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Using sets of behavioral biomarkers to assess short-term effects of pesticide: a study case with endosulfan on frog tadpoles

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Abstract

Pesticides and other chemicals often have detrimental effects at environmental concentrations. Many amphibian species are particularly threatened because of their susceptibility but also because wetlands are often polluted. Behavioral assessments of toxicity have the advantage of showing sublethal effects but quantitative measures at varied scales of integrations are rarely considered together. In this study, we aimed at showing that these behavioral endpoints could be differently affected across time and concentrations, and be biomarkers of toxicity. To this end, we tested the effects of an organo-chlorine pesticide (endosulfan) on amphibians during a standard 96 h test. We evaluated possible lag effects in continuing the analyses after removal of the pesticide. The study was based on 240 tadpoles (4 pesticide treatments: 0.4, 3, 22, and 282 µg/l, 1 control and 1 solvent-control). Abnormal behaviors such as lying and swirling rapidly were exhibited only in the presence of the pesticide. Essential functions such as breathing and feeding were deeply affected by the pesticide: contaminated tadpoles breathed and fed less than control tadpoles. They also moved less and occupied a more central position in the aquariums in the presence of the pesticide. A higher mortality was only found at the highest concentration. These results suggest that endosulfan is toxic to amphibians at environmental concentrations. Behavioral markers showed potential as early warning systems. They should thus be used in complement to other markers to detect sublethal effects only a few days after application of the pesticide and at concentrations where mortality does not occur.

Keywords: Amphibian decline - Behavioral markers - Endosulfan - Mass - Sublethal effects - Survival

Introduction

The most common evaluation of pesticide toxicity is based on short-term (e.g., 96 h) acute lethality tests of continued exposure such as those providing LC50 values (Carlile 2006). Whereas this method has the advantage of giving a quantitative and comparable assessment of mortalities, it does not account for sublethal effects. To cope with this, short-term early embryo-larval assays (FETAX) have been recognized as toxicity tests (Bromhall 2005). Longer studies involving chronic exposure to chemicals until metamorphosis (Newman et al. 2006; Relyea and Hoverman 2006), post-exposure tests at the larval stage (Berrill et al. 1998; Jones et al. 2009) but also through the entire life cycle (Hayes et al. 2010; Kvarnryd et al. 2011) allowed researchers to examine the effects of pollutants at low concentrations on the long term. Both short and long term studies provided fine scale data such as gene or protein signature (Gillardin et al. 2009a), cytology (Marquis et al. 2010), physiology (Gillardin et al. 2009b), morphology (Bernabò et al. 2008), and behavior (Bromhall 2005; Giusi et al. 2010; Egea-Serrano et al. 2011). Both short and long-term methods are complementary, but it would be useful to develop more sublethal markers for short-term tests.

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Endosulfan is an organochlorine pesticide that has been used since the fifties in many parts of the world over a large variety of crop fields (Jia et al. 2009). It is now found in soil, water and air at long distances from application sites (Weber et al. 2010). It can bioaccumulate over the food chain (Kelly et al. 2007) and is toxic not only for the targeted insects and acarids, but also for non-target animals such as crustaceans (Dorts et al. 2009; Tu et al. 2009), fish (Stanley et al. 2009; Carriger et al. 2011), amphibians (Brunelli et al. 2009; Jones et al. 2009), and mammals, including humans (Saiyed et al. 2003; Caride et al. 2010). At environmental concentrations, it can cause mortalities (Brunelli et al. 2009) and sublethal effects, such as inhibition of cholinergic neurotransmission (Tu et al. 2009), alterations of hormonal and pheromonal profiles (Park et al. 2001; Thangavel et al. 2010), sex reversal (Palma et al. 2010), and varied behavioral alterations such as feeding, swimming, breathing, and activity patterns (Tu et al. 2010). The toxicity and environmental persistence of endosulfan conducted numerous national authorities to ban it and to propose its inclusion as persistent organic pollutant in the Stockholm convention (Kelly et al. 2007; U.S.EPA 2010). However, despite these regulations and/or limitations, it is still largely used at a world scale, particularly in some developing countries.

The last U.S. Environmental Protection Agency report (U.S.EPA 2010) on endosulfan highlighted the need for more work to consider amphibians as model species. Amphibian populations have been identified to be at major risk on a global scale, in part because of their high sensitivity to environmental contamination (Stuart et al. 2004; Boone et al. 2007), which may make them good biological models (Hopkins 2007). The biphasic life of many species often constrains amphibians to reproducing in waters close to agricultural or urban fields, and their skin and egg membranes are highly permeable to pollutants (Wells 2007; Croteau et al. 2008). Previous studies on the effects of endosulfan on amphibians showed detrimental effects on survival at environmental concentrations during and after exposure (See e.g. Jones et al. 2009 for a multi-species analysis). At lower concentrations, varied effects were found, including morphological deformities (Kang et al. 2008) and gill structure alterations (Bernabo` et al. 2008; Brunelli et al. 2010). At the behavioral level in contaminated tadpoles, Berrill et al. (1998) observed a lack of avoidance response to a simulated predator, feeding suppression and lag effects in two ranid species (*Rana clamitans* and *R. sylvatica*), whereas Westman et al. (2010) found longer times to quiescence after induced stress in one treefrog (*Pseudacris regilla*) and spadefoot toad (*Spea intermontana*). In their study on toad tadpoles, Brunelli et al. (2009) documented more immobile behavior and also reported irregular swimming.

