Modelling effects of diquat under realistic exposure patterns in genetically differentiated populations of the gastropod *Lymnaea stagnalis*

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Pesticide use leads to complex exposure and response patterns in non-target aquatic species, so that the analysis of data from standard toxicity tests may result in unrealistic risk forecasts. Developing models that are able to capture such complexity from toxicity test data is thus a crucial issue for pesticide risk assessment. In this study, freshwater snails from two genetically differentiated populations of *Lymnaea stagnalis* were exposed to repeated acute applications of environmentally realistic concentrations of the herbicide diquat, from the embryo to the adult stage. Hatching rate, embryonic development duration, juvenile mortality, feeding rate and age at first spawning were investigated during both exposure and recovery periods. Effects of diquat on mortality were analysed using a threshold hazard model accounting for time-varying herbicide concentrations. All endpoints were significantly impaired at diquat environmental concentrations in both populations. Snail evolutionary history had no significant impact on their sensitivity and responsiveness to diquat, whereas food acted as a modulating factor of toxicant-induced mortality. The time course of effects was adequately described by the model, which thus appears suitable to analyse long-term effects of complex exposure patterns based upon full life cycle experiment data. Obtained model outputs (e.g. no-effect concentrations) could be directly used for chemical risk assessment.

Keywords: hazard models; full life cycle experiment; time-varying concentration; pesticides; freshwater gastropods; ecological risk assessment

1. INTRODUCTION

Among toxicants, pesticides are challenging compounds for ecotoxicological studies in aquatic environments because their use leads to complex patterns of exposure of field populations. Indeed, treatments often involve repeated sprayings, which cause several delayed pulses of pesticides, mainly owing to spray drift, drainage and run-off from the treated areas (Official Journal of the European Union L 309 1 2009). Resulting time-varying exposure generates a variety of complex biological responses in non-target aquatic organisms, as described in Reinert *et al.* (2002). Such a complexity in exposure and effects is generally not taken into account in standard toxicity tests, in which exposure occurs under constant concentration and during a short part of the life cycle of model species (mostly juveniles). Therefore, the analysis of standard toxicity test data may lead to unrealistic risk forecasts of pesticides.

Diquat (bipyridylium chemical class) is a contact herbicide which is mainly used as a pre-harvest

desiccant/defoliant of selected vegetable seed crops, and as a photosynthetic inhibitor to control weeds in water bodies (United States Environmental Protection Agency 1995). Regarding its aquatic use, adverse effects on freshwater invertebrates are not clearly characterized yet. Indeed, existing studies mostly refer to short-term toxicity tests and provide conflicting results (Emmett 2002). This led to inconsistent regulation of this product across countries: diquat was banned in France in 2001, whereas it was re-authorized in the USA in 2002, and is still widely used there (110 000 pounds per year), as in several European countries, excluding EU member states. Complementary data on long-term effects should be produced before 2012 when diquat will be re-evaluated (Emmett 2002). In order to avoid unrealistic risk forecasts, it is recommended to implement repeated exposures for compounds with short half-lives and long inter-treatment intervals, such as diquat (Reinert *et al.* 2002). Partial or full life cycle experiments are also recommended to ensure that sensitive windows in the life cycle of the studied species are actually exposed to the compound. However, analysing the results of such toxicity tests using current standard ecotoxicological methods does not allow proper linking of the variations in exposure concentrations to the time course of effects and does not provide any mechanistic description of effects.

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In this study, we developed novel experimental test designs and data analysis tools in order to assess long-term effects of a pesticide in a long-lived species under a realistic scenario. We investigated the development pattern, mortality, feeding rate and reproduction in two genetically differentiated populations of the freshwater snail *Lymnaea stagnalis*, exposed to regularly repeated applications of diquat. *Lymnaea stagnalis* was chosen as the studied species because this long-lived herbivorous snail reproduces during the plant growth period which is targeted for herbicide applications. Egg clutches are laid on weeds, which provide both shelter and food to newborn snails. Therefore, repeated exposures to diquat over one treatment season can be integrated by those snails, and the corresponding effects can be expressed through the variations of snail individual performances. The influence of feeding rate and genetic characteristics (i.e. diversity, differentiation and genetic load) on the biological responses was evaluated because genetic and environmental factors may have joint effects on fitness-related traits, thus determining individual performances in polluted environments. Such interactions should thus be taken into account when assessing long-term responses to contaminants (Kammenga & Laskowski 2000; Coutellec & Lagadic 2006).

Mortality data were analysed using a toxicokinetics–toxicodynamics (TK–TD) model. Indeed, models that combine TK and TD assumptions to describe the time course of effects have been identified as relevant tools to analyse such data (Forbes *et al.* 2008). Various TK–TD models have already been used to describe the mortality pattern of organisms exposed to time-varying concentrations of pesticides, as reviewed in Ashauer *et al.* (2006). Yet, only some of them are relevant to properly describe and/or predict long-term effects of complex exposure patterns (Hommen *et al.* 2010). They are based either on the dynamic energy budget (DEB) theory (Kooijman 2000), or on the critical body residues theory (McCarty & Mackay 1993). In these models, death is assumed to be partly stochastic, although dependent on toxicant concentration or damage (Hommen *et al.* 2010). Among these approaches, we chose the threshold hazard model derived from the DEB theory, as proposed by Kooijman & Bedaux (1996). Indeed, this model has already been successfully applied to assess biological effects and recovery after one pulse exposure (24 h) to the pyrethroid insecticide fenvalerate under various feeding conditions in *Daphnia magna* (Pieters *et al.* 2006). It has also been used to describe survival in *L. stagnalis* in unpolluted waters (Zonneveld 1992). In our study, we extended existing approaches in order to analyse, over the long term, cumulated mortality owing to repeated applications of diquat in this snail.

