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Environmentally relevant concentrations of endosulfan impair development, metamorphosis and behaviour in *Bufo bufo* tadpoles

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ABSTRACT

Endosulfan is a widely used organochlorine pesticide with well-documented neurotoxic effects in both humans and laboratory animals (mammals and fish). Neurotoxicity has been implied also in amphibians after short-term exposure to endosulfan. Little is known about effects of chronic exposure of endosulfan in amphibians. Previously, we examined the short-term toxicity of endosulfan in common toad (*Bufo bufo*) tadpoles and determined the LC50 value to 0.43 mg/L. In the present study, we investigated the effects of endosulfan on *B. bufo* tadpoles after chronic exposure to ecologically relevant concentrations. Tadpoles were exposed in a static renewal test, from shortly after hatching (Gosner stage 25) to completed metamorphosis, to 0.01, 0.05 and 0.1 mg endosulfan/L (nominal). The exposure period lasted 43–52 days. Mortality, larval growth (mass), development (reached Gosner stage at various times and deformities presence), metamorphosis and behaviour (swimming activity) were monitored regularly over the entire course of larval development. Our results show that 0.05 and 0.1 mg endosulfan/L caused impaired behaviour, prolonged time to metamorphosis, increased incidences of mouth and skeletal malformations as well as mortality, and reduced body weight (observed also at 0.01 mg/L) in *B. bufo* tadpoles. Behavioural effects occurred at exposure day 4, before any other effects occurred, indicating a neurotoxic effect. Endosulfan levels found in groundwater and surface water range from 0.1 to 100 μg/L and after extraordinary runoff events, concentrations exceed 0.5 mg/L in surface water.

Our results indicate that endosulfan may negatively affect wild frog populations in agricultural areas.

1. Introduction

Amphibian populations are declining globally with a drastic rate (Houlahan et al., 2000; Blaustein and Kiesecker, 2002; Stuart et al., 2004). In a recent report McCallum (2007) estimates, using a modelling approach, that the current extinction rate of amphibians is 211 times the background amphibian extinction rate. Several hypotheses have been proposed to explain the amphibian decline (Kiesecker et al., 2001; Beebee and Griffiths, 2005) which, in most cases, implicate anthropogenic alterations of the environment (Carey and Bryant, 1995). From the outset, habitat destruction and climate change were suggested as major factors involved in amphibian decline (Blaustein and Wake, 1990; Wake, 1991). More recently other possible causes have been recognized, e.g. increased exposure to ultraviolet radiation, emerging diseases, presence of alien species, exposure to environmental pollutants and fertilizers (Sparling et al., 2000; Blaustein et al., 2003; Little et al., 2003; Davidson et al., 2001, 2002; Pettersson and Berg, 2007).

Decreased species richness, reduced populations, and high deformity incidences have been reported in agroecosystems, which might be linked to the extensive use of pesticides (Berrill et al., 1994, 1997; Bonin et al., 1997; Davidson, 2004; Bridges et al., 2004; Knutson et al., 2002; Relyea, 2005). As outlined by Boone et al. (2005) there is a lack of data for many commonly used pesticides with regard to their effects on amphibians.

Endosulfan is a broad spectrum organochlorine insecticide used extensively on a variety of crops, including fruits, vegetables, rice, grains, tea, coffee, cotton and also in forestry. Endosulfan reaches aquatic systems through direct application, as well as spray drift and runoff from agricultural areas (Leonard et al., 1999, 2000, 2001; Broomhall, 2002; Jergentz et al., 2004). Endosulfan levels found in groundwater and surface water range from 0.1 to 100 μg/L (Dalvie et al., 2003). After extraordinary runoff events, concentrations exceeding 0.5 mg/L have been found in surface water (EPA, 2001). Endosulfan levels of 1.7 and 0.04 mg/L were found in water

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bodies in the vicinity of the spraying area, and 200 m away, respectively (Ernst et al., 1991).

Currently, endosulfan is subjected to a number of regulations and action plans, and it is proposed to be added to the annex of the Stockholm Convention on Persistent Organic Pollutants (POPs) by the European Commission (German Federal Environment Agency, 2007). Nevertheless, results from a global monitoring network for POPs reveal that endosulfan is abundant in the environment and that its use is increasing (Pozo et al., 2006; Harner et al., 2006).

Due to their persistence, toxicity and tendency to bioaccumulate, organochlorine environmental contaminants might pose a threat towards amphibians (Bishop et al., 1999; Glennemeier and Denver, 2001; Klemens et al., 2003; Fagotti et al., 2005). Endosulfan was implicated in the drastic population decline of anuran amphibians in western USA (Sparling et al., 2001). As of yet, however, there is a lack of experimental data on the chronic effects of endosulfan on amphibians as available studies focus on short-term effects (Berrill et al., 1998; Broomhall, 2002, 2004; Broomhall and Shine, 2003). We recently demonstrated that short-term exposure to 0.2 mg/L of endosulfan disrupts gill morphology and function in *Bufo bufo* tadpoles (Bernabò et al., 2008).

