Environmental Pollution 157 (2009) 1597–1602

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Environmental conditions enhance toxicant effects in larvae of the ground beetle *Pterostichus oblongopunctatus* (Coleoptera: Carabidae)

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Combined negative effects of nickel and chlorpyrifos on carabid beetles depend on ambient temperature.

ARTICLE INFO

Article history:
Received 9 October 2008
Received in revised form
19 December 2008
Accepted 23 December 2008

Keywords:
Multiple interactions
Pterostichus oblongopunctatus
Metals
Pesticides
Temperature

ABSTRACT

The wide geographical distribution of ground beetles *Pterostichus oblongopunctatus* makes them very likely to be exposed to several environmental stressors at the same time. These could include both climatic stress and exposure to chemicals. Our previous studies demonstrated that the combined effect of nickel (Ni) and chlorpyrifos (CHP) was temperature (T)-dependent in adult *P. oblongopunctatus*. Frequently the different developmental stages of an organism are differently sensitive to single stressors, and for a number of reasons, such as differences in exposure routes, their interactions may also take different forms. Because of this, we studied the effects of the same factors on the beetle larvae. The results showed that all factors, as well as their interactions, influenced larvae survival. The synergistic effect of Ni and CPF was temperature-dependent and the effect of Ni $\times$ T interaction on the proportion of emerged imagines indicated stronger toxicity of Ni at 25 °C than at 10 °C.

1. Introduction

Decades of studies on the toxicity of various chemicals to a range of organisms have yielded reasonably abundant and reliable data for dozens of organic and inorganic pollutants. More recently, however, ecotoxicologists have realized that pollutants are only seldom present in nature as single chemicals, and that organisms are often exposed to mixtures of different chemicals. Consequently, it has been suggested that good risk assessment methodologies should not rely on single-chemical ecotoxicological data but rather need to consider the effects of mixtures of chemicals actually (or potentially) present in an area of interest (De Zwart and Posthuma, 2005). The toxicity of a chemical and the interaction between chemicals may be additionally modified by natural factors, such as temperature or moisture fluctuations. The importance of extreme climatic events is believed to be increasing over the years due to global climate changes (Easterling et al., 2000). Unfortunately, the combined effects of chemical and non-chemical factors are still relatively poorly studied, especially for terrestrial invertebrates (Holmstrup et al., 2000; Spurgeon et al., 2005). Identifying the effects of such multiple factors on the ground beetle *Pterostichus oblongopunctatus* was the main line of the present study.

The nature and concentrations of chemicals in a mixture, the exposure routes, and the sensitivity ranges of receptor organisms are all important factors determining the type and intensity of response. Because of its wide geographic distribution (Brunsting, 1981) and large populations, *P. oblongopunctatus* is an important predatory species in many Palearctic forests. At the same time, and for the same reasons, in many industrial regions its populations may suffer from exposure to metal pollution originating from industry and from the use of pesticides in forestry and agriculture. Simultaneous exposure to metals and pesticides may result in additive effects (Steevens and Benson, 2001), but significant deviations from strict additivity have also been observed. For example, Forget et al. (1999) found synergism (greater than additive interaction) between a metal (arsenic, copper, or cadmium) and a pesticide (carbofuran, dichlorvos or malathion) in the marine microcrustacean *Tigriopus brevicornis*, while van der Geest et al. (2000) observed antagonism (less than additive interaction) after exposure of mayfly *Ephoron virgo* larvae to copper and diazinon. Broerse et al. (2007) also observed less-than-additive effects after exposure of the springtail *Folsomia candida* to nickel and chlorpyrifos.