Behavioral ecotoxicology is an emerging field providing multiple ways to quantify responses from contaminated organisms (Bromhall 2005; Denoël et al. 2010; Giusi et al. 2010). Studying behavioral endpoints is particularly relevant because they could link varied physiological disorders with ecological processes and because they could be exhibited before other traits, with possible direct consequences on the organisms (Tu et al. 2010). Because such endpoints are quantitative, there are available behavioral markers to assess toxicity in complement to other standard markers (Scott and Sloman 2004). The observed neutotoxic effects and behavioral alterations suggest that endosulfan is a good candidate to test the effectiveness of behavioral markers. However, tests on sets of behavioral markers in the framework of a single experiment remain scarce. In this perspective, we aimed to determine (1) if endosulfan affects six different behavioral endpoints at varied levels of behavioral integrations, including activity patterns, abnormal behaviors, feeding, breathing, and space use; (2) if these endpoints are effective markers, i.e. if they are affected before any noticed effects on survival or in situations not impacting mortality at identical concentrations; and (3) if lag effects are expressed for all the endpoints. To this end, endpoints were quantified two times a day since the beginning of exposure to endosulfan.

Materials and methods

Field and laboratory maintenance

We used 240 tadpoles of a ranid frog (*Rana temporaria*) at their early larval stage (Gosner stage 26: Gosner 1960) from a stock of eggs which hatched in the laboratory. The eggs came from four freshly laid clutches sampled at La Mare aux Joncs (Liege Province, Belgium, 50°34'18"N–5°30'35"E, elevation 250 m a.s.l.) on March 2011. The pond was not contaminated by endosulfan, as this pesticide was not both historically and recently used close to the pond and was not detected by chromatography (see hereafter for the technique). Endosulfan is also now locally forbidden (E.U. decision 2005/864/EC5). The tadpoles were randomly distributed among 24 3-l aquaria with 10 individuals/aquarium (15 cm x 24 cm x 8 cm high). Individuals from the four clutches were placed in different aquariums in order to take into account the clutch of origin in the analyses. The walls of the aquariums were semi-transparent. Soft water was reconstituted from deionized tap water following APHA recommendations (APHA 1985): NaHCO3: 48 mg/l, CaSO4•2H2O: 30 mg/l, MgSO4•7H2O: 61 mg/l, KCl: 2 mg/l. Water was renewed every evening (at the end of each 24 h periods) with a fresh stock during the 8 day of the experiment to keep similar conditions daily (Hoke and Ankley 2005). Tadpoles were manipulated gently during water change that took only a few minutes per aquarium. They were placed in the same model of tank, filled with the same water but without pesticide, as their experimental tank during water change.

Values up to 1.7 mg/l of endodulfan have been reported in polluted waters whereas amphibian survival was usually lowered at concentrations above 500 µg/l (see e.g., Ernst et al. 1991; Brunelli et al. 2009; Jones et al. 2009; Srivastava et al. 2009). Preliminary observations also suggested mortalities at 500 µg/l in *Rana temporaria* (Denoël M.& D'Hooghe B., unpublished data). As our aim was to determine sublethal effects as well as LC50 and LC10 values, we used four nominal concentrations of endosulfan pesticide (0.5, 5, 50 and 500 µg/l) including the solvent (pure ethanol), and also one control (only reconstituted water) and one second control with reconstituted water and the solvent only. The actual mean concentrations (±SE) of endosulfan during the experiment were 0.4 ±0.1, 3 ±0.2, 22.3 ±1.9 and 281.6 ±34.6 µg/l (n =39 samples, with 3 or 4 samples per treatment at times 0 and 24 h, the same tanks being sampled both times), henceforth referred to as 0.4, 3, 22, and 282 µg/l respectively (see hereafter for details on the chemical analyses). The difference between nominal and actual concentrations of endosulfan is mainly due to the low affinity of endosulfan in water at high concentrations (Guerin 2001; Jones et al. 2009). No endosulfan was detected in the controls. Endosulfan and ethanol were analytical "Dr Ehrenstorfer" grade purchased from

