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Effects of Cadmium on the Survival of Three Life-Stages of the Freshwater Pulmonate *Lymnaea stagnalis* (Mollusca: Gastropoda)

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As a complement to chemical analysis, numerous tests of toxicity using bacteria, plants or animal species are used to evaluate the biological quality of freshwater ecosystems. For practical purposes, it is recommended to use tests based on organisms of different trophic levels for ecological risk assessment. However, the ability of such experiments to detect adverse effects of contaminants depends on several factors including toxicological, environmental and biological characteristics. Among invertebrates, crustaceans are commonly used because of the standardisation of the tests (e.g. ISO/DIS 10706). In addition, substantial toxicological databases exist for freshwater biota and the US EPA (2000) has recently proposed some standardised designs using benthic and annelids to assess the toxicity of sediments in laboratory test.

Few toxicological studies are devoted to the effects of metals on freshwater gastropods (Wier and Walter 1976, Khangarot and Ray 1988, Ravera 1991). However, aquatic gastropods can represent 20 to 60% of the global macroinvertebrate abundance and biomass in freshwater ecosystems (Habdija *et al.* 1995). They have an important function in transfer of material and energy (Habdija *et al.* 1995). Reynoldson and Day (1998) noticed that "While no formal protocols have been proposed, recent studies suggest that molluscs (including pulmonate gastropods) can be used as test organisms … there is no reason why a mollusc test should not be developed". So, we undertook toxicological studies on freshwater pulmonates in order to (1) document various demoecological effects of pollutants on this group of invertebrates; (2) propose experimental conditions for developing a (or several) standardised mollusc toxicity test(s). We decided to choose the freshwater pulmonate gastropod *Lymnaea stagnalis* to include a gastropod test among the tools available for ecological risk assessment. *L. stagnalis* is an ubiquitous species with a large geographical distribution and is principally present in lentic hydrosystems. It is an efficient grazer of periphytic algae and represents a significant part of the diet of many species of fish, crayfish, mammals, birds, insect larvae and annelids. Moreover, this species is easy to rear in laboratory conditions (Gomot 1998). Some previous experiments on the sublethal effects of Cd on juvenile and adult *L. stagnalis* allowed us to propose some experimental test conditions and to assess Cd toxicity on its growth, reproduction, embryonic development and hatching (Gomot 1998, Coeurdassier *et al.* 2003). Sublethal effects of pollutants on various stages of the life-cycle of an

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organism may have, as mortality, an important impact on population dynamics (Calow and Sibly 1990) and consequently, on community structure and ecosystem. However, to define the demoecological impact of Cd, complementary data about its effects on the survival of different key-stages of *L. stagnalis* are needed. The present study investigates the effects of Cd toxicity on the survival of *L. stagnalis* for three different stages in its ontogenesis, two stages of juveniles (i.e. immature) and adult (i.e. mature) snails for different durations of exposure in laboratory conditions.

MATERIALS AND METHODS

Test animals were obtained from adult *L. stagnalis* collected in an artificial outdoor pond in Besançon (France) and placed in artificial freshwater (A.F.W; pH 7.8, hardness: 250 mg L^{-1} $CaCO_3$, Ca/Mg = 4, $[O_2] > 7$ mg L^{-1}) (Gomot 1998) for reproduction. Egg masses were placed under standard conditions (20 ± 1°C, light/dark cycle of 12h/12h) in A.F.W. New hatched snails were reared under the same conditions and fed *ad libitum* with lettuce.

Table 1. Characteristics of life-stages of *L. stagnalis* used in the test.

Life stage	Age (weeks)	Wet wt (mg)		Shell height (mm)	
		Mean	Range	Mean	Range
Juv-S1	4	12	7-20	6	5-7
Juv-S2	9	500	200-700	19	15-22
Adult	20	1900	1300-2700	28	25-32

Cd toxicity was measured on three life-stages of *L. stagnalis*: 4-week-old (juvenile stage 1: Juv-S1), 9-week-old (juvenile stage 2: Juv-S2) and 20-week-old individuals (adult) described in Table 1. Experiments were conducted in Pyrex containers, the total volume of test solution in each container depending of the studied life-stages: 10 ml of test solution in 50 ml container for Juv-S1, 500 ml of test solution in 1000 ml container for Juv-S2 and 1000 ml of test solution in 2000 ml container for adults. Series of seven Cd concentrations, 0, 1000, 1250, 1500, 1750, 2000 and 2500 µg L^{-1} were prepared. Cd concentrations in water samples taken at 0, 3 and 8 d were acidified with HNO_3 (analytic quality, Aldrich) and measured using a flame AAS (Perkin-Elmer AAS 3100). Owing to the preference of *L. stagnalis* for lentic systems, static bioassays were conducted over 8 d under the conditions described above. Stock solutions were prepared by dissolving reagent grade cadmium chloride ($CdCl_2$ Aldrich) in ultrapure water (Milli Q water, 18.2 µS.cm^{-1}) and were not changed during the test.