2. MATERIAL AND METHODS

(a) Experimental populations

Two populations of *L. stagnalis* (thereafter named A and B), differing in their evolutionary history, were used. They were founded in 2003 from different initial

population sizes and levels of genetic relatedness, as described in Coutellec *et al.* (2008a): population A was small and included related snails (eight founders from two lineages, resulting in an effective population size of 4.67), whereas population B was large and included only unrelated snails (80 founders from 80 different lineages, resulting in an effective population size of 80). Sensitivity to diquat was supposed to be higher in the initially smaller population, owing to lower genetic variability, genetic load accumulation and inbreeding (Lynch & Gabriel 1990; Willi *et al.* 2006). Snails were then reared independently in outdoor pond mesocosms during seven generations (average duration between two successive generations: 174 days) in order to allow stochastic evolution in natural conditions. The resulting populations exhibited significant differences in both genetic characteristics (diversity, differentiation and genetic load) and fitness-related trait expression (survival and reproductive parameters), as described in Coutellec *et al.* (2008a). In 2007, 30 adults were sampled from each population and acclimated in the laboratory for one month. When acclimation ended, egg clutches (83 ± 18 eggs per clutch) aged less than 24 h were collected and immediately exposed to diquat in a full life cycle experiment (336 days; average snail lifespan in the field: 424 days; Brown 1979).

(b) Treatments and diquat concentration monitoring

The snails were maintained in dechlorinated, charcoal-filtered tap water, which was repeatedly treated with acute doses of diquat. The first dose was delivered at the very beginning of the life cycle (snails were aged from 0 to 24 h), in order to ensure exposure in the development stage which was expected to be the most sensitive to diquat based upon previous experiments (V. Ducrot 2007, unpublished data). The herbicide was then applied every six weeks until four treatments had occurred. Such timing was chosen because it corresponds to the worst-case realistic scenario for weeding with diquat in Florida (USA), which is the main user of this herbicide for aquatic weeding (Ritter *et al.* 2000). Applied nominal concentrations were 0, 5, 10, 20, 40, 80, 160 and 320 $\mu\text{g l}^{-1}$ (maximal recommended application rate is 224 $\mu\text{g l}^{-1}$; field measured mean concentration after spraying varies between 200 and 370 $\mu\text{g l}^{-1}$; Emmett 2002). The commercial preparation Reglone 2 (200 g l^{-1} diquat; Syngenta Agro SAS, Guyancourt, France) was used to prepare the treatment solutions. Each nominal concentration was obtained by dispensing the appropriate amount of Reglone 2 in plastic tanks containing dechlorinated, charcoal-filtered tap water. The treatment solutions were then agitated and distributed among exposure vessels. In those vessels, water was regularly renewed to ensure appropriate physico-chemical characteristics for the snails regarding O_2 , NH_4^+ , NO_2^- and HPO_4^{2-} contents. To avoid a sudden and unrealistic drop of exposure concentration when renewing water, renewal water also contained diquat, except in the controls (0 $\mu\text{g l}^{-1}$). Renewal water was prepared at the same time as treatment

solutions and stored until use in plastic tanks that contained neither snails nor food. Diquat concentration was measured (by high performance liquid chromatography, according to Coutellec *et al.* 2008b) in treatment solutions, renewal water and exposure water which was sampled from exposure vessels 15 min after a treatment or a water renewal occurred, thus allowing monitoring of the exposure profile.

(c) Full life cycle experiment design and measured endpoints

Controls ($0 \mu\text{g l}^{-1}$) and exposed snails ($5\text{--}320 \mu\text{g l}^{-1}$) from populations A and B were studied using an identical full life cycle experiment design (see figure S1, electronic supplementary material). Freshly laid clutches (less than 24 h) were individually exposed to diquat in plastic six-well plates ($V = 10 \text{ ml}$). The hatching rate was estimated after every embryo had either hatched or died (i.e. lost their mobility). The mean duration of embryonic development was also calculated, based upon data obtained by monitoring the number of newly hatched snails every other day. Newborns that hatched after 28 days of exposure were collected and transferred to 100 ml Petri dishes (five snails per dish and 12 replicates per concentration and population). They were fed with weighted slices of organic lettuce, which were provided only if no leftover remained. The total weight of food provided per replicate was determined in order to assess snail feeding rate. Mortality was monitored daily, dead snails being removed from the exposure vessels and counted. Because feeding behaviour is assumed to depend on the number of snails per exposure vessel (Brown 1979), this number had to be kept constant in order to avoid an experimental bias in the measurement of the feeding rate. Therefore, dead snails were replaced by siblings of similar age (± 3 days) and size ($\pm 0.5 \text{ mm}$), which had been collected from the same previously exposed clutches, and subsequently batch-reared under the same feeding and exposure conditions to snails used in the life cycle experiment. Temperature ($21 \pm 1^\circ\text{C}$), photoperiod (14 L:10 D) and number of snails per replicate (five) were kept constant, whereas exposure vessel volume, food input and frequency of water renewal increased along the snail life cycle, proportionally to individual growth (as shown in figure S1, electronic supplementary material), in order to avoid density-dependent effects owing to increasing competition for space and food. At day 168, snails were transferred to clean water in order to assess post-exposure mortality. The full life cycle experiment stopped after 336 days, when control survival fell below 75 per cent (i.e. upper limit of the survival threshold for validity of toxicity test data; American Society for Testing and Materials 1999).