Cluzeau Info-Labo (France). The solvent-control was used because of endosulfan's low solubility in water (Marquis et al. 2006; Jones et al. 2009). It was the lowest concentration to allow solubilisation of the pesticide. The amount of ethanol added was the same as that one used in the endosulfan concentration treatments (33 µl/l). Endosulfan was added daily just after water change and before to replace tadpoles in their tanks. After this 4-day experiment, tadpoles were maintained in reconstituted water (without the pesticide and the ethanol) for another 96 h-period. Organic spinach leaves previously boiled, frozen and thawed to increase digestibility by tadpoles, were given ad libitum (2 leaves of 2 cm^2/tank) every evening, i.e. after the daily observations. This food resource is typical in laboratory experiments on ranid tadpoles and has the advantage of being easily quantified, particularly for behavioral observations (Denoël et al. 2010). Photoperiod followed the natural cycle of the capture place, i.e. 12 h 30 light–11 h 30 dark. Water temperature and dissolved oxygen were maintained at a mean ±SE of 13.99 ± 0.05°C and 9.68 ± 0.04 mg/l respectively (n = 32, taken randomly during the experiment). Temperature and oxygen concentration did not vary between exposure and post-exposure periods (t_{30} = 1.112, P = 0.30 and t_{30} = -0.048, P = 0.96 respectively).

Quantification of endosulfan in water

To determine the actual endosulfan concentrations in the tanks, water samples were analyzed by gas chromatography with time-of-flight mass spectrometer (GC-TOFMS). Endosulfan and Mirex (internal standard) were analytical "Dr Ehrenstorfer" grade purchased from Cluzeau Info-Labo (France). Chemicals solvent were obtained from Sigma-Aldrich (Germany) for isooctane and VWR (USA) for ethanol and ethyl acetate. The water samples were first extracted following a solid phase extraction method as described by De la Colina et al. (1996). In this purpose, Supelco Supelclean™ ENVI-18 SPE cartridges were used (1 g, 6 ml) (Supelco, Bellefonte, PA, USA) with a 5 ml volume of isooctane/ethyl acetate (v:v/50:50). The elution fraction was concentrated to 50 ll using a gentle stream of nitrogen, after which 50 llof Mirex were added as internal standard. The purified extracts were injected on a LECO Pegasus 4D GC-TOFMS (LECO corp., St Joseph, MI, USA) using a 30 m x 0.18 mm x 0.2 µm Rtx-5ms column (Restek, Bellefonte, USA). The gas chromatography oven ramp temperature was started at 110°C during 1 min, then increased to 200°C with a rate of 30°C/min, then to 260° with a rate of 5°/min and held for 5 min. The mass spectrometry transfer line temperature was 250°C. The ion source temperature was 230°C with electron ionisation (EI) energy of 70eV. The collected mass range was 35–600 amu with a scan rate of 20 spectra/s and detector voltage of 1650 V.

Measures and statistical tests

Behavioral observations were made two times a day at similar hours (10:00, 14:00, local time) during 2 min-periods by aquarium by the same observer. The observer moved slowly in front of the tanks and remained stationary during the observation periods. This did not cause changes in the behavior of tadpoles. The six behavioral endpoints were recorded and were based on the proportion of surviving tadpoles in each tank exhibiting the following behaviors at least once during the time period:

(1) swirling, i.e. abnormal fast rotations which are a sign of neurotoxic stress (Brunelli et al. 2009).

(2) lying on the lateral or dorsal side, i.e. an abnormal immobile behavior with the lateral surface area of the tail more or less parallel to the substratum (hereafter, lying on the lateral side). The normal posture is with the dorsal side upward. Lack of correct equilibrium posture is considered to be a good biomarker of toxicity (Fordham et al. 2001).

(3) air surface breathing, which complements aquatic breathing in active tadpoles (Gdovin et al. 2006; Wells 2007).

(4) feeding, which is a sensitive indicator of toxicity as food is a requirement for growth and other physiological functions (McWilliam and Baird 2002; Egea-Serrano et al. 2009).

(5) activity, i.e. presence of swimming patterns, a typical behavior assessed in ecotoxicological studies on tadpoles (Brunelli et al. 2009; Egea-Serrano et al. 2011).

(6) space use of the peripheral area of the tanks, i.e. within two cm from the edges (this distance is slightly larger than tadpole size). Depending on space use, tadpoles could be differently exposed to predation (Laurila 2000; Eterovick et al. 2010). We expect that tadpoles would be more visible to predators in open areas than along "walls".

These six behavioral endpoints are not necessarily mutually exclusive as the tadpoles can exhibit several acts during the sampling period. The number of surviving tadpoles was recorded at the same time; dead tadpoles were removed from the aquariums. This design was used during the two successive periods of 4 days (except for the highest concentration treatment which killed almost all tadpoles at the end of the first period). At the end of the experiment, the surviving tadpoles were weighed on an electronic balance (Pioneer PA64, Ohaus, NJ, USA). We present only mass data because length data gave similar results. All tadpoles were euthanatized in a Benzocaine solution (250 mg/l) at the completion of the experiment, conforming with the recommendations of the European Commission (Close et al. 1996) and as approved by the ethical committee of the university.