Organisms living in the natural environment are not only exposed to mixtures of chemicals but are also subjected to non-chemical stressors (climate, food shortage, pathogens, etc.) which are likely to interact with chemicals either directly, for example by changing their bioavailability, or indirectly, changing the

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doi:10.1016/j.envpol.2008.12.027

organism's behavior and physiology and thereby its sensitivity to the toxicants (Holmstrup et al., 1998; Sjursen and Holmstrup, 2004). One of the most significant factors that can affect an organism's response to toxicants is temperature (Holmstrup et al., 1998). Studies using soil invertebrates have shown that temperature can influence the sensitivity of species to toxicants (Bednarska and Laskowski, 2008; Donker et al., 1998; Spurgeon et al., 1997), but there is a lack of research focusing on interactions of mixtures of dissimilar chemicals at different temperatures. Our previous work (Bednarska et al., in press) demonstrated that the interaction between the organophosphorus (OP) insecticide chlorpyrifos (CPF) and a metal, nickel (Ni), which can potentially occur in the environment concurrently, was temperature-dependent for the survival of adult *P. oblongopunctatus*. However, different developmental stages of beetles may be differently sensitive to such multiple interactions. As predators, both larvae and adult beetles are potentially exposed to high levels of pollutants accumulated in tissues of their prey, but regardless of sharing a similar diet, the food demand and thus the amount of metal assimilated from food may differ between the two life stages. In addition, larvae have to moult regularly in order to grow, and this process can help in the removal of metals incorporated into the exoskeleton (Lindqvist and Block, 1995). Therefore the elimination of metal may be more efficient in larvae than in adults. The influence of soil on toxicant effects also seems far greater in the soil-dwelling larvae than in epigeal adults. Unlike adult beetles, which spend most of the time at the soil surface when not hibernating in rotting branches on the ground or under mosses, the desiccation-sensitive larvae spend their life digging in soil (Brunsting, 1981). Adults may easily be exposed to pesticide sprays topically; the more probable pathway of pesticide exposure for larvae is through the soil.

To mimic some of the environmental conditions, we designed our experiment with continuous exposure to Ni through food and with a single application of CPF to the soil. As in our previous study on adults (Bednarska et al., in press), temperature was the non-chemical factor. Chlorpyrifos is one of the widely used and best-studied pesticides in terms of its effects on soil invertebrates in the laboratory and in the field (Jänsch et al., 2006), and interest in the effects of nickel on soil-dwelling invertebrates has increased in recent years as production of the metal has risen (Bednarska and Laskowski, 2008; Lock and Janssen, 2002; Scott-Fordsmand et al., 1998; Scott-Fordsmand et al., 1999). Except for the work of Broerse et al. (2007), however, who reported that CPF mitigated Ni-induced mortality and growth reductions in *Folsomia candida*, we have located no other data on Ni and CPF interaction for soil invertebrates. Thus, the extent to which the interaction between Ni and CPF depends on the chemicals themselves or is species-specific remains largely an open question. Even less is known about how temperature or other natural stressors influence the effects of mixtures of such dissimilar chemicals. The effects of Ni, CPF and temperature on soil-dwelling larvae of *P. oblongopunctatus* were quantified in terms of mortality and the proportion of emerged imagines.

2. Materials and methods

2.1. *Pterostichus oblongopunctatus* culture

The larvae for the experiment were taken from ca. 100 adult beetles collected from an uncontaminated forest near Krakow, southern Poland. The beetles were cultured to obtain larvae as described by Bednarska and Laskowski (2008). The concentration ranges of Ni and CPF were chosen based on range-finding experiments performed before this study. Temperatures of 10, 20 and 25 °C were selected on the basis of larva performance. Because development from larva to immature beetle is inhibited below 10 °C and above 25 °C, we took these two temperatures as the minimum and maximum, and 20 °C as optimal (cf. also Metge and Heimbach, 1998).