(d) Data analysis and modelling

The time to dissipation of half the nominal concentration (DT_{50}) and the bioconcentration factor (BCF) of diquat were calculated from concentration measurements in both the water and snails. Normality and homoscedasticity of data were checked using Kolmogorov–Smirnov and Levene tests, respectively,

with $\alpha = 0.05$. Significant effects of diquat on the hatching rate, embryonic development duration, juvenile mortality, feeding rate and age at first spawning in populations A and B were tested at $\alpha = 0.05$ using Mann–Whitney U (MW– U) tests, as recommended in the guidance documents for toxicity test data analysis (Organisation for Economic Cooperation Development 2006, ch. Hypothesis testing). Original and replacement snails were pooled in the statistical analysis because they could not be distinguished from each other in our experiment. Statistical tests were performed with the STATISTICA 6.0 software (StatSoft Inc., Tulsa, OK, USA).

Data were also analysed using the threshold hazard model of Kooijman & Bedaux (1996). In this model, effects are related to diquat internal concentration, as assessed using a TK model. We chose to use the simplest suitable approach in aquatic invertebrates (Ashauer *et al.* 2006), which is the linear one-compartment kinetics model

$$\frac{dc_q(t)}{dt} = \dot{k}_a(c(t) - c_q(t)), \quad (2.1)$$

where $\dot{k}_a$ is the diquat elimination rate (d^{-1}), c the actual diquat concentration in exposure water ($\mu\text{g l}^{-1}$), and c_q ($\mu\text{g l}^{-1}$) the internal concentration of diquat in the tissues scaled by the bioconcentration factor (BCF, which corresponds to the ratio of the absorption and elimination rates at TK steady state). Using such a parsimonious formalization, the uptake rate constant is a hidden parameter, the elimination rate drives the TK and the scaled internal concentration directly relates to the concentration of toxicant in water. Dilution by growth was not accounted for in the modelling, because juvenile growth is very slow in our experimental conditions during the period under consideration in the model (Ducrot *et al.* 2008).

Toxic effects are supposed to occur only above an external concentration threshold, called the no-effect concentration (NEC), which corresponds to the maximal toxicant concentration level that can be handled by physiological regulation systems without generating detectable effects on individuals. Mortality probability in exposed organisms is thus described by $c_q(t)$, which drives TD. Based upon these assumptions, the mean number of dead snails per replicate (N) between observation dates t_n and t_{n+1} , accounting for the replacement of dead snails between observations, was

$$N = N_0 \int_{t_n}^{t_{n+1}} \dot{h}(\tau) d\tau, \quad (2.2)$$

where N_0 is the constant number of snails per replicate ($N_0 = 5$ in our study) and $\dot{h}(\tau)$ is the hazard rate at time τ (day). For a small time interval $d\tau$, $\dot{h}(\tau) d\tau$ represents the probability of dying between τ and $\tau + d\tau$, for an organism that has survived until time τ . This probability depends on both background mortality (i.e. the mortality level in control snails) and diquat toxicity, which is assumed to linearly increase with the difference between $c_q(t)$ and the

threshold value for effect (NEC), as described in

$$\begin{cases} \dot{h}(t; c) = \dot{\lambda} & \text{when } c_q(t) < c_0 \\ \dot{h}(t; c) = \dot{k}_t(c_q(t) - c_0) + \dot{\lambda} & \text{when } c_q(t) > c_0, \end{cases} \quad (2.3)$$

where $\dot{\lambda}$ is the background mortality rate (d^{-1}), $\dot{k}_t$ is the killing rate (a descriptor of the snails' sensitivity to diquat, in $1 \mu\text{g}^{-1} \text{d}^{-1}$) and c_0 corresponds to the maximal external concentration (in $\mu\text{g l}^{-1}$) that does not cause mortality in exposed organisms (i.e. NEC, a descriptor of the snails' responsiveness to diquat).

Toxicokinetics/dynamics parameter values were estimated based upon both diquat aqueous concentration measurements and mortality data, obtained from the full life cycle experiment. Regarding TK, linear interpolation between data points was first used to describe the variation of diquat water concentration between sampling dates. Outputs of those linear functions over time were then used to feed the kinetics part of the model. As described in Kooijman & Bedaux (1996), three different approaches were tested in order to account for TK: (i) kinetics is assumed to be rapid, and equation (2.1) simplifies to $c = c_q$, (ii) kinetics is assumed to be so slow that it corresponds to a linear increase of c_q as a function of c , and (iii) equation (2.1) cannot be simplified. Regarding TD, the model was calibrated using only the mortality data obtained for the first 84 days of post-hatching exposure because no snail exposed to nominal concentrations exceeding $20 \mu\text{g l}^{-1}$ survived longer than this (see §3).

Differential equations were integrated using the Euler method with a step of 0.01 day. Parameter values were estimated through the least square method, by minimizing the sum of the squares of the differences between actual data and model predictions. This sum was calculated for all possible combinations of parameters (c_0 , $\dot{k}_t$, $\dot{\lambda}$ and $\dot{k}_a$) with c_0 ranging from 0.5 to $3.5 \mu\text{g l}^{-1}$ with a step of 0.05, $\dot{k}_t$ ranging from 0.003 to $0.0051 \mu\text{g}^{-1} \text{d}^{-1}$ with a step of 0.0001, $\dot{\lambda}$ centred on the mean control value, ranging from this value minus 0.00002 d^{-1} and this value plus 0.00002 d^{-1} with a step of 0.000002, and the three approaches for kinetics, with $\dot{k}_a$ ranging from 0.01 to 0.5 d^{-1} and a step of 0.01 for case (iii). We then used global mapping to find the global best fit and to check for local minimums.