Experiments were started by randomly placing ten individuals of each stage in the different series of containers. The snails were not fed during the test. They were observed at 12h intervals for 120 h and at 6 d and 8 d and the number of dead snails recorded. In adults, death was determined when stimulation of the foot with a spine led to no reaction from the animal. For Juv-S1 and Juv-S2 snails, the absence of heart contractions was observed through the translucent shell with a stereomicroscope. Dead specimens were removed from the test recipient.

Given that the Cd concentrations declined over the experiment (Table 2), the use of "Time Weighted Mean Concentration" (TWMC) is relevant since it takes into account the variation of instantaneous Cd concentrations in water over time (as proposed in ISO/DIS 10706, 1999). The T.W.M.C is calculated by:

$$\frac{(\text{conc } 0 - \text{conc } 1)*d}{\text{Ln (conc 0)} - \text{Ln (conc 1)}}$$

Where conc 0 and conc 1 are the measured concentrations at the start of experiment and at each observation time respectively; d is the number of d between measurements; Ln (conc 0) and Ln (conc 1) are the natural logarithms of conc 0 and conc 1 respectively.

Table 2. Nominal, measured and time-weighted mean (TWMC) Cd concentrations (µg L^{-1}) of artificial freshwater at different times during the test.

D0		D3	**D0-D3**	D8	**D0-D8**
Nominal	Measured	Measured	**TWMC**	Measured	**TWMC**
0	0	0	**0**	0	**0**
1000	910	680	**789**	580	**732**
1250	1150	880	**1009**	763	**943**
1500	1380	1050	**1207**	861	**1100**
1750	1575	1270	**1414**	1105	**1324**
2000	1830	1510	**1664**	1375	**1592**
2500	2430	2010	**2213**	1865	**2135**

Concentrations / lethal response curves (% survivors versus Cd concentrations) were established for each stage and duration of exposure. Median lethal concentrations (LC50) were determined by probit analysis. LC50 were calculated with measured concentration at t=0 and also with TWMC data when at least one Cd concentration differed by more than 20% from the measured concentration at a given time, as recommended in ISO/DIS10706 (1999).

RESULTS AND DISCUSSION

All the experimental Cd concentrations measured at the beginning of the tests (D0) were within 10% of nominal concentrations (Table 2). During the experiment, measured concentrations decreased progressively and the measurements performed at the third (D3) and eighth day (D8) allowed an evaluation to be made of the TWMC for both periods in the static system (Table 2). This could be due to two non-exclusive phenomena. At first, the most important one was probably the adsorption of Cd^{2+} at the surface of the container and on the shell and the body of the animals. The formation of unsoluble species of Cd is unlikely in the present experimental conditions (pH of test solution = 7.8) by considering the range of pH for the precipitation of Cd hydroxides, generally reported to be between 9 and 11. Secondly, such as numerous aquatic gastropods (Gundacker 2000), *L. stagnalis* is able to accumulate high concentration of Cd in its tissues, particularly in the hepatopancreas, (262 < bioconcentration factor < 662; Coeurdassier *et al.* 2003). Consequently, this storage capacity might also

contribute to remove Cd concentration from the test solution. The use of TWMC allowed to integrate the decrease of the exposure concentration during the experiment. The calculation of LC50-TWMC values can present important variations with those obtained from nominal Cd concentrations. The percentage of variation between LC50 and LC50-TWMC values ranged from 10% to more than 30% (Table 3). This underlines the importance to consider variation of exposure concentrations during all the experiment to improve the assessment of species sensitivity in toxicity bioassay.

Table 3. LC50 (µg L^{-1}) for different life stages of *L. stagnalis* exposed to Cd.