Generalized mixed models (GLMM), assuming binomial error distributions, were used to evaluate the effects of the six treatments on the frequency of behavioral endpoints and survival during the two 96-h periods. Binomial models are more appropriate to these data and provide more statistical power, comparing to using the percentage of individuals as dependent variable (Venables and Ripley 2002). Similarly, we used GLMMs assuming normal error to evaluate the effects of the treatment on the body mass of tadpoles at the end of the experiment. Body mass was log-transformed prior to perform this analysis to achieve normality of residuals (Shapiro–Wilk's test for normality: P = 0.37). In all these models, endosulfan concentration was included as a fixed factor. Clutch of origin and aquarium nested within clutch were included as random factors. In all GLMMs we used treatment contrasts to identify which treatments significantly differed from controls (Venables and Ripley 2002). We assessed significance of GLMMs using likelihood ratio tests. Correlations between behavioral endpoints at day 1 and survival at day 4 were computed with Spearman tests. Correlations using behavioral endpoints at day 2 gave the same results (not shown). To estimate the LC10 and LC50 at 4 days, we used probit analysis to fit a sigmoid-shaped curve to the data, using log-transformed endosulfan concentration as independent variable. Only one more tadpole died

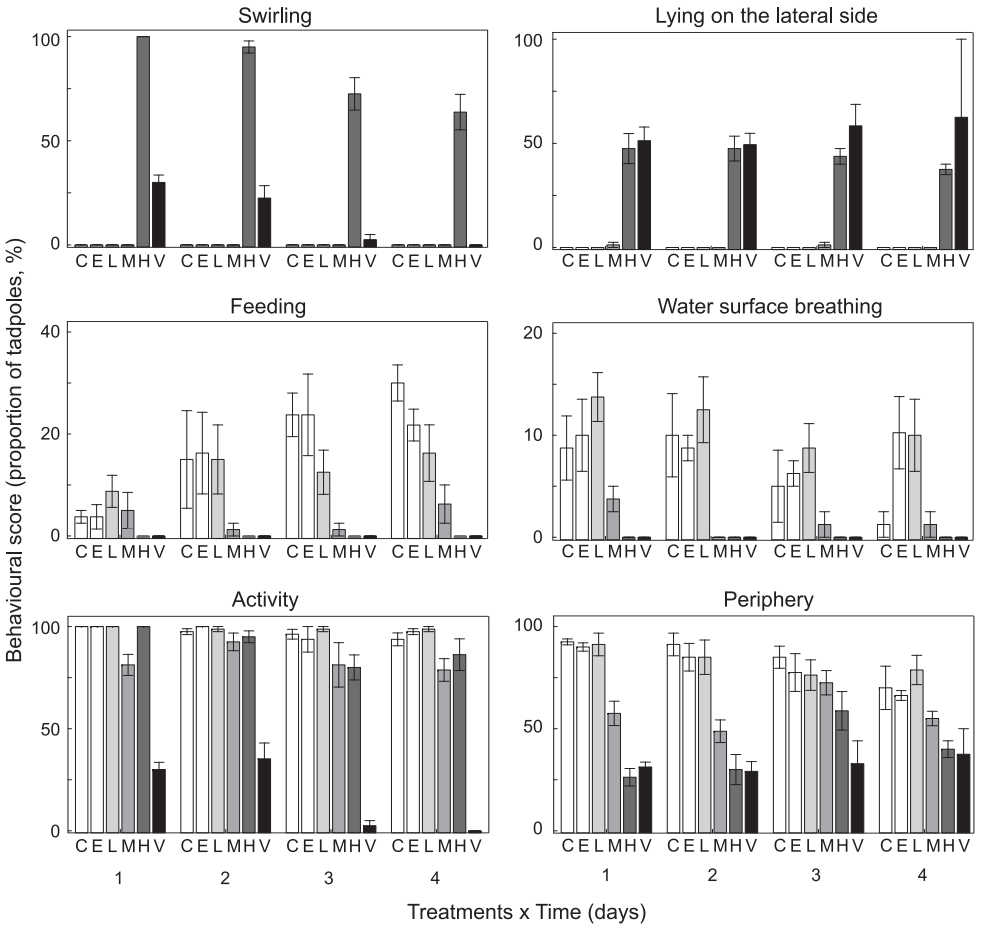

Fig. 1. Behavioral endpoints of tadpoles (mean ± SE proportions of individuals) as a function of endosulfan concentration over time: swirling (fast circular swimming movement), lying on the lateral side (abnormal posture), feeding, surface air breathing, activity (swimming), and space use of the peripheral area. See Table 1 and text for the results of statistical tests. *C* control (*left white bar*), *E* ethanol-solvent control (*right white bar*), *L* low concentration (0.4 µg/l, *light grey bar*), *M* medium concentration (3 µg/l, *grey bar*), *H* high concentration (22 µg/l, dark grey bar), and *V* very high concentration (282 µg/l, black bar)

between 4 and 8 days from the beginning of the experiment, therefore we did not estimate LC10/LC50 at 8 days. Mixed models were performed in R 2.12 (www.r-project.org) using LME4 and NLME (Pinheiro and Bates 2000; Bates and Maechler 2010). Probit analysis was performed in SPSS 19.