Confidence intervals were obtained by bootstrapping, as follows. Based upon data from the full life cycle experiment, 1000 experimental outputs (i.e. cumulated mortality at a given observation date, for the different exposure concentrations) were simulated. One experimental output corresponded to data for 12 replicates per exposure concentration and observation date. Therefore, 12 values of cumulated mortality were randomly sampled among the actual data, with possible resampling, for each concentration and observation date. Model parameters were then estimated for each sampled set of data, as previously described. The 95 per cent confidence intervals were derived as bounded by the 2.5 and 97.5 percentiles among the parameter estimate distributions. The parameter estimation and bootstrapping program was written in C++ (Visual C++, Microsoft, Redmond,

WA, USA). It is provided in the electronic supplementary material.

The model was first fitted to data from populations A and B, separately, and then to the pooled data. The Chow test was used in order to highlight significant differences in model parameter values between those two populations. The global goodness-of-fit for our model was analysed by implementing lack-of-fit tests on data from populations A and B. The principle of these tests is to decompose the sum of square of residuals (i.e. differences between test data and model predictions) into two components, the lack-of-fit sum of squares and the pure error sum of squares, and to compare them. The ratio of these components (previously divided by their degrees of freedom) follows an *F*-distribution. This statistic is used in order to test the null hypothesis meaning that lack-of-fit sum of squares is inferior to the pure error sum of squares, which indicates a good fit to the data.

3. RESULTS

(a) Exposure profile

Because no embryo survived at nominal concentrations exceeding $80 \mu\text{g l}^{-1}$, actual diquat concentration was monitored only for the exposures to 5, 10, 20, 40 and $80 \mu\text{g l}^{-1}$. The corresponding mean actual peak concentrations (and their 95% confidence intervals), calculated from concentrations measured in samples obtained 15 min after each treatment, were, respectively, 3.2 (2.5; 3.9), 6.7 (6.2; 7.3), 13.7 (8.8; 18.6), 27.7 (14.3; 41.0) and 55.6 (43.1; 68.1) $\mu\text{g l}^{-1}$. These actual values for peak concentrations will be referred to in the following. For each concentration, the diquat level measured in the exposure vessels before water renewal was not significantly different from that measured in the renewal water (MW-*U* tests, $p > 0.17$ for all studied concentrations), so that renewing water did not lead to a significant increase in exposure concentration. DT_{50} of diquat linearly increased from 14 to 32 days, for peak concentrations increasing from 3.2 to $13.7 \mu\text{g l}^{-1}$. DT_{50} could not be calculated from the experiments conducted with 22.7 and $55.6 \mu\text{g l}^{-1}$, because more than 50 per cent of diquat remained in the water just before the next applied dose. DT_{50} values were thus extrapolated from the values obtained at lower diquat peak concentrations, using the following relation: $\text{DT}_{50} = 1.72 \times c + 8.49$ ($R^2 = 0.99$), with c the peak diquat concentration, which gave DT_{50} values of 56 and 104 days, respectively. For concentrations exceeding $6.7 \mu\text{g l}^{-1}$, diquat did not entirely dissipate between successive applications. Yet, this did not lead to a build-up of diquat concentration in exposure vessels over the experiment because the dose of pesticide applied at each treatment was added to clean water, not to the water in which snails were already exposed. Therefore, at each treatment, peak concentrations were similar over the whole experiment. Estimates of the model parameters were based upon the actual values of external concentrations, as previously described. Measured and modelled variations of external concentrations are shown in figure 1.

Figure 1. Evolution of diquat exposure concentration ($\mu\text{g l}^{-1}$) in water during the first part of the full life cycle experiment with *L. stagnalis* (the second part corresponds to recovery in clean water): example for the nominal concentration of $5 \mu\text{g l}^{-1}$. Lines are model fits, and points are actual concentrations measured in water ($\pm$ s.e.). T and R indicate diquat treatment and water renewal, respectively.

(b) Effects of diquat on embryonic survival and development

The mean hatching rate and mean duration of embryonic development were similar in controls from populations A and B (MW-*U* tests, d.f. = 18, $p > 0.1$; table 1). When compared with the controls (figure 2), the mean hatching rate was significantly reduced in snails exposed to diquat peak concentrations equal to or above $13.7 \mu\text{g l}^{-1}$ in population A (MW-*U* tests, d.f. = 18, $p = 0.00004$), whereas significant reduction of the hatching rate occurred above $6.7 \mu\text{g l}^{-1}$ in population B (MW-*U* tests, d.f. = 18, $p = 0.0288$; table 1). This suggests that embryos responsiveness might be higher in population B than in population A. In both populations, no embryo survived at $55.6 \mu\text{g l}^{-1}$, or at higher concentrations. The mean hatching rates at a given exposure concentration were similar in populations A and B (MW-*U* tests, d.f. = 18, $p > 0.48$ for all studied concentrations), suggesting that the sensitivity of embryos to diquat was equivalent in those populations. In dead embryos, development was interrupted at specific stages, as defined by Lalah *et al.* (2007): all the embryos reached the hippo stage (i.e. fully formed stage) before dying when the diquat peak concentration was between 6.7 and $13.7 \mu\text{g l}^{-1}$, but died at the morula stage (i.e. after the very first cellular divisions) when concentration exceeded $13.7 \mu\text{g l}^{-1}$.