	LC50 Juv-S1		LC50 Juv-S2		LC50 adult	
Test duration (d)	Measured Cn*	**TWMC**	Measured Cn	**TWMC**	Measured Cn	**TWMC**
1	> 2500		> 2500		> 2500	
2	1183		2430		> 2500	
3	920	**633**	1515	**1303**	1771	**1610**
4	752		1515		1585	
5	All dead		1324		1549	
8	All dead	All dead	761	**658**	1261	**912**

* Measured Cn: measured Cd concentrations

Freshwater gastropods, particularly pulmonates, are scarcely considered in toxicological research. Indeed, most previous studies into the effects of metals have concerned the survival of arthropods and fishes. In addition, those laboratory experiments that investigated contaminant toxicity in freshwater gastropods focused mainly on the sublethal effects of metals (Ravera 1991; Gomot 1998; Coeurdassier *et al.* 2003) and on the lethal and of sublethal effects of organic pollutants. The present results bring some original data on the acute toxicity of Cd on different stages of the pulmonate gastropod *L. stagnalis*. Survival of controls ranged from 80 to 100% for 8 d of exposure. For the three stages, the responses showed a general decrease in survival as the time of exposure increased (Figures 1A, B and C). The Juv-S1 was the most sensitive stage. They responded to Cd particularly strongly during the first three d of exposure (Figure 1A) with the LC50 values decreasing from 1183 to 752 µg L^{-1} after 48 hours and 96 hours. All Juv-S1 died before the fifth day of exposure at all the tested Cd concentrations (Table 3, Figure 1A). Juv-S2 response was intermediate compared to other stages. LC50 decreased respectively from over 2430 µg L^{-1} at 48 hours to 1324 µg L^{-1} at 5 d. After 8 d of exposure, 80 and 70% of the juvenile died at 1000 and 1250 µg L^{-1} respectively and 100% at 1500 µg L^{-1}. The corresponding LC50-8 d was 761 µg L^{-1} (Table 3, Figure 1B). In adults, test durations of at least 3 d may be required to accurately assess the toxicity of Cd. This stage was the least sensitive with LC50 that decreased progressively from 1771 µg L^{-1} at 72 hours to 1261 µg L^{-1} at 8 d (Table 3, Figure 1C). According to Stoeppler (1991), the concentrations of dissolved and particle-bound Cd in unpolluted water are below 0.1 µg L^{-1}. He also reported that Cd concentrations around 1 µg L^{-1} can initiate toxic effects in freshwater organisms and that Cd concentrations above 2 µg L^{-1} are lethal for certain species. To be related to environmental concentrations, these LC50 for