Results

First 96-h endosulfan exposure

Tadpoles exhibited two behavioral endpoints that only appeared in the endosulfan treatments (Fig. 1; Table 1): swirling and lying on the lateral side. These behaviors were observed more frequently in the two highest endosulfan concentrations (control contrasts, both $P \leq 0.001$), but not at the lowest ones (swirling, all $P > 0.9$; lying: all $P > 0.09$). Surface air breathing was significantly lower (Fig. 1; Table 1) in the three highest endosulfan concentrations than in the controls (control contrasts, all $P \leq 0.002$), and was slightly higher than in the control at the 0.4 µg/l concentration ($P = 0.038$). Tadpoles fed less frequently (Table 1) in the three highest concentrations (all $P \leq 0.001$), but frequency of feeding in the lowest concentration was similar to that in the controls ($P = 0.13$). The use of the peripheral area of the aquariums was lower in the three highest concentrations than in the controls (all $P \leq 0.001$), while it was not different from controls at the 0.4 µg/l concentration ($P = 0.59$). Tadpoles were less active in the three highest concentrations than in the controls ($P \leq 0.05$), but all showed a similar activity at the 0.4 µg/l concentration ($P = 0.10$).

For most parameters, the interaction between treatment and day was not significant. However, a significant day x treatment interaction for periphery and activity indicates that effect of treatment on these behaviors was not constant through time. Both swirling and lying appeared at the first day of exposure in the two highest concentrations, feeding was lower than in controls at day 1 in the two highest concentrations, and at day 2 at the 3 µg/l concentration, breathing was lower than in controls in the three highest concentration since the first day, periphery use was lower than in controls in the three highest concentration since the first day, activity was lower than in controls at the 282 µg/l concentration at day 1, and at day 3 at the 3 and 22 µg/l concentrations (Fig. 1; Table 1).

The solvent (ethanol) had no significant effect on the six behavioral endpoints (control contrasts: swirling, $P > 0.9$; lying on the lateral side, $P > 0.9$; breathing, $P = 0.59$, feeding, $P = 0.22$; periphery: $P = 0.19$; activity: $P = 0.49$, Fig. 1).

Tadpole mortality was significantly affected by treatment ($\chi 2_5 = 21.5$, $P \leq 0.001$). All tadpoles survived in both controls and in most of the lowest endosulfan concentrations. After 4 days, 2.5% tadpoles died at a concentration of 22 µg/l of endosulfan and 92.5% at a concentration of 282 µg/l (Fig. 2). Only mortality at the highest concentration was significantly higher than in the controls ($P \leq 0.001$). At the fourth day, the concentration

Table 1. GLMMs evaluating the effect of treatment (4 concentrations of endosulfan, 1 control and 1 solvent-control), days and their interaction on six behavioral endpoints during the first and last four days of the experiment (i.e. before and after removal of the pesticide and solvent): swirling (fast circular swimming movement), lying on the lateral side (abnormal posture), feeding, surface air breathing, activity (swimming), and space use (periphery of tanks)

Variables	Factors	Days 1-4			Days 5-8		
		d.f.	χ^2	*P*	d.f.	χ^2	*P*
Swirling	Treatment	5	72.78	**<0.001**			
	Days	3	67.16	**<0.001**			
	Treatment x Days	15	4.62	>0.9			
	Clutch	3	7.46	0.059			
Lying on the side	Treatment	5	62.28	**<0.001**	4	18.77	**<0.001**
	Days	3	2.27	0.518	3	4.63	0.201
	Treatment x Days	15	5.35	>0.9	15	0.1	>0.9
	Clutch	3	0.58	0.900	3	0.1	0.9
Feeding	Treatment	5	53.16	**<0.001**	4	25.63	**<0.001**
	Days	3	32.00	**<0.001**	3	33.99	**<0.001**
	Treatment x Days	15	17.95	0.265	15	28.35	0.005
	Clutch	3	15.78	0.001	3	0.79	0.851
Air breathing	Treatment	5	60.47	**<0.001**	4	34.37	**<0.001**
	Days	3	4.78	0.189	3	5.42	0.144
	Treatment x Days	15	9.57	0.846	15	7.97	0.787
	Clutch	3	<0.1	>0.9	3	0.1	>0.9
Activity	Treatment	5	45.43	**<0.001**	4	35.16	**<0.001**
	Days	3	35.65	**<0.001**	3	6.79	0.079
	Treatment x Days	15	31.48	**0.008**	15	18.46	0.103
	Clutch	3	11.88	**0.008**	3	1.031	0.794
Space use	Treatment	5	49.75	**<0.001**	4	18.05	**0.001**
	Days	3	17.67	**<0.001**	3	45.97	**<0.001**
	Treatment x Days	15	57.10	**<0.001**	15	34.59	**0.001**
	Clutch	3	<0.1	>0.9	3	1.97	0.58