Short-term tests are useful to investigate the potency of chemicals to induce overt toxicity. Chronic toxicity studies are, however, needed to assess more subtle and indirect effects of chemicals on life-history functions which may have long-term consequences. The effects of environmental pollutants on amphibians vary considerably depending on the timing of exposure during the life cycle (Greulich and Pflugmacher, 2003). Chronic exposure during development may seriously impact population dynamics by influencing characteristics such as growth and development, swimming ability, metamorphic traits, foraging success, reproductive organ development, and reproductive performance of exposed individuals (Relyea and Mills, 2001; Relyea, 2003, 2004; Boone and Semlitsch, 2002; Broomhall, 2004; Pettersson et al., 2006; Gyllenhammar et al., 2009).

Common toad (*B. bufo*) lives in a variety of habitats including agricultural sites and is a widespread species in Europe. During recent decades a marked decrease in *B. bufo* populations has been observed locally in Europe (Pavignano and Giacoma, 1990; Hilton-Brown and Oldham, 1991) and it was suggested that intensive agricultural practices and/or alteration of breeding habitats contributed to the decline (Pavignano and Giacoma, 1990; Carrier and Beebee, 2003).

The aim of the present study was to investigate effects of chronic larval exposure to ecologically relevant concentrations of endosulfan on growth, development, metamorphosis and behaviour in *B. bufo* tadpoles.

2. Materials and methods

2.1. Tadpole maintenance

B. bufo tadpoles were hatched from eggs collected from a permanent pond in a regional natural reserve located near Cosenza in Calabria, Southern Italy (16°00′E, 39°33′L). All the animals were held under controlled laboratory conditions of 12:12 h light–dark cycles, water temperature of 22° ± 1 °C, and median pH of 7.3. Water quality parameters (pH, dissolved oxygen (DO), conductivity, alkalinity, and hardness) were monitored before and following renewal of the test solutions in all tests. The developmental stage was determined by the presence of distinctive morphological features according to an appropriate developmental chronological table (Gosner, 1960). Tadpoles were fed boiled lettuce or spinach *ad libitum* three times a week throughout the exposure period until the start of metamorphosis (Gosner stage 41). When the front legs emerged (Gosner stage 42), tadpoles were removed from exposure tanks and housed in 5-L plastic tanks containing both wet (with treatment solution) and dry areas; the tadpoles were not fed until tail had resorbed (metamorphosing tadpoles live off of fat stored in their tails).

2.2. Exposure conditions

Exposure solutions were prepared by dissolving commercial-grade endosulfan (purity 99%, Chem Service Inc., West Chester, PA, USA) in dechlorinated tap water to obtain the nominal concentrations: 0.01, 0.05, and 0.1 mg/L, from now on referred to as the low, medium and high concentration group, respectively. The control group was kept in tap water. For each experimental units (control, low, medium and high) 30 tadpoles of comparable body dimensions at Gosner stage 25 (Gosner, 1960) were assigned to 30-L glass tanks (1 tadpole/L). A static-renewal exposure system was used according to standard procedure guidelines (ASTM, 1997) with complete renewal of the water volume every third day. Since endosulfan has an aqueous half-life of several weeks, nominal concentration was expected to remain constant for 72 h (Miles and Moy, 1979; Broomhall, 2004). The endosulfan concentrations were chosen based on the LC50-96 h value of 0.43 mg/L determined in our previous study (Bernabò et al., 2008). The experiments were conducted for 2 consecutive years using the same methods and experimental conditions thus resulting in two replicates.

2.3. Growth, development, deformities, mortality and metamorphosis

The experimental period was approximately 48 days: from Gosner stage 25 to the reaching of Gosner stage 46 (complete tail resorption). The completion of metamorphosis in control and low concentration groups represented the end of the experiment. During this time individuals in high and medium concentration groups did not reach metamorphosis due to their poor growth.

Body weight of tadpoles was measured at the beginning of the experiment and every 8th day during the whole test period. Each tadpole was towel-dried and weighed to the nearest milligram. The developmental stage was recorded weekly on a subsample of at least 5 randomly selected tadpoles per tank. The presence of deformities and mortality were monitored daily and dead animals were removed. Deformities, mass and date of completed metamorphosis (Gosner stage 46) were recorded for all individuals.

2.4. Behaviour

Behavioural observations were conducted after 4, 12, 20, and 28 days of exposure. The behaviours noted were: regular swimming, irregular swimming, and immobility. Irregular swimming was defined as erratic swimming, body twisting and convulsions. Immobility was defined by complete stillness for the whole observation period (60 s). For the observations five tadpoles were randomly selected from the control and each treatment group and transferred to observation chambers containing 5 L of tap water or the respective endosulfan concentration. After 10 min of acclimatization the tadpoles in each chamber were recorded using a Panasonic NV-GX7 video camera for 30 min and the videos downloaded to a computer. The behaviour of each tadpole was observed, during a 30-min period, every 60 s resulting in a score number (0–30) assigned evaluating how many times each behaviour was observed during a 30-min interval. A mean score value was then calculated per observation day and exposure group. The observa-