In surviving snails, when compared with the controls, a significant increase in development duration was observed for all the studied concentrations in population A (MW-*U* tests, d.f. = 18, $p = 0.0185$), whereas this increase became significant only for peak concentrations equal to or above $13.7 \mu\text{g l}^{-1}$ in population B (MW-*U* tests, d.f. = 17, $p = 0.00002$; table 1). This suggests that embryos from population A might be more responsive to diquat than those from population B. This is in contrast with the above results regarding the hatching rate. When comparing populations A and B, the mean durations of embryonic development at a given exposure concentration

were similar (MW-*U* tests, d.f. = 18, $p > 0.10$ for all studied concentrations), confirming that embryos from both populations were equally sensitive to diquat. This duration (in days) linearly increased with the actual diquat peak concentration, based on the following relations: duration = $0.64 \times c + 15.85$ ($R^2 = 0.99$) and duration = $0.55 \times c + 15.94$ ($R^2 = 0.97$) in populations A and B, respectively.

(c) Effects of diquat on juvenile survival and development

The number of hatchlings that survived exposure to diquat peak concentrations exceeding $13.7 \mu\text{g l}^{-1}$ was too low to implement a sufficient number of replicates to study juvenile survival properly. Therefore, the life cycle experiment went on only for snails that were exposed to 0 – $13.7 \mu\text{g l}^{-1}$. In this concentration range, we had enough siblings to replace dead snails over the duration of the experiment, so that the bias owing to differences in the number of snails per exposure vessel was avoided. Background mortality, as estimated through the cumulated number of dead snails in controls, was similar in populations A and B (MW-*U* tests, d.f. = 22, $p = 0.75$; table 1).

When compared with controls, both populations experienced a significant increase in juvenile mortality when exposed to diquat (figure 3). In population B, mortality increased after each pesticide application, as shown by the increase in cumulated mortality during the 24 days that followed the application. This was only observed after the first application in population A, since cumulated mortality did not increase faster after the second and following diquat applications. Between applications, the cumulated mortality increased slower after the renewal than before in both populations. Thus, the time course of lethal effects was in accordance with the variation of pesticide concentration in time.

In both populations, no juvenile survived longer than 84 days after hatching when exposed to $13.7 \mu\text{g l}^{-1}$. The cumulated mortality at 84 days in exposed snails was significantly higher, when compared with the controls, for peak concentrations superior or equal to $6.7 \mu\text{g l}^{-1}$ (MW-*U* tests, d.f. = 22, $p = 0.0002$ and $p = 0.0083$ in populations A and B, respectively; table 1). These results suggest that juvenile responsiveness to diquat is equivalent in those two populations. When comparing the two populations, the cumulative number of dead snails at day 84 was significantly higher in population B than in population A when snails were exposed to $3.2 \mu\text{g l}^{-1}$ (MW-*U* tests, d.f. = 22, $p = 0.0083$), equivalent in both populations when snails were exposed to $6.7 \mu\text{g l}^{-1}$ and significantly higher in population A than in population B when snails were exposed to $13.7 \mu\text{g l}^{-1}$ (MW-*U* tests, d.f. = 22, $p = 0.0009$). Therefore, mortality in exposed snails did not provide any clear trend regarding the relative sensitivity of juveniles from the two populations to diquat exposure. Immediate and full restoration of background mortality rates was observed in both populations when survivors were transferred to clean water (day 168).

Figure 2. Mean hatching rate ($\pm$ s.e.) in clutches from the two experimental populations of *L. stagnalis* after the first application of diquat (peak concentrations ranging from 0 to 55.6 $\mu\text{g l}^{-1}$). For each data point, the ratio of the number of hatched embryos to the total number of eggs in the clutch, cumulated over the 10 studied clutches per concentration, is indicated. Significant differences between control and exposed snails, as identified by MW–U tests, are indicated by * $p < 0.05$ and *** $p < 0.001$.

Table 1. Overview of the effects of diquat, as observed in the full life cycle experiment (mean $\pm$ s.e.) and modelled (parameter value $\pm$ 95% CI) using a threshold hazard model for juvenile mortality, in the two experimental populations of *L. stagnalis*. As an indication of responsiveness, threshold diquat concentration ($\mu\text{g l}^{-1}$) refers to the lowest peak concentration inducing a significant effect on the considered endpoint when comparing controls and snails that have been repeatedly exposed to diquat.

experimental endpoints	control value for both populations (mean $\pm$ s.e.)	threshold diquat concentration ($\mu\text{g l}^{-1}$)		relative sensitivity of the two populations
		population A	population B	
hatching rate (%)	98.1 $\pm$ 0.2 ($n = 20$)	13.7	3.2	A $\approx$ B
embryonic stage duration (days)	16.1 $\pm$ 1.7 ($n = 20$)	3.2	13.7	A $\approx$ B
juvenile cumulated mortality (over 84 days)	0.7 $\pm$ 0.9 ($n = 24$)	6.7	6.7	3.2 $\mu\text{g l}^{-1}$: A < B 6.7 $\mu\text{g l}^{-1}$: A $\approx$ B 13.7 $\mu\text{g l}^{-1}$: A > B
age at first spawning (days)	167.2 $\pm$ 4.2 ($n = 16$)	3.2	3.2	3.2 $\mu\text{g l}^{-1}$: A > B $\geq 6.7 \mu\text{g l}^{-1}$: A < B
amount of food consumed (mg lettuce/juvenile/84 days)	120.5 $\pm$ 26.0 ($n = 24$)	3.2	3.2	3.2 $\mu\text{g l}^{-1}$: A < B $\geq 6.7 \mu\text{g l}^{-1}$: A $\approx$ B
model outputs		population A	population B	model reliability
kinetics NEC ($\mu\text{g l}^{-1}$)		rapid 1.1 (0.85; 1.7)	rapid 1.75 (1.40; 2.60)	error frequency 0–4%
killing rate (1 $\mu\text{g}^{-1} \text{d}^{-1}$)		0.0045 (0.0044; 0.0046)	0.0041 (0.0038; 0.0045)	lack-of-fit tests passed
background mortality (d^{-1})		0.00002 (0.000010; 0.000032)	0.00002 (0.000010; 0.000032)	Chow test passed