2.2. Experimental design

The possible interactions between chemicals and temperature were studied in a full factorial test design. The newly hatched larvae were transferred individually to 30 ml plastic vials filled ca. 3/4 with moistened peat (80% of water holding capacity, WHC), contaminated with 0, 0.5, 1 or 2 mg CPF kg^{-1} dry weight (dw) (CPF-0, CPF-0.5, CPF-1 or CPF-2, respectively) and randomly assigned to one of three artificial foods spiked with 0, 600 or 1200 mg Ni kg^{-1} dw (Ni-0, Ni-600 or Ni-1200, respectively). The food was ground frozen *Tenebrio molitor* larvae mixed with ground apple, with 1 g sodium benzoesate ($C_7H_5NaO_2$, Fluka, Germany) per kilogram of food as preservative. The routes of exposure to the two chemicals were different since the most probable way to acquire Ni by carnivorous larvae living in a metal polluted environment is through feeding on metal-contaminated prey. In contrast to metals, organic pesticides, except for systemic ones, act mostly through direct contact with body surfaces. Thus, we assumed that shortly after spraying, the highly toxic but gradually degrading CPF is toxic to soil-dwelling larvae mostly through soil rather than through food. The larvae were fed ad libitum every second day; the food was placed on the soil surface. When fresh food was supplied, the remains of old food were taken out to keep the vials as clean as possible. The larvae were cultured at three different temperatures: 10, 20 or 25 °C (T-10, T-20 or T-25, respectively), in darkness and 75% relative humidity (RH). Each individual larva was treated as a replicate, with 10–18 replicates used for each treatment. Altogether, 492 larvae were used in the experiment. Nickel chloride hexahydrate ($NiCl_2 \cdot 6H_2O$, Eurochem BGD, Poland) was added to the food as aqueous solution, and CPF (minimum 98% technical, Cheminova, Denmark) was added to the dry peat as an acetone solution, mixed and left overnight under a fume board to let the acetone evaporate. The following day the CPF-contaminated peat was mixed with deionized water to reach 80% WHC. An additional acetone-only control was prepared and run at each temperature.

The survival of larvae was checked every day during the first week of the experiment and on each feeding day until pupation. Just before emergence they were checked daily to determine the exact emergence date. The emerged adults were transferred to uncontaminated peat and were fed uncontaminated food every second day. Consequently we were sure that effects observed in adults resulted from exposure in the larval stage. The experiment was ended after 125 days, when all larvae had either pupated or died. The recorded endpoints were the lifetime followed for 125 days and the proportion of imagines emerged in each treatment.

2.3. Chemical analyses

To determine the actual nickel concentrations in food, a dry sample (ca. 0.1 g) of each batch was analyzed by flame atomic absorption spectrometry (AAnalyst 800; Perkin-Elmer, USA). At least three blanks accompanied every run of analysis. Additionally, samples of certified reference material (dogfish muscle DORM-2, Canada) were included to check analytical precision. The detection limit (0.68 mg l^{-1}) was calculated as three standard deviations of the mean measurements of the calibration blanks. The measured concentrations were within ±13% of the certified reference value. Nickel concentration was expressed in milligrams per kilogram dry weight.

2.4. Statistical analyses

For treatments in which mortality during the experiment was high enough, LT_{50} (median survival time) was estimated from survival analysis (Kaplan and Meier, 1958). If a test of all treatments together detected statistically significant differences between survival curves ($p \leq 0.05$), they were then analyzed separately within each stressor (Ni, CPF or T) by pair-wise comparisons using the log-rank test (Mantel, 1966). All beetles used in the experiments were incorporated in survival analyses, but incomplete (censored) survival data for beetles that escaped before the end of the experiment were excluded from GLM analysis (see below). The lifetime of the beetles that survived to the end of the experiment was set equal to the test duration (125 days). This approach might lead to underestimation of the effects on survival, because the differences in lifetime between treatments in which beetles died during the experiment and those in which no mortality occurred would be larger if the experiment had been prolonged.

To quantify the relationship between the endpoints (lifetime and proportion of emerged imagines) and the factors (Ni, CPF, temperature), the data were analyzed with the general linear model (GLM) method. Because the data were not normally distributed (Kolmogorov–Smirnov test), they were all rank-transformed. Rank transformation was chosen because the variance of rank data is automatically stable.

Because preliminary inspection of the data revealed an approximately linear relationship between temperature and both survival and proportion of emerged imagines, the following model was tested:

$$Y = a1 + a2 \times T + a3 \times \mathrm{Ni} + a4 \times \mathrm{CPF} + a5 \times T \times \mathrm{Ni} + a6 \times T \times \mathrm{CPF} + a7 \times \mathrm{Ni} \times \mathrm{CPF} + a8 \times T \times \mathrm{Ni} \times \mathrm{CPF}$$

where Y is the dependent variable studied and $a1$ to $a8$ are the estimated parameters. After running the full model, that is, testing of all the main factors and interactions for significance ($p \leq 0.05$), the non-significant terms were consecutively