Clutch is introduced as a random effect in the models. See Fig. 1 and text for differences between treatments and during the two time periods

Bold values are statistically significant

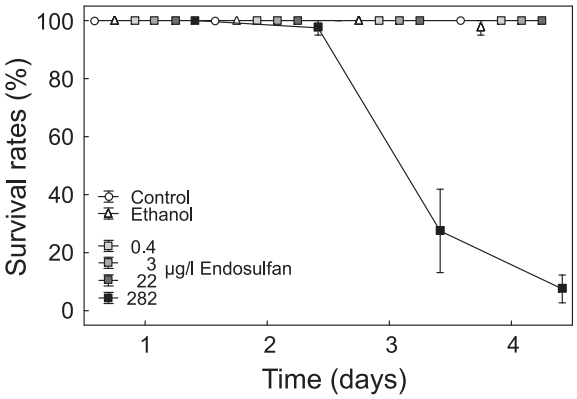

Fig. 2. Survival rates of tadpoles (mean ± SE values) as a function of endosulfan concentration over time. See text for the results of statistical tests. *Open circles* controls, *open triangles* solvent-controls, *squares* endosulfan (*light shaded* 0.4 µg/l, *shaded* 3 µg/l, *dark shaded* 22 µg/l, *black* 282 µg/l)

that killed 10 and 50% of tadpoles was 54 µg/l (95% CI: 12–95 µg/l) and 115 (95% CI: 49–168 µg/l) respectively.

Average survival in a batch at the end of the experiment was strongly correlated to several behavioral endpoints showed by individuals at day 1: survival was significantly higher in tadpoles exhibiting less lying on the flank (r_s -0.521, $P \leq 0.01$, $n = 24$), higher activity (r_s 0.669, $P < 0.001$) and more periphery behaviors (r_s 0.407, $P < 0.05$). Furthermore, survival tended to be negatively related to swirling (r_s -0.383, $P = 0.064$) and positively related to breathing (r_s -0.379, $P = 0.067$) at day 1. Correlation between survival and feeding was not significant (r_s 0.282, $P = 0.181$).

Second 96-h period:lag effects

Removing the contaminant from the aquarium allowed the tadpoles to partially recover at the behavioral level, but some significant differences persisted for several parameters (Table 2). Tadpoles at the 22 µg/l concentration displayed more lying, less

Table 2. Behavioral endpoints of tadpoles (mean ± SE proportions of individuals) as a function of three endosulfan concentration over time after the removal of pesticide and solvent: lying on the lateral side (abnormal posture), feeding, surface air breathing, activity (swimming), and space use of the peripheral area

Variables	Control	Solvent	Endosulfan		
			0.4 µg/l	3 µg/l	22 µg/l
Lying on the side	0	0	0	0	0.002±0.006
Feeding	0.138±0.027	0.137±0.021	0.134±0.017	0.122±0.031	0.019±0.001
Air breathing	0.059±0.008	0.013±0.021	0.118±0.013	0.075±0.005	0.013±0.005
Activity	0.950±0.005	0.973±0.010	0.974±0.008	0.969±0.013	0.639±0.132
Space use	0.806±0.052	0.794±0.035	0.874±0.015	0.800±0.034	0.606±0.048

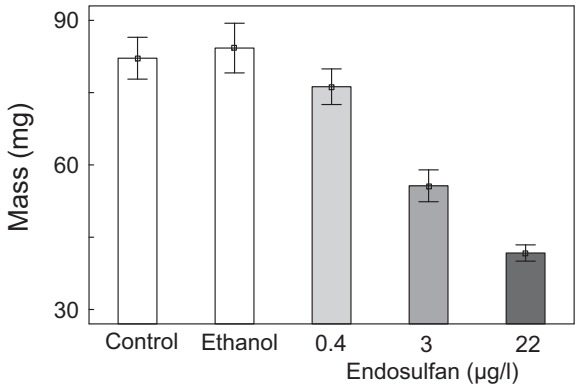

Fig. 3. Body mass (mean ± SE values) of tadpoles at the end of the experiment (8 days) as a function of endosulfan concentration

feeding, less breathing, less periphery use and less activity than controls (contrasts, all $P \leq 0.002$). Tadpoles at lower concentration showed behavioral patterns not significantly different from the controls (all $P > 0.1$). Swirling was almost not exhibited after the removal of the contaminant (one observation the first day at 22 µg/l concentration). Only one tadpole died during this second period (at 22 µg/l endosulfan).