(d) Effects of diquat on juvenile feeding rate and age at first spawning

The cumulated amount of food eaten per juvenile in 84 days was similar in the controls of both populations (MW–U tests, d.f. = 20, $p = 0.10$; table 1). Exposure to diquat significantly affected food consumption, when compared with controls, in both populations (MW–U tests, d.f. = 20, $p < 0.0001$ for all studied concentrations). Indeed, snails exposed to 3.2 and 6.7 $\mu\text{g l}^{-1}$ ate more food than the controls, whereas snails exposed to 13.7 $\mu\text{g l}^{-1}$ ate less than the controls (figure 4). However, this response was more intense in population A than in population B at 3.2 $\mu\text{g l}^{-1}$ (MW–U tests, d.f. = 20, $p = 0.0003$). On the contrary, it was more intense in population B than in population A at 6.7 and 13.7 $\mu\text{g l}^{-1}$ (MW–U tests, d.f. = 20, $p = 0.0018$ and $p = 0.0387$ at 6.7 and 13.7 $\mu\text{g l}^{-1}$,

respectively). These results suggest that feeding behaviour was modified in exposed snails, in a way that depended on exposure concentration. However, the relative intensity in the change in feeding behaviour of snails from populations A and B provided no clear trend about their plasticity for this life cycle trait.

First reproduction occurred after the last diquat application, at a similar age in the controls of both populations (MW–U tests, d.f. = 17, $p = 0.92$; table 1). Age at first reproduction was significantly higher in exposed snails than in the controls, for all tested concentrations and for both populations (MW–U tests, d.f. = 17, $p < 0.0001$). For example, organisms exposed to 6.7 $\mu\text{g l}^{-1}$ exhibited a one-month delay at first spawning when compared with the controls. The mean age at first reproduction was higher in population B, when compared with

Figure 3. Time course of juvenile cumulated mortality ($\pm 95\%$ confidence interval, as simulated by bootstrap over 12 replicates of five snails) within the next 84 days post-hatching in the two experimental populations of *L. stagnalis* repeatedly exposed to diquat. Lines are model fits, and points are actual data. Diquat treatment (peak concentrations ranging from 0 to $13.7 \mu\text{g l}^{-1}$) and water renewal occurring during the juvenile stage are indicated by the symbols T and R, respectively. Significant differences between control and exposed snails, as identified by MW-U tests, are indicated by $**p < 0.01$ and $***p < 0.001$. Diamonds, $0 \mu\text{g l}^{-1}$; squares, $3.2 \mu\text{g l}^{-1}$; triangles, $6.7 \mu\text{g l}^{-1}$; crosses, $13.7 \mu\text{g l}^{-1}$; dash-dotted line, $0 \mu\text{g l}^{-1}$; green line, $3.2 \mu\text{g l}^{-1}$; red line, $6.7 \mu\text{g l}^{-1}$; blue line, $13.7 \mu\text{g l}^{-1}$.

population A, for snails exposed to diquat concentrations below $6.7 \mu\text{g l}^{-1}$ (MW-U tests, d.f. = 20, $p < 0.012$). Yet, it was similar in both populations for snails exposed to $6.7 \mu\text{g l}^{-1}$ (MW-U tests, d.f. = 19, $p < 0.082$). Therefore, there was no clear trend in the relative impact of diquat on age at first reproduction in the studied populations, indicating no difference in their sensitivity.

(e) Modelling mortality with the hazard model

Mortality data analysis in juvenile snails from population A provided the following model parameter estimates (and 95% confidence intervals): $c_0 = 1.1$ (0.85; 1.7) $\mu\text{g l}^{-1}$, $k_t = 0.0045$ (0.0044; 0.0046) $\text{l } \mu\text{g}^{-1} \text{d}^{-1}$, $\lambda = 0.00002$ (0.000010; 0.000032) d^{-1} . Optimal fittings were computed assuming that the elimination rate was infinite in the TK part of the model. This assumption implies that elimination/detoxication of the absorbed toxicant should rapidly occur in exposed snails. The predicted background mortality rate in population A was low. The NEC value was inferior but close to the lowest actual peak concentration ($3.2 \mu\text{g l}^{-1}$). This was expected because no significant mortality occurred at this concentration.

Figure 4. Cumulated amounts of food ($\pm \text{s.e.}$) eaten by juveniles from the two experimental populations of *L. stagnalis* exposed to diquat (peak concentrations ranging from 0 to $13.7 \mu\text{g l}^{-1}$) within the next 84 days post-hatching. Significant differences between control and exposed snails, as identified by MW-U tests, are indicated by $***p < 0.001$. White bar, population A; grey bar, population B.

With these parameter values, 96 per cent of the predicted cumulated mortalities remained in the 95 per cent confidence interval of data. Only one value (at day 28 for snails exposed to $6.7 \mu\text{g l}^{-1}$) was slightly overestimated, as shown in figure 3.

Mortality data analysis in population B provided the following estimates and 95 per cent confidence intervals: $c_0 = 1.75$ (1.40; 2.60) $\mu\text{g l}^{-1}$, $k_t = 0.0041$ (0.0038; 0.0045) $\text{l } \mu\text{g}^{-1} \text{d}^{-1}$, $\lambda = 0.00002$ (0.000010; 0.000032) d^{-1} . As in population A, optimal fittings were computed under the assumption of rapid kinetics. The predicted background mortality rate was low and similar to that of population A. The killing rate values in populations A and B were also close, with overlapping 95 per cent confidence intervals. The NEC was slightly higher in population B, when compared with population A, with overlapping 95 per cent confidence intervals. All the predicted cumulated mortalities remained in the 95 per cent confidence interval of data (figure 3).

