previous studies (Baird *et al.* 1991; Barata *et al.* 1998). The higher levels of Zn toxicity observed in relation to the predicted values (Figure 3B), could indicate that chronic mortality caused by Zn could be mediated by toxic anorexia. Previous studies have shown that low concentrations of Cd, Zn, and Cu (Flickinger *et al.* 1982; Allen *et al.* 1995; Crane 1995) inhibit feeding. Thus, for metals such as Zn, which typically have a low toxicity to daphnids and a general mode of toxic action (Stohs and Bagghi 1995), mortality in long-term or chronic exposure scenarios could be driven by toxic anorexia.

From the above discussion, it is evident that concentration-time-response surfaces offer the potential to assess the effect of time on toxicity, which is desirable to credibly extrapolate from acute to chronic scenarios. Furthermore, by determining lower mortality thresholds (*i.e.*, LC_{10}) at different times, concentration-time-response surfaces were able to emulate the mode of action of xenobiotics on chronic exposures, which has potential application in environmental risk assessment.

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