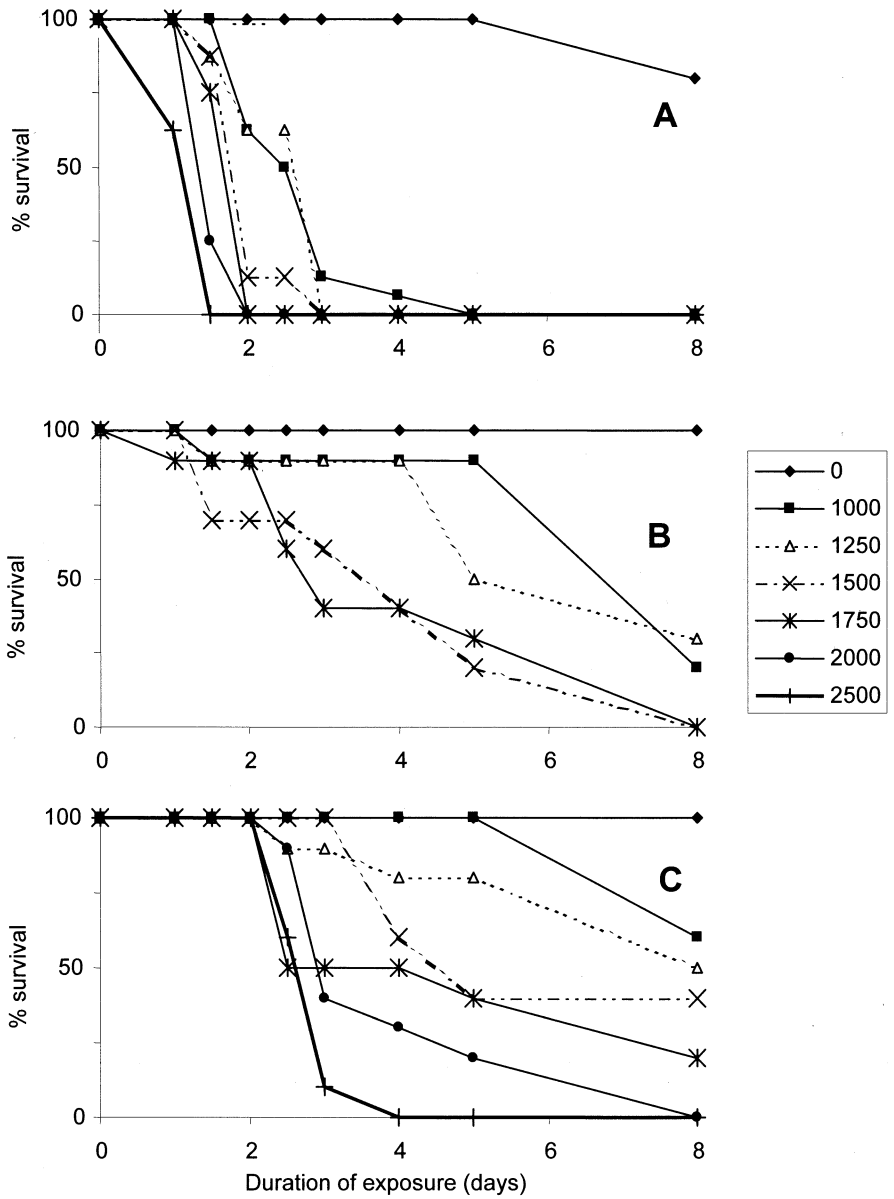

Figure 1. Percent survival of different life-stages of *L. stagnalis* exposed to increasing Cd concentrations (µg L^{-1}) in time (A: Juv-S1; B: Juv-S2; C: adult).

different life-stages of *L. stagnalis* are divided by 1000 (i.e. the safety factor used to determine predictive no-effect concentrations (PNEC) from LC50 of a single acute toxicity test, EEC 1994). The effective environmental Cd concentrations reported by Stoeppler (1991) are in the same range that PNEC determined from *L. stagnalis* LC50.

Generally, in invertebrates, larval and juvenile stages are reported to be more sensitive than later stages. This has been observed in other freshwater snails such as *Physa gyrina* (Wier and Walter 1976), in different groups of arthropods such as isopods (*Asellus aquaticus*, Green *et al.* 1986) or insects (*Chironomus riparius*, Williams *et al.* 1986) and in leeches (*Nephelopsis obscura*, Wicklum *et al.* 1997). However, in vertebrates, newly hatched tilapia *Oreochromis mossambicus* were seven times less sensitive to Cd than 3-day-old individuals (Hwang *et al.* 1995). Juvenile rainbow trout *Onchorynchus mykiss* were found to be three times less sensitive to metals than adults (Stubblefield *et al.* 1999). Moreover, the same study pointed out that acclimation of juvenile and adult trout to sublethal Cd concentrations increased their tolerance to Cd by a factor of 25 and 4.8 respectively (Stubblefield *et al.* 1999). Similar experiments should be envisaged to study this phenomenon in aquatic snails because acclimation, consequent genetic adaptation or physiological tolerance represent compensatory responses playing an important role in the maintenance of populations in polluted areas.

Numerous authors consider that long-term studies involving sublethal effects (fertility, sexual maturity…) of toxicants at low levels of contamination are more instructive than those which measure survival in an acute test during 24 to 96 hours. Owing to their higher sensitivity, sublethal endpoints are relevant for the determination of no-effect concentrations and to try to define a safety limit for the future use or release of dangerous substances. Previously, reproduction of mature *L. stagnalis*, embryogenic development and hatching and growth were shown to be sensitive to Cd in chronic tests performed in similar laboratory conditions but during four weeks (Gomot 1998, Coeurdassier *et al.* 2003). This opens some encouraging perspectives to integrate this species in risk assessment procedures (acute and/or chronic tests) using a battery of relevant species of different trophic levels. However, as previously demonstrated (Kammenga *et al.* 1996), the intensity of the population response to toxicants is not always determined by the most sensitive individual trait and the life-history strategy should be taken into account when predicting effects on populations. Evaluation of both lethal and sublethal responses in different stages of a species is necessary to project individual responses to population level. The advantage of introducing this population endpoint in laboratory toxicity testing is to integrate demoecological effects, both lethal and sublethal, on several life-stages in a single value which takes into account the relative "weight" of each life-trait (generally called elasticity, see Hansen *et al.* 1999) and its sensitivity to a toxicant in the expression of a global, i.e. population, response. In other organisms, such as the terrestrial snails *Helix aspersa* (Laskowski 1996), with a *r*-demographic strategy closed to those of *L. stagnalis*, survival of immature individuals (the most sensitive life-stages to Cd) is the life-trait with the highest elasticity. This indicates that small decrease in the survival probability of Juv-S1 and Juv-S2 may affect extensively the population dynamics. These remarks are some solid arguments to use population endpoints, which integrate life-history strategies in risk assessment rather than basing results on a single individual effect.

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