Significant differences in parameter values between populations were investigated using the Chow test. Fitting the model to pooled data from populations A and B led to the following parameter estimates: $c_0 = 1.65 \mu\text{g l}^{-1}$, $k_t = 0.0044 \text{ l } \mu\text{g}^{-1} \times \text{d}^{-1}$, $\lambda = 0.00002 \text{ d}^{-1}$. With three model parameters and 18 data points per population, the Chow test statistics followed the *F*-distribution with three and 30 degrees of freedom. It highlighted no significant difference between the parameter values (c_0 , k_t , λ) estimated in populations A and B ($p > 0.05$). The lack-of-fit test was implemented in populations A and B for each studied diquat concentration. All the *F*-statistics were inferior to one (thus leading to $p > 0.05$), showing that the error owing to the lack of fit was not significantly superior to the background pure error. Therefore, our model provided good fits to the data.

4. DISCUSSION

(a) Effects of diquat in *L. stagnalis*: comparison with other studies

Repeated exposures to diquat induced significant mortality in *L. stagnalis* even at low concentrations. Only

65 per cent of the exposed embryos survived to peak concentrations of $13.7 \mu\text{g l}^{-1}$, and their development time almost doubled. Juveniles further exposed to $13.7 \mu\text{g l}^{-1}$ died before they could reproduce. Moreover, snails repeatedly exposed to $6.7 \mu\text{g l}^{-1}$ suffered large mortalities (30–40% within three months), grew slower and did not reproduce in contaminated water. Effects were less pronounced, but still statistically significant, when the snails were repeatedly exposed to $3.2 \mu\text{g l}^{-1}$. This suggests that early development stages of *L. stagnalis* are very sensitive to diquat.

Based upon model descriptions, no deleterious effect on juvenile survival was expected in *L. stagnalis* for actual diquat concentration below $0.85 \mu\text{g l}^{-1}$. This is consistent with the results of feeding experiments with diquat-contaminated leaves, which showed reduced survivorship in juveniles of *Lymnaea elodes* at $1.0 \mu\text{g l}^{-1}$ (Fronza & Kendrick 1986). However, another study with *L. stagnalis* showed that 8 days of exposure to diquat-treated water led to an LC_{50} (i.e. lethal concentration for 50% of the exposed snails) of $1.0 \mu\text{g l}^{-1}$ (development stage and experimental conditions not reported; Carter 1971 in Emmett 2002). This discrepancy is probably due to differences in experimental conditions, with respect to both exposure pattern and genetically based sensitivity of *L. stagnalis* strains. Indeed, it has been shown that intermittent exposure to peak concentrations might lead to more or less pronounced effects than continuous exposure (mean daily concentration being equal), depending on the species, toxicant and treatment patterns (Reinert *et al.* 2002).

Published data regarding effects of diquat on the life cycle of aquatic organisms only consist of the results of 21-day tests with *D. magna*. They highlighted significant reproductive effects above $0.057 \mu\text{g l}^{-1}$ (Emmett 2002). Macrophytes and algae are among the most sensitive aquatic organisms to diquat. Indeed, the lowest observed effect concentration-96h ($\text{LOEC}_{-96\text{h}}$) for the duckweed *Lemna minor* and the algae *Selenastrum capricornutum* has been estimated at 0.018 and $0.039 \mu\text{g l}^{-1}$, respectively (Emmett 2002). Yet, those results are currently not taken into account for the purposes of registration because plants are the intended targets of aquatic herbicides containing diquat. Based upon our present results, the long-term sensitivity of *L. stagnalis* to diquat might be lower than the sensitivity of standard species for toxicity tests. The relevance of including these results when reassessing the safety of diquat on aquatic organisms remains to be evaluated.

(b) Relevance of the modelling

Survival models in ecotoxicology usually assume either stochastic death or individual selection for tolerance. It is not clear when these hypotheses apply, and which factors they depend on (Ashauer & Brown 2008). However, in our study, individual selection for tolerance would imply that the most sensitive organisms would die after the first diquat application, selecting tolerant snails, so that mortality rates should progressively decrease with successive applications. Our data do not support this hypothesis because the second and

further applications did induce substantial mortalities, and systematic decrease in mortality rates did not occur. Therefore, the use of the proposed model was appropriate to assess effects of diquat on the snails.

Regarding TK and according to the estimates of k_a , the elimination/detoxication of the compound was assumed to be rapid (i.e. low values for depuration half-life). According to its pharmacokinetic properties (reviewed in Emmett 2002), this latter assumption may apply to diquat. Indeed, diquat is a highly charged cation, very water soluble ($\log K_{ow} = -4.6$) and highly lipid insoluble. Moreover, it is minimally metabolized and not bioaccumulated. Therefore, unbound molecules entering the body are mainly distributed throughout the organs in the aqueous phase, sequestered in tissues towards eventual excretion by the kidney. Excretion is assumed to occur within 2 days in vertebrates (Emmett 2002). This result is in agreement with previous studies on fishes, daphnids, oysters, clams and mayflies (Emmett 2002), which exhibited depuration half-lives of between 1 and 3 days.

Regarding TD, the hazard rate directly relates to the internal concentration in our model. Therefore, effects are supposed to occur as soon as $c_q(t) > c_0$, which rapidly happened (according to the TK part of the model). Effects of diquat are known to result from the production of the superoxide free radical, which may lead to lipid peroxidation and cytotoxicity (reviewed in Stohs 1995). The onset of such effects at the cell level might occur within the first days of peak exposure to diquat in *L. stagnalis* (Lefeuvre-Orfila *et al.* 2005). Based upon this study, the underlying hypothesis regarding the onset of toxic effects in our model may thus be verified. Furthermore, the proposed model also assumes full and instantaneous recovery occurring once exposure ceases. The present restoration of background mortality rates data support this assumption for *L. stagnalis* exposed to diquat.