At the end of the experiment, mass was significantly different among treatments ($F_{4,187} = 23.0$, $P \leq 0.0001$; Fig. 3). Tadpoles were lighter than controls at the two highest concentrations (control contrast: $P \leq 0.0001$), while differences were not significant at a concentration of 3 µg/l or less (all $P > 0.57$).

Discussion

Global use of a tremendous number of chemicals makes it difficult to assess them all efficiently beyond typical LC50 tests (Walker et al. 2006). This has important conservation implications as toxic chemicals that have only sublethal or chronic effects are released into the environment and could have health implications for a large variety of species (Relyea and Hoverman 2006; Boone et al. 2007). Here, we confirm and extend results of previous studies (e.g. Berrill et al. 1998; Brunelli et al. 2009) in showing that a large set of behavioral endpoints can help in evaluating sublethal toxicity during the time period of short-term toxicity tests. The advantage is twofold. On one hand, such studies provide data on the effects at low contaminant concentrations, such as those not causing death of individuals but often present in the environment (De Lange et al. 2006). On another hand, because assessing behavior would be done on the same individuals used for typical toxicity tests, this approach will result in the reduction of the use of animals in research, an important point for both ethical and conservation considerations (Wolfenson and Lloyd 2003).

The strongest effects on behavior were found at the same endosulfan concentration (282 µg/l) that caused mortality during the first 96-h experiment but appeared earlier. There were also significant effects at concentrations that did not affect survival during the timeframe of the experiment. Two abnormal behavioral endpoints were found only in the presence of the pesticide. They can be easily recognized during the observation sessions: the tadpoles either exhibited very incoherent fast and short movement (swirling) or were just lying on their lateral or dorsal side (normal posture is on the belly). Abnormal reactions caused by endosulfan have also been reported for invertebrates (Tu et al. 2010), fish (Stanley et al. 2009), and amphibian species, for which both Berrill et al. (1998) and Brunelli et al. (2009) documented irregular and convulsive swimming but also more immobility (paralysis) in bufonid and ranid tadpoles. Similarly, Westman et al. (2010) observed a longer time to quiescence after simulated predatory stress in spadefoot toad and treefrog tadpoles. Altered behavioral endpoints, and particularly convulsions such as swirling suggest a neurotoxic effect (Tu et al. 2010). The physiological basis of endosulfan action is not yet known in detail, but there is evidence of different modes of actions. First, inhibition of acetylcholinesterase disturbs neurotransmission, particularly in muscles, and thus could affect locomotory performance such as found in shrimp (Tu et al. 2009). Second, neuronal degeneration in cerebral targets, such as the mesenencephalon and hypothalamus, was also highlighted with associated altered binding levels at major histamine receptors in fish (Giusi et al. 2005).

Previous studies on the effects of endosulfan on amphibian tadpoles hypothesized that lower activity could affect fitness as this might imply lower food acquisition (Brunelli et al. 2009). In looking directly into both feeding behavior and activity in this study, we indeed found that both components were similarly inhibited. These results also confirmed those of Broomhall and Shine (2003) who found an inhibition of feeding on treefrog tadpoles exposed to endosulfan. On another hand, the smaller mass of contaminated tadpoles at the end of the experiment can be at least in part explained by their reduced feeding rates.

Air breathing was also affected: contaminated tadpoles of the three highest concentrations gulped surface air less often than control tadpoles. Atmospheric oxygen is important for long term survival,

particularly in case of hypoxia or during sustaining physical activities, but the exact benefits of air breathing in oxygenated waters remain to be determined (McIntyre and McCollum 2000). The absence of expression of this behavior is probably associated with the mortality found at the highest concentration. Similarly, Gdovin et al. (2006) found that both young and old ranid tadpoles that were prevented to access to air surface had higher mortalities than those able to breath at water surface. The lower activity of tadpoles caused by endosulfan is expected to be at the basis of such a reduction of air breathing. Egea-Serrano et al. (2011) concluded that stress caused by chemicals, such as nitrits, may induce an increase of air-breathing, but as long as tadpoles were able to exhibit locomotor activity up to water surface. In our experiment, a significant higher air breathing rate was found at the lowest endosufan concentration in line with these conclusions whereas the three highest concentrations of endosulfan might have altered too deeply tadpoles to allow them to compensate stress by taking more atmospheric air. There is also some evidence of an effect of endosulfan on gas exchange property of blood in fish (Rangaswamy and Naidu 1999). In bufonid amphibian tadpoles, morphological analysis of the gills also showed that endosulfan altered this aquatic breathing organ (Bernabò et al. 2008; Brunelli et al. 2010). Consequently, as contaminated tadpoles moved less to breathe at the water surface than control individuals in our study, they might particularly suffer from oxygen depletion.