The model adequately expressed the impact of changes in exposure concentrations on effect trajectories for snails from populations A and B. Indeed, predicted survival rates remained in the 95 per cent confidence interval of data even several weeks after the treatment, when such changes in concentration were subtle. Even though the time course of effects in relation to the time-varying exposure was well described, the number of dead snails in populations A and B were slightly underestimated at $20 \mu\text{g l}^{-1}$, whereas it was slightly overestimated at $5 \mu\text{g l}^{-1}$ in population A (figure 3). Those discrepancies resulted from a constant bias in the predicted values. This result suggests that some of the model assumptions were not verified for all the exposure concentrations. In particular, control and exposed snails are supposed to be submitted to similar environmental conditions, except the presence of the toxicant. This was verified for temperature and light, but not for food at the concentration where discrepancies between model predictions and data were found. Indeed, we observed that the cumulated amount of food eaten per snail after 84 days corresponded to 363 and 18 per cent of the control values in snails from population A exposed to 3.2 and $13.7 \mu\text{g l}^{-1}$, respectively. The ingestion

rate of snails from population B exposed to $13.7 \mu\text{g l}^{-1}$ of diquat was only 62 per cent of the control value. Therefore, food consumption might have acted as a modulating factor in the biological response of the snails.

(c) Food consumption and evolutionary history as modulating factors of diquat toxicity

Food consumption was significantly affected by diquat exposure. At low concentrations ($3.2 \mu\text{g l}^{-1}$), increased food consumption probably constitutes a behavioural response enabling the snails to compensate for the extra amount of energy which was required to cope with reactive oxygen species (ROS) generated during exposure. Indeed, depletion of glycogen reserves and subsequent energy allocation to detoxication of ROS have been observed in *L. stagnalis* exposed to fomesafen, a peroxidizing herbicide (Jumel *et al.* 2002). Higher diquat concentrations led to an opposite behavioural response: snails exposed to $13.7 \mu\text{g l}^{-1}$ avoided the food which was spoiled by the herbicide as shown by a change in tissues structure and colour. This confirmed previous results on diquat-induced changes in food palatability for *L. elodes* (Fronza & Kendrick 1986). However, in our study, reduced food intake was not directly responsible for the death of exposed snails. Indeed, independent feeding experiments in similar conditions of temperature and light showed that unexposed juvenile snails could survive up to $49 (\pm 7)$ days without food before dying due to starvation (V. Ducrot 2008, unpublished data). However, food shortage in diquat-exposed snails may have impaired ROS detoxication owing to reduced energy input, leading to high mortalities. Similar modulation by food was observed in *D. magna* exposed to fenvalerate in *ad libitum* and food-limiting conditions (Pieters *et al.* 2006), but modulation pathways were different: since food was the major exposure route, it was concluded that modulation was mainly due to differences in TK. However, both studies confirm that effects of pesticides would depend on the feeding rate of the exposed organisms, and this may have important implications in both standard laboratory toxicity tests and field populations. Further studies are required to investigate this hypothesis in *L. stagnalis* exposed to diquat. For instance, using feeding rate and diquat concentrations in the food as additional dependant variables in the survival model might allow accounting for a cross-effect between food and diquat. This could not be achieved in this study owing to the lack of data regarding diquat concentration in the food.

Considering the role of evolutionary history as a modulating factor of survival, no clear trend appeared from the statistical analysis of data regarding the relative sensitivity and responsiveness in the studied populations (table 1). Furthermore, the Chow test pointed out that these populations could not be distinguished based upon the parameter values of the survival model (c_0 , k_t and λ), thus suggesting that they exhibited similar background mortality, sensitivity and responsiveness to diquat with respect to survival. These results suggest that, in the present study, evolutionary history had no significant influence

on snail response to diquat (regarding survival and stage duration in embryos and juveniles). Additional comparative studies on growth and reproduction in populations A and B or in populations of different geographic origins are required in order to investigate whether or not the evolutionary history of exposed snails might influence their biological responses to diquat.

5. CONCLUSIONS

The proposed full life cycle experimental design was suitable to assess and model long-term effects of repeated exposures to diquat, and to study the role of food consumption and snail evolutionary history as potential confounding factors of the responses to this herbicide. Effects of diquat on snail survival were described using a threshold hazard model. Incorporating TK in this model allowed accounting for the dynamics of effects over exposure duration. Because the NEC has been recognized as a relevant effect criterion, the present results could be directly used for chemical risk assessment, by following instructions provided in guidance documents (Organisation for Economic Cooperation Development 2006, ch. Biology-based methods). They highlighted that low diquat concentrations (greater than or equal to $1 \mu\text{g l}^{-1}$) may induce significant adverse effects on hatching rate, embryonic stage duration, juvenile mortality rate and age at maturity in the freshwater snail *L. stagnalis*. However, mortality effects immediately ceased when clean conditions were restored. The present study also provided preliminary information on the modulating factors of the biological responses to a repeated toxic stress in *L. stagnalis* experimental populations. The evolutionary history of exposed snails had no consequence on their survival and development pattern. Yet, diquat led to changes in the feeding rate of exposed snails, which may have influenced their responses to the herbicide. Further modelling, with an energy-based approach (e.g. based on the DEB theory; Kooijman 2000) and measurements of diquat concentration in food, could allow accounting for the role of this factor on diquat kinetics and effects.

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