Predation by fish or invertebrates is the primary cause of mortality of tadpoles in a natural environment (Alford 1999). Although pesticides do not necessarily kill directly tadpoles, their action on behaviors could indirectly affect their success because of ineffective response to predators (Scott and Sloman 2004; Relyea and Hoverman 2006). The present study did not test for predatory avoidance, but the observed results suggest that more work is needed on interactions between predation risk and the effect of endosulfan. Indeed, the exhibition of swirling behavior and an occupation of an open rather than a peripheral area by the contaminated individuals could make them much more visible to predators (Laurila 2000).

The six studied behavioral biomarkers were valuable to assess toxicity on the short term at all the tested concentrations, except for the 0.4 µg/l concentration for which there were no detected effects. The effects were immediate (i.e., after 1 day) for all behaviors at the highest concentration (Table 3). The effects at the other concentrations appeared from the first to the third day. The effects on behavioral endpoints occurred before the first signs of survival rate alterations. Three of these behavioral endpoints (lying on the lateral side, activity, and space use) at day 1 were correlated with mortality events at day 4. This indicates they are early indicators of alterations to survival. Moreover, all six behavioral endpoints were also found at concentrations that did not cause mortality in our experiment (as low as 3 instead of 282 µg/l). Preliminary observations suggest that mortalities would also occur in the long term at concentrations under 282 µg/l (M. Denoël & S. Libon, unpublished data). Mortality rates due to endosulfan in the present study were within the range of previously published studies (see e.g., Brunelli et al. 2009; Jones et al. 2009).

An important component, often missing from 96-h LC50 studies, is lag effects. Berrill et al. (1998) and more recently Jones et al. (2009) noticed that tadpoles died after pesticide removal following this test period, thus lowering real LC50 values. In the present study, we did not find any mortality increase but the behavioral disturbance caused by the pesticide was maintained after pesticide removal and this was particularly true at the highest concentration at which tadpoles survived the first 96-h test period (22 µg/l). As pointed out by Jones et al. (2009) differences between studies may be due to different tolerance between species. Only Berrill et al. (1998) evaluated possible lag effects for avoidance behaviors. Similarly to us, they found persistence of effects. In a natural situation, this shows that aberrant behaviors may remain even when the concentration of the pesticide is diluted or completely removed from the habitat. Consequently, even if pesticides degrade rapidly in the environment, their immediate effects can be long-lasting and have detrimental consequences over the long term, i.e. during tadpole life up to metamorphosis. Tadpoles exhibiting abnormal behaviors, feeding and breathing less often, and occupying possibly more risky habitats may have little chance of survival over the long term, particularly as this might expose them to predation, but mesocosm or field enclosures are needed to quantify this effect (Relyea and Hoverman 2006).

Conclusions

This study extends results of previous studies showing that endosulfan is a harmful substance at multiple levels at environmental concentrations (see e.g., Brunelli et al. 2009). It has significant effects on behavior and survival of amphibian tadpoles not only on the long term but also since the first day following the contact with the pesticide. Because of its toxicity, it should never been used in close proximity to water bodies, an obligate reproductive and feeding habitat for many amphibian species (Stebbins and Cohen 1995). The current presence of endosulfan in wetlands (Ernst et al. 1991; Srivastava et al. 2009; Weber et al. 2010) indicates that it can have a negative effect on natural populations and thus be one of the numerous agents responsible, in part, for declining amphibian populations (Stuart et al. 2004).

Because pesticide restrictions are often too strongly based on LC50 values, quantifying sublethal effects with repeatable and comparable designs is needed and should be prioritized as strongly as are

Table 3. First day of significant effect of endosulfan concentrations for the six tested behavioral endpoints (contrast test)

Concentrations	Swirling	Lying	Feeding	Air breathing	Periphery	Activity
0.4 µg/l	-	-	-	-	-	-
3 µg/l	-	-	2	1	1	3
22 µg/l	1	1	1	1	1	3
282 µg/l	1	1	1	1	1	1

mortality tests (see also Venturino et al. 2003; Scott and Sloman 2004; Giusi et al. 2010; Egea-Serrano et al. 2011). Behavioral markers can now be rigorously quantified in experiments that can be replicated and thus are powerful tools to complement other traditional survival analyses. New developments of analytical methods of behaviors, such as image analysis (Friberg-Jensen et al. 2010) and video-tracking of locomotor patterns (Denoël et al. 2010; Winandy and Denoël 2011), but also analyses of more complex aspects of behaviors such as sensory perception (Mandrillon and Saglio 2007) and learning processes (Eddins et al. 2010), are encouraged because of the complex and often over-looked actions of chemicals. Going in-depth with such techniques would also put behavioral assessment closer to physiological, histological and molecular studies in explaining the mechanisms of toxicity and the action of chemicals at very low concentrations.

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